Agnès Ricroch · Dennis Eriksson
Dragana Miladinović · Jeremy Sweet
Katrijn Van Laere · Ewa Woźniak-Gientka
Editors

A Roadmap for Plant Genome Editing

A Roadmap for Plant Genome Editing

Agnès Ricroch • Dennis Eriksson
Dragana Miladinović • Jeremy Sweet
Katrijn Van Laere • Ewa Woźniak-Gientka
Editors

A Roadmap for Plant Genome Editing

Editors
Agnès Ricroch
The Jean-Monnet Faculty. Idest Laboratory
Paris-Saclay University
Sceaux, France

Dragana Miladinović
Institute of Field and Vegetable Crops
National Institute of Republic of Serbia
Novi Sad, Serbia

Katrijn Van Laere
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO)
Melle, Belgium

Dennis Eriksson
Department of Plant Breeding
Swedish University of Agricultural Sciences
Alnarp, Sweden

Jeremy Sweet
Sweet Environmental Consultants
Cambridge, UK

Ewa Woźniak-Gientka
Institute of Bioorganic Chemistry
Polish Academy of Sciences
Poznań, Poland

ISBN 978-3-031-46149-1 ISBN 978-3-031-46150-7 (eBook)
https://doi.org/10.1007/978-3-031-46150-7

This work was supported by Cost Association

© The Editor(s) (if applicable) and The Author(s) 2024. This book is an open access publication.
Open Access This book is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.
The images or other third party material in this book are included in the book's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the book's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, expressed or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Paper in this product is recyclable.

Editorial

Genome editing is a technology that has been embraced swiftly and enthusiastically by plant researchers, innovators and breeders across the world in the past decade. Its impact can hardly be overestimated. A quick search for “plant genome editing” in the Web of Science shows over 8300 scientific publications in 2013–2023, with a steady and considerable growth in numbers per year. The COST Action PlantEd (CA18111), funded by the European Cooperation in Science and Technology programme (www.cost.eu), represents a European-based network of more than 600 experts on plant genome editing that has monitored, and led the progress in this field since 2019. When it started, highly controlled CRISPR/Cas-based advancements such as base editing and prime editing were barely more than conceptual ideas in plants whereas these are now being used for various applications (Betül Kaya). Novel delivery methods have been developed (Doyle Prestwich et al.), guide RNA design and validation is steadily improving (Dorso) and the screening as well as functional prediction of resulting mutations are increasingly efficient (Vereecke et al.). This technology now also allows for the editing of entire gene families (Avci and Sipahi) as well as avoiding linkage drag and other undesired side effects (Wind), which are considerable improvements to conventional plant breeding. All these novel methods and improvements of genome editing offer to plant scientists and breeders multiple strategies for enhancing crop production, crop protection, food quality and climate change adaptation (citing Part V, Future Outlook).

To date, genome editing has been applied in a wide range of crops, including wheat (Nigro et al.; Smulders et al.), barley (Jakobson et al.; Pouramini and Hensel), maize (Varotto), legumes (Das and Acharjee; Dervishi et al.), soybean (Oztolan Erol), oilseed rape (Boniecka), tomato (Scarano and Santino; García-Caparrós), chicory (Cankar et al.), various horticultural plants (Mahna and Nayeri), fruit trees (Claessen et al.), forest trees (Bruegmann et al.) and also algae (Opsahl-Sorteberg and Evju; Evju and Opsahl-Sorteberg).

The plant breeding sector stresses the importance of innovations in breeding with genome editing for contributing to solving many important societal challenges (Jorasch and Vangheluwe). At the same time, the technology is surrounded by controversy, particularly in Europe (Kuntz), and the number of concerned EU citizens

seems to be increasing (Ichim). In addition, acceptability of collaborative licensing platforms to overcome the difficulties created by the complex patent landscape of CRISPR technology is lacking (Ricroch). A survey conducted in Poland identified knowledge gaps and low awareness on genome editing (Molodziejko et al.); however, a study in Germany suggests that strong moral convictions may hinder effective science communication (Waldhof). One result of this societal controversy is that plant genome editing remains regulated in the EU to the extent that innovations are hindered (Zimny; Sprink and Wilhelm). It is therefore important to discuss what types of reforms are necessary to allow society to reap the benefits that this technology can bring. In this context, it is important to compare the EU views with the approaches to genome editing and other novel gene technologies being taken in other parts of the world (Sprink and Wilhelm; Rosado).

This book represents a snapshot of the latest advancements in the field of plant gene editing, together with perspectives from many other disciplines such as social sciences and law. It is our hope that this book will contribute to illuminate the road ahead for this very important technology and that it will serve as inspiration and guidance for a wide range of stakeholders.

The Jean-Monnet Faculty. Idest Laboratory
Paris-Saclay University
Sceaux, France
agnes.ricroch@universite-paris-saclay.fr
Agnès Ricroch

Department of Plant Breeding
Swedish University of Agricultural Sciences
Alnarp, Sweden
Dennis Eriksson

Institute of Field and Vegetable Crops
National Institute of Republic of Serbia
Novi Sad, Serbia
Dragana Miladinović

Sweet Environmental Consultants
Cambridge, UK
Jeremy Sweet

Flanders Research Institute for Agriculture,
Fisheries and Food (ILVO)
Melle, Belgium
Katrijn Van Laere

Institute of Bioorganic Chemistry
Polish Academy of Sciences
Poznań, Poland
Ewa Woźniak-Gientka

Contents

Part I Plant Breeding and Technological Advances

1 **Genome Editing of Gene Families for Crop Improvement** 3
Utku Avci and Hülya Sipahi

2 **Base Editing and Prime Editing** 17
Hilal Betul Kaya

3 **Novel Delivery Methods for CRISPR-Based Plant Genome Editing** 41
Barbara Doyle Prestwich, Teodoro Cardi, Allah Bakhsh, Alessandro Nicolia, and Kaushal Kumar Bhati

4 **Balancing Trait Improvement with Tradeoff Side-Effects Using Genome Editing Technology** 69
Julia Johanna Wind

5 **CRISPR/Cas Mutation Screening: From Mutant Allele Detection to Prediction of Protein Coding Potential** 79
Elke Vereecke, Katrijn Van Laere, and Tom Ruttink

6 **Methods and Techniques to Select Efficient Guides for CRISPR-Mediated Genome Editing in Plants** 89
Fabio D'Orso, Valentina Forte, Simona Baima, Marco Possenti, Daniela Palma, and Giorgio Morelli

Part II Applications of Genome Editing

7 **Genome Editing of a Macroalgae with Possible Global Impacts** 121
Hilde-Gunn Opsahl-Sorteberg and Espen Evju

8 A Short Review of Advances in Plant-Based Antigen Production Strategies and the Production of Viral Vaccine Antigens Derived from CRISPR/Cas9 Genome Edited *N. benthamiana* Plants for Enhanced Vaccine Efficacy 131
Espen Evju and Hilde-Gunn Opsahl-Sorteberg

9 Precise Gene Editing of Cereals Using CRISPR/Cas Technology 141
Pouneh Pouramini and Goetz Hensel

10 Implementing Genome Editing in Barley Breeding 151
Liina Jakobson, Signem Oney Birol, and Ljudmilla Timofejeva

11 Current Status and Future Prospective of Genome Editing Application in Maize 165
Serena Varotto

12 Using Gene Editing Strategies for Wheat Improvement 183
Domenica Nigro, Mark A. Smedley, Francesco Camerlengo, and Sadiye Hayta

13 Gene Editing of Wheat to Reduce Coeliac Disease Epitopes in Gluten 203
Marinus J. M. Smulders, Luud J. W. J. Gilissen, Martina Juranić, Jan G. Schaart, and Clemens C. M. van de Wiel

14 Genome Editing in Horticultural Plants: Present Applications and Future Perspective 223
Nasser Mahna and Shahnoush Nayeri

15 Application of CRISPR/Cas-Mediated Genome Editing Techniques in Leguminous Crops 247
Debajit Das and Sumita Acharjee

16 Genetic Improvement in Leguminous Crops Through Genome Editing 259
Aida Dervishi, Muhammad Waqas Choudry, Rabia Riaz, and Allah Bakhsh

17 Soybean Improvement and the Role of Gene Editing 271
Nihal Öztolan Erol

18 CRISPR/Cas-Based Precision Breeding of Oilseed Rape (*Brassica napus* L.) – Recent Improvements 291
Justyna Boniecka

19 Targeted Gene Editing in Pome Fruit Genetics and Breeding: State-of-the-Art, Application Potential and Perspectives 309
Hanne Claessen, Pollien Aert, and Nico De Storme

20 Genome Editing in Forest Trees 347
Tobias Bruegmann, Alexander Fendel, Virginia Zahn, and Matthias Fladung

21 Genome Editing for Reduction of Bitterness and for Production of Medicinal Terpenes in *Cichorium* Species 373
Katarina Cankar, Katrijn Van Laere, and Dirk Bosch

22 Engineering Phytonutrient Content in Tomato by Genome Editing Technologies 385
Aurelia Scarano and Angelo Santino

23 Breeding for Yield Quality Parameters and Abiotic Stress in Tomato Using Genome Editing 395
Pedro García-Caparrós

24 Genome Editing-Based Strategies Used to Enhance Crop Resistance to Parasitic Weeds 411
Kubilay Yıldırım, Musa Kavas, Melekşen Akın, and İlkay Sevgen Küçük

Part III Policies and Regulations

25 Genome Editing in Biotech Regulations Worldwide 425
Thorben Sprink and Ralf Wilhelm

26 Interpreting Precision Breeding: Key Legal Concepts Under International Law and Current Domestic Regulatory Approaches in the Global South 437
André Rosado

27 CRISPR Processes Patents in Green Biotechnology: Collaborative Licensing Models 453
Agnès Ricroch

Part IV Public and Stakeholder Perceptions

28 The View of the European Seed Sector on Genome Editing Tools in Plant Breeding 471
Petra Jorasch and Nick Vangheluwe

29 The Awareness of the Polish Society on New Genomic Techniques 481
Wiktoria Mołodziejko, Justyna Nowakowska, and Anna Linkiewicz

30 Improving Science Communication About Genome Editing – Mitigating Strong Moral Convictions Through Shared Moral Goals 493
Gabi Waldhof

31 The Citizens' Awareness and Concerns During the Transition from Genetically Modified to Genome Edited Plants in Europe About Their Use in Agriculture and Food Production 519
Mihael Cristin Ichim

32 What Is the Problem with Europe in a Philosophical Point of View? 533
Marcel Kuntz

33 NGT Plant Products in the EU. The Postulates, The Outlooks, and Possible Consequences of a Regulatory System Reform in the Context of Legislative Reforms in Third Countries and Detection Requirements 545
Tomasz Zimny

Part V Future Outlook

34 Prospects for Plant Genome Editing 557
Agnès Ricroch, Dennis Eriksson, Dragana Miladinović, Jeremy Sweet, Katrijn Van Laere, and Ewa Woźniak-Gientka

Part I
Plant Breeding and Technological Advances

Chapter 1
Genome Editing of Gene Families for Crop Improvement

Utku Avci and Hülya Sipahi

Abstract Crop improvement has been a long-standing focus of agricultural research, aiming to enhance nutritional richness, aroma, visual appeal, and yield to meet the growing global food demand. Recent advances in molecular biology and genetic engineering, particularly genome editing, offer precise and targeted tools for modifying crop genomes. Traditional plant breeding methods, while successful in the past, are time-consuming, and techniques like mutagenesis and transgenesis have limitations. Genome editing techniques provide unprecedented precision and enable scientists to make desired modifications to a plant's DNA. This chapter explores the role of genome editing, specifically in gene families, for crop improvement, highlighting its potential benefits and challenges.

Gene families are crucial for important crop traits like yield, disease resistance, and environmental adaptation. However, conventional breeding methods often struggle to effectively manipulate gene families due to their complex nature. Genome editing offers a promising solution by allowing targeted modifications to specific gene family members. The precision of genome editing tools can help unravel the functions of gene family members in diverse plant species.

With the challenges posed by climate change, global conflicts, and population growth, the conventional food system falls short of meeting future demands sustainably. Genome-edited crops hold promise in obtaining elite genotypes with desirable traits, contributing to a resilient and sustainable agriculture and food system. Moreover, genome editing facilitates the study of genetic diversity that governs desirable crop characteristics, benefiting both genome-edited and conventionally bred crops.

U. Avci (✉) · H. Sipahi
Faculty of Agriculture, Agricultural Biotechnology Department,
Eskisehir Osmangazi University, Eskisehir, Türkiye

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_1

1 Introduction

Crop improvement has been the main target of agricultural research for centuries in comprehensive areas such as increasing the nutritional richness of foods, obtaining aromatic foods with pleasant smell and taste, producing decorative products that appeal to visual pleasures, while increasing the yield, especially to meet the increasing food demand of the global population. In recent years, advances in molecular biology and genetic engineering have provided new tools for crop improvement. Genome editing, in particular, has emerged as a promising technique for modifying the genome of crops in a precise and targeted manner [1, 2].

The process of conventional plant breeding is time-consuming despite its significant contribution to the first green revolution in the 1960s. The introduction of molecular marker-assisted selection has helped to speed up the process of plant breeding. Techniques such as mutagenesis and transgenesis have helped to reduce the time required to develop new varieties. However, mutagenesis is a random process that can have undesirable side effects or can result in incomplete and off-target disruption of genes. Genetically modified transgenic plants can also face regulatory challenges. Unlike traditional genetic engineering methods, which involve the insertion of foreign DNA into an organism's genome, genome editing allows scientists to make precise modifications to the existing DNA sequence. Genome editing can be accomplished through different techniques, including CRISPR-Cas9, TALENs, and Zinc Finger Nucleases (ZFNs) [2]. These tools offer an unprecedented level of precision, enabling scientists to make precise, targeted changes to a plant's DNA, resulting in desired traits [3].

Gene families are groups of genes that share a similar DNA sequence and function. They often play critical roles in crop traits such as yield, disease resistance, and environmental adaptation. Although members of a gene family have different specialized functions, they share a common purpose. Therefore, it is important to understand and define the functions of gene family members involved in important plant development or stress resistance in different plant species. Traditional breeding methods are often unable to effectively target and manipulate gene families, as they may contain many members that have overlapping functions or redundancies [1].

The food system faces challenges such as sustainability and food supply stemming from climate change, global conflicts and increasing population [4]. Conventional breeding and agricultural practices come short in terms of meeting the current and future food demand. Therefore, it is necessary to have different approaches to ensure a more resilient and sustainable agrifood system. In this sense, genome-edited crops have the potential to play a role in obtaining elite genotypes with desirable traits in plant breeding. Besides, genome editing has another advantage, as it enables the study of genetic diversity that governs desirable crop characteristics. The knowledge that comes out of such studies could be used in the development of a genome-edited crop as well as in conventional breeding.

In this chapter, we explore the role of genome editing of gene families in crop improvement, its potential benefits, and its challenges.

2 Benefits of Genome Editing in Crop Improvement

Genome editing has several potential benefits for crop improvement. One of the most significant advantages is its precision. Unlike traditional genetic engineering techniques, which often involve the insertion of foreign DNA into an organism's genome, genome editing allows scientists to make precise modifications to the existing DNA sequence. This precision means that genome editing can be used to introduce desirable traits into crops without introducing unwanted traits or disrupting existing genes [1, 3].

Another advantage of genome editing is its efficiency. Traditional breeding methods can take many years to develop new crop varieties with desirable traits. In contrast, genome editing can produce changes in the DNA sequence in a matter of weeks or months. This speed and efficiency mean that genome editing can be used to rapidly develop new crop varieties that are better adapted to changing environmental conditions, such as drought or disease resistance.

According to a survey of experts on the added potential benefits of genome-edited crops compared to those developed through genetic modification and conventional breeding, there is a consensus among experts on the enhanced agronomic performance and product quality of genome-edited crops over alternatives [5]. The majority of experts indicated that genome editing enables faster trait development, lower R&D costs, and trait innovation [6]. High-oleic soybean in the US and tomato with increased φ-aminobutyric acid (GABA) levels in Japan are two current genome edited crops in the market. New ones are expected to follow in the coming years.

In recent studies, the functions of members of gene families have been investigated by many different types of mutation, including knock-out, base editing and allele exchange with multiplex sgRNAs for targeted genes with CRISPR/Cas9 technology. Their role in crop improvement has been summarized in Table 1.1 and discussed below in terms of improved agronomic traits, disease resistance, quality, and nutritional content.

2.1 Improved Agronomic Traits

Genome editing can be used to enhance the yield of crops by improving their resistance to environmental stressors, such as drought, heat, and cold. By modifying specific genes, scientists can increase the plant's ability to cope with adverse conditions, leading to improved yield.

Allelic variants of the *ARGOS8* gene belonging to the ARGOS gene family were obtained by CRISPR gene editing in maize. These allelic variants were the negative regulator of ethylene responses [7]. The ARGOS8 variants had high levels of *ARGOS8* transcripts relative to the native allele and increased grain yield under flowering stress conditions and had no yield loss in arid conditions.

Table 1.1 Summary of gene families targeted by genome editing for crop improvement in terms of improved agronomic traits, disease resistance, quality, and nutritional content

	Gene family	Observed phenotypes	Methods	Plant	References
Agronomic traits	ARGOS	Improved grain yield	CRISPR-Cas9	*Zea mays*	[7]
	Phospholipase D	Impaired agronomic traits in *PLD1* mutants	CRISPR-Cas9	*Brassica napus*	[8]
	SUMO proteases	Reduced root and shoot biomass in mutant lines	CRISPR-Cas9	*Oryza sativa*	[9]
	The basic helix–loop–helix (bHLH)	Improved salt stress tolerance	CRISPR-Cas9	*Oryza sativa*	[10, 11]
	The cytochrome P450	Higher grain width, grain length, and grain fragrance, increase in 1000 grain weight.	CRISPR/Cas9	*Oryza sativa*	[12]
	Promoter-binding protein-like (SPL) TFs	Improved agronomic traits	CRISPR/Cas9	*Triticum aestivum*	[13]
	Cytokinin oxidase/dehydrogenase	Effected plant development, seed quality, and starch composition.	CRISPR-Cas9 and CRISPR-Cas12a	*Oryza sativa*	[14]
	The Lateral Organ Boundaries Domain (Lbd) proteins	Enhanced drought tolerance	CRISPR/Cas9	*Solanum lycopersicum*	[15]
	Rubisco	Reduced photosynthetic rates and biomass accumulation	CRISPR-Cas9	*Nicotiana tabacum*	[16]
	Pectin methylesterase inhibitors	Reduced plant height, increased growth, improved resistance to Cadmium in mutant lines	CRISPR-Cas9	*Oryza sativa*	[17]
	The transcription factors *bHLH*, *bZIP*, *MYB*, *PHD* and *LBD*	Various effects on male fertility, anther dehiscence, pollen development	CRISPR-Cas9	*Zea mays*	[18]
	Type 2A serine/threonine protein phosphatases (PP2As)	Self-incompatibility in knock-out lines	CRISPR-Cas9	*Brassica napa*	[19]

Disease resistance	miRNA482	Negatively regulated the resistance to tomato wilt disease in mutant lines	CRISPR-Cas9	*Solanum lycopersicum*	[20]
	miR482	Reduced late blight, caused by *Phytophthora infestans*.	CRISPR-Cas9	*Solanum lycopersicum*	[21]
	miR482	A lower disease index for *Verticillium dahliae* infection	CRISPR-Cas9	*Gossypium hirsutum*	[22]
Quality	Starch branching enzymes (SBEs)	A unique starch lacking branching	CRISPR-Cas9 RNP-method	*Solanum tuberosum*	[23]
	Starch branching enzymes (SBEs)	Higher amylose content	CRISPR-Cas9	*Oryza sativa*	[24]
	Lysophosphatidic acid acyltransferase (LPAT)	Decreased oil content, increased size of oil body	CRISPR-Cas9	*Brassica napus*	[25]
	Polyphenol oxidases (PPOs)	Reduced enzymatic browning	CRISPR-Cas9	*Solanum tuberosum*	[26]
	Polyphenol oxidases (PPOs)	Higher polyphenol content in the berries	CRISPR-Cas9	*Solanum melongena*	[27]
Nutritional content	Fatty acid desaturase (FAD2)	Higher oleic acid content	CRISPR-Cas9	*Glycine max*	[28]
	Laccase	Delayed hairy root development, very low lignin content	CRISPR-Cas9	*Salvia miltiorrhiza*	[29]
	Berberine bridge enzyme-like (BBL)	Reduced amount of nicotine	CRISPR-Cas9	*Nicotiana tabacum*	[30]
	α- and γ-gliadin	Decreased gluten immunogenicity	CRISPR-Cas9	*Triticum aestivum*	[31]
	α-amylase /trypsin inhibitors (ATI)	Decreased amount of α-amylase/trypsin inhibitor proteins for less allergenic wheat variety	CRISPR-Cas9	*Triticum durum*	[32]

In order to understand the function of *BnaPLDα1* genes of Phospholipase D Gene Family in *Brassica napus*, *BnaPLDα1* gene was over-expressed and knocked out by CRISPR. Four lines were edited at one sgRNA site and two lines were edited at two sgRNA sites [8]. Compared to the wild type, the PLDα1 protein was found to be more expressed in the over-expressed edited lines while less or not expressed in the knockout lines. When the plants were planted in the field, the plant height was not different between overexpression lines and wild type, but the plant heights of the knocked-out lines were shorter, indicating that the appropriate dose of PLDα1 was required to maintain the normal growth of the plant, indicating that further increase of PLDα1 level had limited effect on it. Many agronomic traits such as plant height, effective branches, silique number in inflorescence, silique seed number were adversely affected in lines with reduced PLDα1 levels [8]. These findings proved that *BnaPLDα1* regulation changes the agronomic traits of *B. napus*.

The loss of function lines of *OsOTS1* gene enhanced sensitivity to salt with reduced root and shoot biomass, suggesting it plays a role in salinity stress. Mutagenesis of *OsOTS1* gene of SUMO proteases gene family to reveal its role against salinity stress was targeted in rice [9]. To investigate the usability of transcription factor (basic helix-loop-helix (bHLH)) sub-U family genes in breeding rice varieties under salinity stress conditions in rice (*Oryza sativa* L.), loss of function lines of *OsbHLH024* and *OsbHLH044* genes was generated by the CRISPR/Cas9 system [10, 11]. The gene editing mutant lines revealed altered morphological and physiological phenotypes. *OsbHLH024* mutant lines had increased shoot weight, the total chlorophyll content, and the chlorophyll fluorescence. They had less reactive oxygen species and stabilized levels of MDA, fewer Na+ but more K+, and a balanced level of Ca^{2+}, Zn^{2+}, and Mg^{2+} in the shoot and root. Therefore, it was concluded that the *OsbHLH024* gene improves salt stress resistance by playing a role as a negative regulator [10]. Considering *OsbHLH044* mutant lines, they showed reduced morphological and physiological parameters, lower antioxidant activities and higher lipid peroxidation and hydrogen peroxide (H_2O_2) accumulation. The loss-of-function of this gene also altered the expression of ion homeostasis-related genes (*OsHKTs*, *OsHAK*, *OsSOSs*, and *OsNHX*) and ABA-responsive gene (*OsLEA3*). The mutant synthesized less stored starch and proteins because of decreased expression of genes coding for starch (*OsAGPL1*, *OsSSIIa*, *OsWx*, and *OsFLO2*) and seed storage proteins (*GluA1* and *Globulin 1*). It was concluded that the *OsbHLH044* gene plays a role as a positive regulator of salt stress and grain quality [11].

In rice, three genes (*Os03g0603100*, *Os03g0568400*, *GL3.2*) of cytochrome P450 family and *OsBADH2 were* multiplex edited with CRISPR/Cas9 and their novel alleles were generated [12]. The high performance of mutant plants homozygous for grain width, grain length, and grain fragrance was recorded. Also, the mutant plants showed high level 2-acetyl-1-pyrroline (2AP). It has been concluded that the edited plant in this study could be used as valuable genetic material for crop breeding to improve rice yield and quality.

Indel mutations in the *TaSPL13* gene of SQUAMOSA promoter-binding protein-like (SPL) transcription factors gene family were created to increase grain yield in bread wheat (*Triticum aestivum*) [13]. The microRNA 156 recognition element

indel mutations in 3′ UTR of *TaSPL13* brought about the abundance of TaSPL13 transcripts and a decrease in flowering time, tiller number, and plant height and the consequent grain size and number increased in the mutant plants.

Eleven members of the Cytokinin oxidase/dehydrogenase gene family (*OsCKX1-OsCKX11*) were knocked out using CRISPR/Cas9 and CRISPR-Cas12a to reveal their functions in rice [14]. CRISPR-Cas12a performed better than CRISPR-Cas9 for generating multigene mutants. The *OsCKX* members showed functional redundancy and affected plant development, seed quality, and starch composition by regulating endogenous cytokinins. *OsCKX1/2* and *OsCKX3/8/10* genes had roles in the control of panicle architecture and grain number. *OsCKX4/5/9* genes regulated the development of roots and plant height and tillers.

Knock-out and over-expression of *SlLBD40* (*Solyc02g085910*) gene of The Lateral Organ Boundaries Domain (Lbd) Protein Family were created to understand its role against drought stress in tomato [15]. The results showed that *SlLBD40* knock-out lines had improved water-holding ability and relieved the physical harm of the photosynthetic system of tomato seedlings, while *SlLBD40* overexpressing plants showed vigorous wilting symptoms under drought stress, suggesting that *SlLBD40* is a negative regulator of drought tolerance.

In order to increase photosynthetic efficiency in tetraploid tobacco (*Nicotiana tabacum*), it was aimed to obtain novel alleles of genes (*rbcS*) coding the small subunit of CO_2-fixing enzyme Rubisco family so that the efficiency of carbon assimilation through better Rubisco enzymes could be increased [16]. The phenotyping analysis of mutant plants showed reduced Rubisco content, photosynthetic rates, and biomass accumulation. In this study, it has been shown that it is possible to produce superior non-native Rubisco enzymes by multiplex gene editing with CRISPR/Cas9 in polyploid species.

The function of the *OsPMEI12* gene, which belongs to the pectin methylesterase inhibitors (*OsPMEI*) gene family for growth, cell wall development, and response to phytohormone and heavy metal stress, was elucidated by mutating it in rice (*Oryza sativa* L.) [17]. It has been revealed that the *OsPMEI12* gene plays a role in the regulation of methyl esterification during growth, thus pectin status is controlled by hormones and cadmium stress.

Discovery of genetic male sterility (GMS) genes will allow to unravel the molecular mechanism of anther development and to develop male sterility systems for crop breeding and hybrid seed production. Therefore, the researchers focused on elucidating the roles of the transcription factors genes (*bHLH*, *bZIP*, *MYB*, *PHD* and *LBD* gene families) responsible for the GMS agronomic trait in highly heterosis maize [18]. Mutation of transcription factor gene families with CRISPR yielded the following results; that *ZmbHLH51* and *ZmbHLH122* are necessary for male fertility, *ZmTGA10* has affected anther dehiscence. *ZmMYB84* is essential for maize pollen development. *ZmPHD11* is responsible for the formation of anther cuticle and Ubisch bodies. Two MYB33 paralogs are important for GMS. *ZmLBD27* has an impact on the reduced ratio of normal pollen grains. ZmPHD11/27 influenced viable pollen formation.

Two homologous genes (*Bra018924* and *Bra014296*) of PP2A, a 55 kDa B regulatory subunit (PR55/B), were edited through the CRISPR/Cas9 system to develop self-compatible lines controlled by type 2A serine/threonine protein phosphatases (PP2As) in Chinese cabbage [19]. The *PR55/B* gene knock-out line did not show self-incompatibility and produced seeds, suggesting that the *PR55/B* gene was responsible for the self-incompatibility.

2.2 Improved Disease Resistance

Plant diseases are a significant threat to crop productivity, with losses estimated to cost billions of dollars annually. Genome editing can be used to enhance the resistance of crops to diseases by editing genes that encode for disease resistance.

MicroRNAs act as regulators of the plant immune system by silencing the genes involved in pathogen virulence or by regulating the expression of target genes. MicroRNAs belonging to the miRNA482/2118 superfamily targeted R-genes of the class NBS-LRR (nucleotide-binding site-leucine rich repeat). In tomato, knockout mutants for *SlymiR482e-3p* gene, a member of the miR482/2118 family, were generated to detect its role against tomato wilt disease resistance [*Fusarium oxysporum* f. sp. *lycopersici* (race 2) (Fol)] [20]. The results showed that *SlymiR482e-3p* gene negatively regulated the resistance to wilt disease. In another study with miR482 gene family in tomato, mutations in the two genes (*miR482b* and *miR482c*) were generated [21]. The expression levels of their target NBS-LRR genes were increased, followed by reduced late blight, caused by *Phytophthora infestans,* disease symptoms in mutant plants. Interestingly, it was concluded that knocking out these two genes could lead to expression perturbation of other miRNAs, suggesting cross-regulation between miRNAs. In cotton (*Gossypium hirsutum* L.), the MIR482 mutant collection was obtained by knocking out *MIR482* genes in miR482 gene family with dozens of members using CRISPR/Cas9. This mutant collection allow us to examine the role of individual *MIR482* genes against pathogen response and to identify miR482-NLR module(s) that respond to, particularly fungal pathogen *Verticillium dahliae* agent for Verticillium wilt and other pathogens. It has also been reported that this collection may be a useful genetic resource for the development of new disease-resistant cotton varieties [22].

2.3 Improved Quality

Quantitative trait loci (QTLs) are genomic regions that are associated with complex traits such as crop quality. QTLs can be used to improve crop quality by identifying genomic regions associated with desirable traits. Many genes in QTLs have been targeted with genome editing.

Starch branching enzymes (SBEs) are involved in the biosynthesis of starch in plants and there have been several studies on the use of genome editing *SBE* genes in crops to modify starch content and properties. For example, one study used CRISPR-Cas9 RNP-method to induce mutations in *SBE* genes to develop a unique potato starch lacking branching [23]. Another study used CRISPR/Cas9 to generate high-amylose rice by targeting *SBEI* and *SBEIIb* genes [24].

In *Brassica napus*, an oilseed plant that is the raw material for both cooking oil and biodiesel production, loss-of function of the *BnLPAT2* and *BnLPAT5* genes belonging to lysophosphatidic acid acyltransferase (LPAT) family allowed to lay out their precise roles in oil biosynthesis. That is, the oil content decreased, the size of oil body increased in mutant lines [25]. The results of this study illustrated that these genes could be used for oil production improvement.

In order to prevent enzymatic browning, negatively affecting tubers during harvest and post-harvest procedures, researchers targeted the Polyphenol Oxidases (PPOs) gene family and mutated the *StPPO2* gene in *Solanum tuberosum* L. [26]. A lower PPO causing oxidation of polyphenols (natural antioxidants) in tuber was observed in mutant lines and enzymatic browning was reduced. Another study with the polyphenol oxidases (PPOs) gene family was carried out in eggplant (*Solanum melongena* L.) [27]. In this study, the researchers stated that it would be possible to develop eggplant varieties that retain a high polyphenol content beneficial to human health in the berries during harvest and post-harvest processes by knocking out the three genes (*SmelPPO4*, *SmelPPO5*, *SmelPPO6*).

2.4 Improved Nutritional Content

Genome editing can also be used to enhance the nutritional content of crops by increasing the levels of essential nutrients, such as oil, phenolic acid, vitamins and minerals [4]. This is particularly important in developing countries, where nutrient deficiencies are prevalent and access to a diverse diet is limited.

The oleic acid content of soybean seeds was modulated by knocking out GmFAD2-1A and GmFAD2 genes belonging to the fatty acid desaturase (FAD2) family [28]. The loss of function of these genes has paved the way for cultivating soybean genotypes with high oleic acid content.

In *Salvia miltiorrhiza*, the *SmLAC*s genes of the laccase family, which has 29 family members with high homology, were multiplex silenced by targeting their conserved sequences to reveal their role in the production of medicinal phenolic acid compounds [29]. It has been concluded that *SmLAC*s play a role in lignin formation and phenolic acid biosynthesis in the roots due to decreased expression of target genes and delayed hairy root development, larger and looser xylem cells, low RA and SAB accumulation, and very low lignin content in edited lines compared to wild types.

By knocking out six genes (*BBLa*, *BBLc*, *BBLd.2*, *BBLb*, *BBLd.1*, *BBLe*) belonging to the berberine bridge enzyme-like (BBL) family using a single sgRNA, a nicotine-free tobacco variety was developed and the amount of nicotine was reduced [30]. In this way, the use of nicotine-free tobacco by smokers may protect their health.

The genes in α- and γ-gliadin gene families were silenced using six sgRNAs, and a less allergenic bread wheat variety containing gluten with fewer immunogenic epitopes, which would be beneficial in the diet of coeliac patients, was obtained [31]. A less allergenic durum wheat variety was produced [32]. The multiplex editing of *CM3* and *CM16* genes belonging to α-amylase/trypsin inhibitors (ATI) family subunit U form was carried out using seven gRNAs targeting the two genes of interest. The editing of these genes decreased the amount of ATI transcripts. It was reported that this might have happened due to nonsense-mediated mRNA decay. Interestingly, it was noted that ATI 0.28 pseudogene were activated after knocking out *CM3* and *CM6* genes.

3 Challenges of Genome Editing in Crop Improvement

Despite its potential benefits, genome editing in crops also faces several challenges. One of the biggest challenges is the regulatory landscape. Many countries have regulations that govern the use of genetically modified organisms (GMOs), and it is not yet clear how gene-edited crops will be regulated. In some cases, gene-edited crops may be subject to the same regulations as GMOs, even though the changes made to the DNA sequence are much smaller and more precise.

Another challenge is the potential for unintended consequences. Although genome editing is a precise technique, there is still a risk of off-target changes in the host genome [4]. These unintended changes could have unforeseen consequences for the crop's performance or safety. To mitigate this risk, scientists must employ rigorous testing and validation processes to ensure that the desired changes are made without causing any unintended effects. Therefore, it is important to carefully assess the safety and efficacy of gene-edited crops before they are released into the environment or consumed by humans.

4 Summary

Genome editing is a promising technique for crop improvement that has the potential to produce crops that are better adapted to changing environmental conditions and have improved nutritional content. However, there are also challenges that need to be addressed, including regulatory issues and potential unintended consequences. With careful consideration and regulation, genome editing has the potential to revolutionize crop improvement.

The use of genome editing in crop improvement also raises ethical considerations. For instance, there are concerns around the ownership and control of the technology, as well as the potential for gene-edited crops to create new monopolies in the seed industry. Inadequate stewardship, enhanced inequity between rich and poor, lack of transparency, an unclear intellectual property landscape, and inadequate public sector institutional infrastructures to support the use of genome-editing technologies are also challenges that need to be addressed [33].

Regulatory policy is another challenge. The global regulatory policy for genome-edited crops is still emerging and will shape the path of genome editing innovation. Genome editing is a relatively new technology, and its regulatory framework is still evolving. The regulation of gene-edited crops varies between countries, with some countries adopting a more stringent approach than others. As genome editing techniques become more widespread, there is a need for a coordinated global approach to regulating their use in agriculture to ensure that the technology is used responsibly and safely.

References

1. Chen, K., Wang, Y., Zhang, R., et al.: CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu. Rev. Plant Biol. **70**, 667–697 (2019)
2. Zhan, X., Lu, Y., Zhu, J.K., Botella, J.R.: Genome editing for plant research and crop improvement. J. Integr. Plant Biol. **63**, 3–33 (2021)
3. Abdelrahman, M., Wei, Z., Rohila, J.S., Zhao, K.: Multiplex genome-editing technologies for revolutionizing plant biology and crop improvement. Front. Plant Sci. **12**, 721203 (2021)
4. Kumar, D., Yadav, A., Ahmad, R., et al.: CRISPR-based genome editing for nutrient enrichment in crops: a promising approach toward global food security. Front. Genet. **13**, 932859 (2022)
5. Lassoued, R., Macall, D.M., Hesseln, H., et al.: Benefits of genome-edited crops: expert opinion. Transgenic Res. **28**, 247–256 (2019)
6. Kalaitzandonakes, N., Willig, C., Zahringer, K.: The economics and policy of genome editing in crop improvement. Plant Genome, **16**, e20248 (2019)
7. Shi, J., Gao, H., Wang, H., et al.: ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol. J. **15**, 207–216 (2017)
8. Lu, S., Fadlalla, T., Tang, S., et al.: Genome-wide analysis of phospholipase D gene family and profiling of phospholipids under abiotic stresses in Brassica napus. Plant Cell Physiol. **60**, 1556–1566 (2019)
9. Zhang, C., Srivastava, A.K., Sadanandom, A.: Targeted mutagenesis of the SUMO protease, Overly Tolerant to Salt1 in rice through CRISPR/Cas9-mediated genome editing reveals a major role of this SUMO protease in salt tolerance. bioRxiv, 555706 (2019)
10. Alam, M.S., Kong, J., Tao, R., et al.: CRISPR/Cas9 mediated knockout of the OsbHLH024 transcription factor improves salt stress resistance in rice (Oryza sativa L.). Plan. Theory. **11**, 1184 (2022a)
11. Alam, M.S., Yang, Z.K., Li, C., et al.: Loss-of-function mutations of OsbHLH044 transcription factor lead to salinity sensitivity and a greater chalkiness in rice (Oryza sativa L.). Plant Physiol. Biochem. **193**, 110–123 (2022b)
12. Usman, B., Nawaz, G., Zhao, N., et al.: Generation of high yielding and fragrant Rice (Oryza sativa L.) lines by CRISPR/Cas9 targeted mutagenesis of three Homoeologs of cytochrome P450 gene family and OsBADH2 and transcriptome and proteome profiling of revealed changes triggered by mutations. Plants (Basel). **9**, 788 (2020)

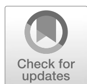
13. Gupta, A., Hua, L., Zhang, Z., et al.: CRISPR-induced miRNA156-recognition element mutations in TaSPL13 improve multiple agronomic traits in wheat. Plant Biotechnol. J. **21**, 536–548 (2023)
14. Zheng, X., Zhang, S., Liang, Y., Zhang, R., et al.: Loss-function mutants of OsCKX gene family based on CRISPR-Cas systems revealed their diversified roles in rice. Plant Genome. **20**, e20283 (2023)
15. Liu, L., Zhang, J., Xu, J., et al.: CRISPR/Cas9 targeted mutagenesis of SlLBD40, a lateral organ boundaries domain transcription factor, enhances drought tolerance in tomato. Plant Sci. **301**, 110683 (2020)
16. Donovan, S., Mao, Y., Orr, D.J., et al.: CRISPR-Cas9-mediated mutagenesis of the rubisco small subunit family in Nicotiana tabacum. Front. Genome Editing. **2**, 605614 (2020)
17. Li, Z., Rao, M.J., Li, J., et al.: CRISPR/Cas9 mutant rice Ospmei12 involved in growth, cell wall development, and response to Phytohormone and heavy metal stress. Int. J. Mol. Sci. **23**, 16082 (2022)
18. Jiang, Y., An, X., Li, Z., et al.: CRISPR/Cas9-based discovery of maize transcription factors regulating male sterility and their functional conservation in plants. Plant Biotechnol. J. **19**, 1769–1784 (2021)
19. Shin, N.R., Shin, Y.H., Kim, H.S., Park, Y.D.: Function analysis of the PR55/B gene related to self-incompatibility in Chinese Cabbage using CRISPR/Cas9. Int. J. Mol. Sci. **23**, 5062 (2022)
20. Gao, Y., Li, S.J., Zhang, S.W., et al.: SlymiR482e-3p mediates tomato wilt disease by modulating ethylene response pathway. Plant Biotechnol. J. **19**, 17–19 (2021)
21. Hong, Y., Meng, J., He, X., et al.: Editing miR482b and miR482c simultaneously by CRISPR/Cas9 enhanced tomato resistance to Phytophthora infestans. Phytopathology. **111**, 1008–1016 (2021)
22. Zhu, Q.H., Jin, S., Yuan, Y., Liu, Q., Zhang, X., Wilson, I.: CRISPR/Cas9-mediated saturated mutagenesis of the cotton MIR482 family for dissecting the functionality of individual members in disease response. Plant Direct. **6**, e410 (2022)
23. Zhao, X., Jayarathna, S., Turesson, H., et al.: Amylose starch with no detectable branching developed through DNA-free CRISPR-Cas9 mediated mutagenesis of two starch branching enzymes in potato. Sci. Rep. **11**, 4311 (2021)
24. Sun, Y., Jiao, G., Liu, Z., et al.: Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. Front. Plant Sci. **8**, 298 (2017)
25. Zhang, K., Nie, L., Cheng, Q., et al.: Effective editing for lysophosphatidic acid acyltransferase 2/5 in allotetraploid rapeseed (Brassica napus L.) using CRISPR-Cas9 system. Biotechnol. Biofuels. **12**, 225 (2019)
26. González, M.N., Massa, G.A., Andersson, M., et al.: Reduced enzymatic Browning in potato tubers by specific editing of a polyphenol oxidase gene via ribonucleoprotein complexes delivery of the CRISPR/Cas9 system. Front. Plant Sci. **10**, 1649 (2020)
27. Maioli, A., Gianoglio, S., Moglia, A., et al.: Simultaneous CRISPR/Cas9 editing of three PPO genes reduces fruit flesh Browning in Solanum melongena L. Front. Plant Sci. **11**, 607161 (2020)
28. Wu, N., Lu, Q., Wang, P., et al.: Construction and analysis of GmFAD2-1A and GmFAD2-2A soybean fatty acid desaturase mutants based on CRISPR/Cas9 technology. Int. J. Mol. Sci. **21**, 1104 (2020)
29. Zhou, Z., Li, Q., Xiao, L., et al.: Multiplexed CRISPR/Cas9-mediated knockout of Laccase genes in Salvia miltiorrhiza revealed their roles in growth, development, and metabolism. Front. Plant Sci. **12**, 647768 (2021)
30. Schachtsiek, J., Stehle, F.: Nicotine-free, nontransgenic tobacco (Nicotiana tabacum l.) edited by CRISPR-Cas9. Plant Biotechnol. J. **17**, 2228–2230 (2019)
31. Jouanin, A., Schaart, J.G., Boyd, L.A., et al.: Outlook for coeliac disease patients: towards bread wheat with hypoimmunogenic gluten by gene editing of α- and γ-gliadin gene families. BMC Plant Biol. **19**, 333 (2019)

32. Camerlengo, F., Frittelli, A., Sparks, C., et al.: CRISPR-Cas9 multiplex editing of the α-amylase/trypsin inhibitor genes to reduce allergen proteins in durum wheat. Front. Sustain. Food. Syst. **4**, 104 (2020)
33. Pixley, K.V., Falck-Zepeda, J.B., Paarlberg, R.L., et al.: Genome-edited crops for improved food security of smallholder farmers. Nat. Genet. **54**, 364–367 (2022)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 2
Base Editing and Prime Editing



Hilal Betul Kaya

Abstract The development of new adaptations of CRISPR-based genome editing platforms, such as base editing and prime editing, made it possible to broaden the scope and applications of genome editing in plants. First base editing and, more recently, prime editing evade the creation of double-stranded breaks in deoxyribonucleic acid (DNA) and the requirement of donor template of DNA for repair while enhancing editing efficiency and product purity over CRISPR/Cas9. As base-pair changes in genomic DNA determine many significant agronomic traits, crop varieties can be developed by precisely converting specific single bases in plant genomes. While base editing can introduce specific nucleotide changes, such as transition and transversion mutations in the targeted region, prime editing can create precise insertions, deletions, and all 12 types of point mutations using the "search-and-replace" method.

This chapter provides the basic principles of base editing and prime editing technologies and their practical applications in plants. The chapter also summarizes the recent breakthroughs in applying base and prime editors in diverse plant species, including their use in improving disease resistance, herbicide resistance, nutritional quality, crop yield, and quality. Finally, this chapter aims to clearly understand base editing and prime editing in plants by outlining potential developments.

1 Base Editing

Base editing is a novel genome editing method that creates transition and transversion mutations at the single-base level without double-stranded DNA breaks, donor templates, or undesirable effects of NHEJ and HDR mechanisms [1, 2] (Fig. 2.1a). Since base editors (BEs) considerably minimize unintended modifications, they show great potential in plant genome editing applications [3]. Base editor combines a catalytically impaired Cas protein with a nucleotide deaminase to convert one

H. B. Kaya (✉)
Department of Bioengineering, Manisa Celal Bayar University, Manisa, Turkey
e-mail: hilalbetul.kaya@cbu.edu.tr

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_2

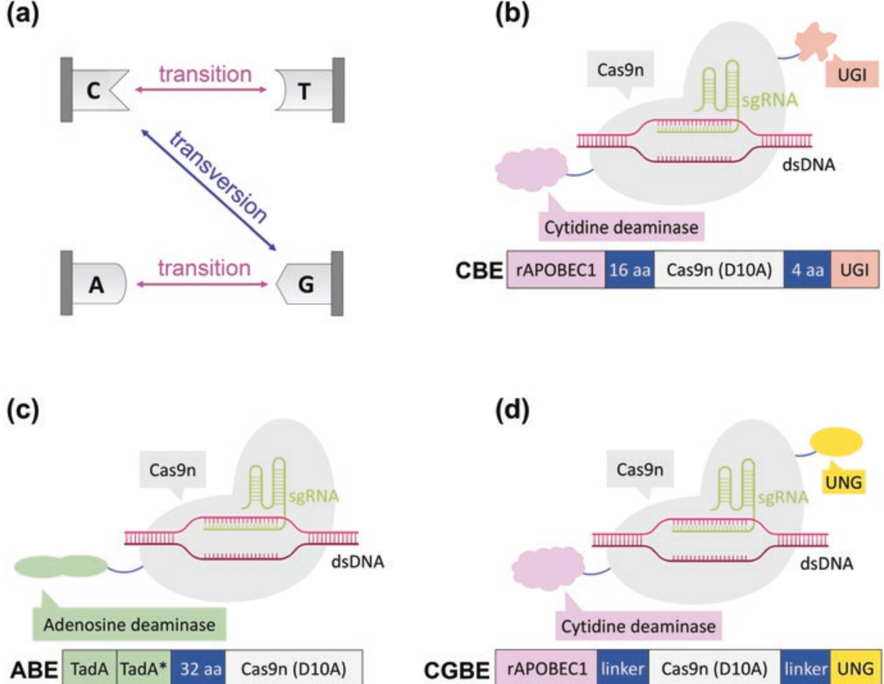


Fig. 2.1 Schematic diagrams of base editors. (**a**) Graphical overview of the transition and transversion base-pair substitutions in base editing, (**b**) Cytosine base editors (CBE) mediate C-to-T conversion, (**c**) Adenine base editors (ABE) mediate A-to-G conversion, (**d**) Glycosylase base editors (CGBE) mediate transversion mutations

base to another at a target locus in DNA or RNA [4, 5]. First, the Cas protein-gRNA complex binds to its target locus in DNA, and then the Cas protein denatures the double-stranded DNA resulting in an R-loop that exposes a small segment of single-stranded DNA [6]. Next, the deaminase enzyme catalyzes the specific base conversion in this single-stranded DNA. Finally, the permanent introduction of single-base substitutions resulted in the target region through DNA repair and replication [5].

The first developed base editors, cytosine base editors (CBEs), convert a cytosine (C) to thymine (T) and guanine (G) to adenine (A) in the opposite strand) in the target region [5] (Fig. 2.1b). In 2016, David Liu group created the first-generation base-editor (CBE1) by fusing a rat cytidine deaminase (rAPOBEC1) to the N terminus of dCas9 (dead Cas9) via a 16 amino acid XTEN linker [5, 7]. Although CBE1 successfully converts C:G to T:A *in vitro*, the base excision repair mechanism (BER) recognizes any G:U base pair as a mismatch and removes the uracils with the help of uracil N-glycosylases (UNGs) in vivo. To address this limitation and improve its efficiency, CBE2 was developed by adding a uracil DNA glycosylase inhibitor (UGI) to the C-terminus of dCas9 (dead Cas9) via a 4-amino acid linker [5, 8]. With this new version, editing efficiency was increased three times compared to CBE1

due to the inhibition of uracil DNA glycosylase (UDG) in the organism by UGI. To increase editing efficiency, CBE3 was developed by replacing the dCas9 with a nCas9 (H840, HNH catalytic domain) nickase variant. In this new version, nCas9 would induce a nick in the G-containing DNA strand, which activated the cellular mismatch repair (MMR) mechanism [5, 7]. This MMR mechanism replaces the G on the nicked strand with an A, forming a U:A pair with the target strand. The U:A pair was later corrected, leading to the desired T:A substitution. While inhibition of BER by using UGI in CBE2 enhanced editing efficiencies approximately threefold, the nicking strategy of CBE3 increased efficiencies by up to sixfold compared to CBE2 in human cells [5]. CBE in plants was first implemented in rice [9–11] and then adapted to various plants species such as wheat [11, 12], *Arabidopsis* [13, 14], maize [11], potato [15, 16], tomato [15, 16], cotton [17], watermelon [18], soybean [19], apple [20], pear [20], strawberry [21], rapeseed [22], *P. patens* [23] and poplar [24] quickly CBE-mediated genome editing used for different purposes, such as obtaining disease and herbicide resistance, accelerating crop domestication, and increasing yield and nutrient use efficiency in various plants, has been summarized in Table 2.1 (also reviewed in [25, 26]).

1.1 Improving the Base Editing Efficiency

With a better understanding of the molecular functions of deaminases, adenine base editors (ABEs) are developed for inducing A•T to G•C conversions with high efficiency [27] (Fig. 2.1c). ABEs use engineered transfer RNA adenosine deaminases (TadA) derived from *E. coli*, which bind to ssDNA and deaminates A into inosine I. The use of ABEs overcomes the limitation of CBEs, which can only edit C or G bases, and provides a broader range of base transformation options. Unlike CBEs, ABEs do not need to suppress the activity of alkyl adenine DNA glycosylase (AAG) [28, 29]. Over time, various optimization strategies were implemented, including TadA mutations and using varying lengths of the linker between TadA and nCas9 (D10A) to enhance the editing efficiencies of ABEs [25, 27, 30]. Various variants (ABE 6.3, ABE 7.8, ABE 7.9, ABEmax, etc.) of ABEs have been developed and implemented in mammalian cells [8, 27] and then rapidly adapted to plant cells. Li et al. [31] used ABE-mediated base editing to edit rice's acetyl-coenzyme A carboxylase (*ACC*) gene to confer herbicide resistance. To create A·T to G·C conversion in *OsMPK6*, *OsSERK2*, and *OsWRKY45* in rice, a florescence-tracking ABE was developed and successfully implemented by obtaining up to 62.3% editing efficiency [32]. In a proof-of-concept study, Kang et al. [33] edited the *PDS* gene in *Arabidopsis thaliana* and *Brassica napus* by creating a single amino acid substitution using ABE. Hua et al. [34] compared two different ABE versions (ABE-P1S and ABE-P1) to increase efficiency in rice. Like CBEs, ABEs were also implemented in various plants, as shown in Table 2.1.

Table 2.1 Selected applications of base editors in plants

Plant species	Targeted gene(s)	Type of base editor	Purpose	Delivery technique	Tissue	Reference
Alfalfa (*Medicago sativa*)	*ALS1* and *ALS2*	CBE	Herbicide resistance	Agrobacterium-mediated	Unspecified	[47]
Arabidopsis	*AtALS*	CBE	Method development	Agrobacterium-mediated	Floral tissues	[13]
Arabidopsis	*AtALS*, *AtPDS*, *AtFT*, *AtLFY*	ABE	Proof of concept	Agrobacterium-mediated	Floral tissues	[33]
Arabidopsis	*AtMTA*	CBE	Proof of concept	Agrobacterium-mediated	Floral tissues	[48]
Arabidopsis	*eIF4E1*	ABE	Enhanced resistance to potyviruses	Agrobacterium-mediated	Floral tissues	[49]
Arabidopsis	*16s rRNA*, *rpoC1*, *psbA*	DdCBE	Proof of concept	Agrobacterium-mediated	Floral tissues	[50]
Citrus	*CsALS*, *CsLOB1*	CBE, ABE	Herbicide resistance, Canker disease resistance	Agrobacterium-mediated	Epicotyl explants	[51]
Cotton	*GhCLA*, *GhPEBPc*	CBE	Method development	Agrobacterium-mediated	Hypocotyl explants	[17]
Cotton	*GhPEBP* and *GhCLA*	G. hirsutum-Base Editor 3 (GhBE3)	Method development	Agrobacterium-mediated	Hypocotyl explants	[52]
Maize	*ZmCENH3*	CBE	Method optimization	Agrobacterium-mediated	Immature embryo	[11]
Nicotiana benthamiana	*NbPDS*	CBE	Proof of concept and increasing gene editing efficiency	Agrobacterium-mediated	Leaves	[53]

Oilseed rape	*BnALS1* 1.8%	CBE	Herbicide resistance	Agrobacterium-mediated	Hypocotyl explants	[22]
Poplar	*PtPDS1* and *PtPDS2*	CGBE	Proof of concept	Agrobacterium-mediated	Shoots	[24]
Potato	*StALS*, *StGBS*	CBE	Proof of principle Herbicide resistance, starch synthesis	PEG-mediated	Protoplasts	[54]
Potato	*StALS1*	CBE	Herbicide resistance	Agrobacterium-mediated	Stem and petiole tissues	[15]
Potato	*StDMR6-1*, *StGBSSI*	CBE	Method development	Agrobacterium-mediated	Stem and petiole tissues	[16]
Rapeseed	*BnALS*, *BnPDS*	ABE	Proof of concept	PEG-mediated	Protoplasts	[33]
Rice	*OsNRT1.1B*, *OsSLR1*	CBE	Method development	Agrobacterium-mediated	Calli	[10]
Rice	*ALS*	CBE	Herbicide-resistant	Agrobacterium-mediated	Calli	[55]
Rice	*OsPDS*, *OsSBEIIb*	CBE	Proof of concept	Agrobacterium-mediated	Calli	[9]
Rice	*OsCDC48*	CBE	Method optimization	Agrobacterium-mediated	Calli	[11]
Rice	*OsCERK1*, *OsSERK1*, *OsSERK2*, *ipa1*, *pi-ta*	CBE	Method development	Agrobacterium-mediated	Unspecified	[56]
Rice	*OsMPK6*, *OsMPK13*, *OsSERK2*, *OsWRKY45*	ABE	Method development	Agrobacterium-mediated	Calli	[32]
Rice	*OsALS*, *OsCDC48*, *OsAAT*, *OsDEP1*, *OsACC*, *OsNRT1.1B*, *OsEV*, *OsOD*	ABE	Method development, Herbicide resistance	Agrobacterium-mediated	Calli	[31]

(continued)

Table 2.1 (continued)

Plant species	Targeted gene(s)	Type of base editor	Purpose	Delivery technique	Tissue	Reference
Rice	*OsRLCK185*, *OsCERK1*, *Pi d2*	CBE	Proof of concept	Agrobacterium-mediated	Calli	[57]
Rice	*OsAAT*, *OsCDC48*, *OsDEP1*, *OsNRT1.1B*, *OsOD*, *OsEV*, *OsHPPD*	CBE	Proof of concept	PEG-mediated	Calli	[54]
Rice	*OsSPL14*, *OsSPL17*, *OsSPL16*, *OsSPL18*, *OsIDS1*, *OsTOE1*, *SNB*, *PMS3*	ABE CBE	Method development	Agrobacterium-mediated, Particle bombardment	Calli	[30]
Rice	*Wx*, *GL2/OsGRF4*, *OsGRF3*	ABE	Method development	Agrobacterium-mediated	Unspecified	[58]
Rice	*OsDEP1*, *OsCDC48*, *OsPDS*	CBE	Proof of concept	PEG-mediated, Agrobacterium-mediated	Protoplasts, Calli	[59]
Rice	*EPSPS*, *ALS*, *DL*	CBE	Proof of concept	Agrobacterium-mediated	Calli	[60]
Rice	*GL1-1*, *NAL1*	CBE	Proof of concept	Agrobacterium-mediated	Calli	[48]
Rice	*sgOs-siteG1*, *sgOs-site2*, *sgOs-site3*, *sgOssite4*	ABE	Method development	Agrobacterium-mediated	Calli	[61]
Rice	*OsMPK9*, *OsMPK17*, *OsCPK5*, *OsMPK15*, *OsMPK16*, *OsCPK6*, *OsCPK7*, *OsCPK8*	hAID-CBE3	Proof of concept	Agrobacterium-mediated	Unspecified	[62]
Rice	*OsACC*	CBE ABE	Herbicide resistance	Agrobacterium-mediated,	Calli	[63]
Rice	*OsALS1*, *OsALS2*, *OsALS3*	CBE ABE	Method development, herbicide resistance	Agrobacterium-mediated, Particle bombardment	Calli	[64]

Rice	*OsMPK2*, *OsMPK5*, *OsMPK5*, *OsALS*, *OsNRT1.1B*	CBE	Proof of concept	Agrobacterium-mediated	Calli	[65]
Rice	*OsWaxy*, *OsEUI1*, *OsCKX2*	CBE ABE	Method development	Unspecified	Unspecified	[66]
Rice	*OsCKX2*, *OsWaxy*, *OsEUI1*, *OsSPL4*, *OsSPL7*, *OsSPL14*, *LF1*, *OsIAA13*, *OsMADS57*, *OsGBSSI*	CBE4	Method development	Agrobacterium-mediated	Calli	[67]
Rice	*OsSPL14*, *SLR1*, *OsSERK2*, *Tms9-1*, *OsNRT1.1B*, *OsACC1*, *OsDEP1*, *SPX-MSF2*, *OsSPL14*, *OsSPL17*, *OsSPL14*, *OsSPL16*, *OsSPL18*, *OsSPL13*, *SNB*	ABE	Method optimization	Agrobacterium-mediated, PEG-mediated	Calli	[34]
Rice	*Pid3*, *WX*	ABE	Method optimization	Agrobacterium-mediated	Calli	[68]
Rice	*OsACC*	STEME	Method development, Herbicide resistance	Agrobacterium-mediated, PEG-mediated	Calli, protoplast	[46]
Rice	*OsALS*, *OsCGRS55*	CGBE	Proof of concept	Agrobacterium-mediated, PEG-mediated	Calli, protoplast	[24]
Rice	*OsCOI2*, *OsBSR*, *OsMPK13*, *OsGS1*, *OsGSK4*	ABE8e	Herbicide resistance	Agrobacterium-mediated transformation	Calli	[69]
Rice	*OsWaxy*, *OsEUI1*	CBE2/CBE3/CBE4	Reduced amylose content	Agrobacterium-mediated	Calli	[70]
Rice	*WX*, *OsCPK6*, *OsMPK9*, *OsALS1*, *OsTubA2*, *OsGS1*, *OsACC*	ABE	Method development	Agrobacterium-mediated	Calli	[71]

(continued)

Table 2.1 (continued)

Plant species	Targeted gene(s)	Type of base editor	Purpose	Delivery technique	Tissue	Reference
Rice	*OsEPSPS*, *OsALS*, *OsWaxy*	ABE8e	Herbicide resistance	Agrobacterium-mediated	Calli	[72]
Rice	*OspsaA*	DdCBE	High photosynthetic efficiency	Agrobacterium-mediated	Calli	[73]
Rice	*OsALS*, *OsBADH2*, *OsLAZY1*, *OsPDS*	pDuBE1	Method development	Agrobacterium-mediated	Calli	[74]
Rice	*OsSPL14*, *OsIAA13*, *OsSPL7*, *OsLF1*, *OsGBSSI*, *OsCKS2*, *OsEUI1*, *OsTS*	ABE8e	Method development	Agrobacterium-mediated	Calli	[75]
Rice	*OsIPA1*, *OsbZIP5*, *OsSLR1*, and *OsALS1*	CGBE	Method development	PEG-mediated	Protoplasts	[39]
Rice	*OsSUS6*, *OsSPS1*, *Os07g0134700*, *OsMSP1*, *Chalk5*, *OsCKX2*	(CGBEs), A-to-Y (T/C)	Method development	Agrobacterium-mediated	Calli	[40]
Rice	*OsAAT*, *OsALS2*, *OsCKX2*, and *OsSPL14*	CGBE	Proof of concept	PEG-mediated	Protoplast	[41]
Rice	*OsPDS*, *OsACC1*	CBE	Method development	Agrobacterium-mediated	Calli	[76]
Rice	*OsGS1*	ABE CBE	Herbicide resistance	Agrobacterium-mediated transformation	Immature embryos	[77]
Soybean	*GmFT2a*, *GmFT4*	CBE	Method development	Agrobacterium-mediated	Unspecified	[19]
Tomato	*DELLA*, *ETR1*	CBE	Proof of concept	Agrobacterium-mediated	Cotyledon explants	[55]
Tomato	*SLALS1 71%*	CBE	Herbicide resistance	Agrobacterium-mediated	Cotyledon segments	[15]
Tomato	*SIALS1*	CBE	Method development	Agrobacterium-mediated	Cotyledon segments	[16]

Plant species	Targeted gene(s)	Type of base editor	Purpose	Delivery technique	Tissue	Reference
Tomato	*AGO7*	CGBE	Proof of concept	PEG-mediated	Protoplasts	[24]
Watermelon	*Als* *23%*	CBE	Herbicide resistance	Agrobacterium-mediated	Cotyledons	[18]
Wheat	*TaLOX2*	CBE	Method optimization	Particle bombardment	Immature embryos	[11]
Wheat	*TaDEP1*, *TaGW2*	ABE	Method optimization	Particle bombardment	Immature embryos	[31]
Wheat	*TaALS*, *TaMTL*, *TaLOX2*, *TaDEP1*, *TaHPPD*, *TaVRN1*	CBE	Proof of principle	PEG-mediated	Immature embryos	[54]
Wheat	*TaALS-P174*	CBE	Herbicide tolerance	Particle bombardment	Immature embryos	[12]

Since CBEs and ABEs mainly generate base transitions, C-to-G base editors (CGBEs) were developed by modifying CBEs to generate a new tool suitable for C·G to-G·C transversion (Fig. 2.1d). Instead of the UGI inhibitor used in CBEs, CGBEs include Uracil-N-glycosylase (UNG), which locates U in the DNA and eliminates it [35], promoting the BER pathway and improving uracil glycosylation. CGBEs were first developed in human cells, including a UNG fused to nCas9 nickase (D10A) and a cytidine deaminase rAPOBEC1 or its engineered form rAPOBEC1 (R33A). Because of the promising editing results of CBEs and ABEs in plants, Sretenovic et al. [24] modified the CGBE method for plants which is proven to work in human cells [36–38]. They tested three versions of CGBEs in rice, tomato, and poplar and obtained different editing efficiencies from CGBEs in different plants. Tian et al. [39] developed CGBEs for rice by optimizing the codon of UNG and by using three highly active deaminases, hAID, hA3A, and Anc689. They tested optimized CGBEs in five different rice genes and obtained successful C-to-G conversions with an average frequency of 21.3% [39]. Another CGBE-mediated genome editing in rice was reported by Zeng et al. using the highly active cytidine deaminase evoFENRY and the PAM-relaxed Cas9-nickase variant Cas9n-NG with rice and human UNG [40]. Although their CGBEs achieved C-to-G conversions up to 27.3% in rice, they did not achieve significant C-to-G performance, contrary to previous studies on mammalian cells [40]. Recently, monocot plant-compatible CGBEs were developed in rice protoplasts, and low editing efficiency was obtained [41]. Similar to previous CGBE studies in plants, this study also emphasizes that further improvements are necessary to enhance the editing efficiency of plant CGBEs for versatile applications [41].

As a result of several attempts to increase base editing efficiency, dual base editing technology which combines both ABEs and CBEs into a single base editor, was developed. Dual base editors convert C-G to T-A and A-T to G-C mutations simultaneously in the target site using a single gRNA [42]. Various dual-base editing platforms have been developed for mammalian cells (SPACE, A&C-BEmax, Target-ACEmax, and ACBE) [42–45] and plants (STEMEs) [46]. STEMEs (saturated targeted endogenous mutagenesis editors), a fusion of nCas9 with both deaminases, APOBEC3A/ecTadA, was first tested in the OsACC gene to obtain herbicide-resistant rice mutants [46].

Applications of base editors in plants are presented in Table 2.1 by highlighting the plant species, target genes, type of BE, purpose of the targeted mutation, and delivery technique of the reagents.

2 Prime Editing

In 2019, David Liu's group introduced prime editing, a 'search and replace' tool that can perform any intended changes, including all 12 possible base-to-base conversions, insertions, and deletions without requiring DSBs or donor DNA templates [78] (Fig. 2.2a). Prime editor is a versatile, precise genome editing tool that is

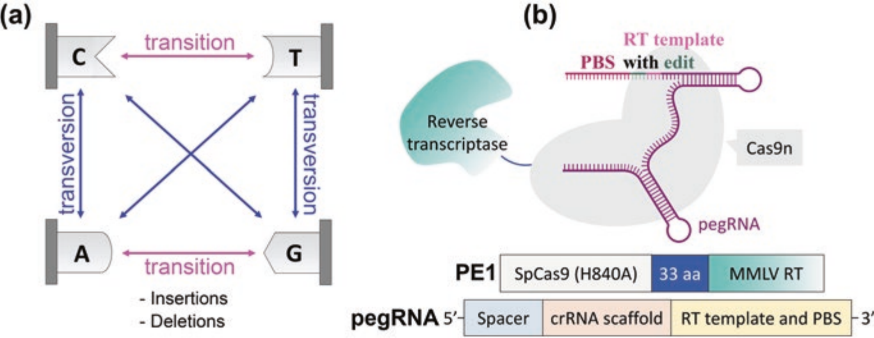


Fig. 2.2 Schematic diagrams of prime editors, (**a**) Graphical overview of the edits including insertions, deletions, transition, and transversion base-pair substitutions in prime editing, (**b**) Prime editors consist of a Cas9 nickase domain fused to a reverse transcriptase domain

composed of a Cas9 nickase (nCas9; H840A mutation) fused to an engineered reverse transcriptase (RT) and a prime editing guide RNA (pegRNA) containing a primer binding site (PBS) and an RT template (Fig. 2.2b). When pegRNA is delivered into a cell, Cas9 nickase (nCas9; H840A mutation) recognizes and breaks the non-complementary strand of the DNA three bases upstream of the PAM site. The PBS hybridizes with the bases upstream of the nCas9 (H840A)-generated nick and RT template encodes desired edits and directs reverse transcription. Then the new DNA containing the desired edit is integrated, and the unedited strand is repaired to match the edited strand by a cellular DNA repair system [78].

Anzalone et al. [78] presented three versions of prime editing system in their first article on prime editing. The first prime editor (PE1) incorporates wild-type reverse transcriptase from commercial Moloney murine leukemia virus (M-MLV) with Cas9 (H840A) nickase and a pegRNA. Various RT mutations have been investigated to increase the efficiency of prime editing by altering thermostability, processivity, DNA–RNA substrate affinity, and RNaseH activity. PE1 efficiency was increased by harboring engineered M-MLV-RT pentamutant (M-MLV RT (D200N/ L603W/T330P/T306K/ W313F)) after which it is called “prime editor 2” (PE2). PE2 enhanced the editing efficiency by 1.6- to 5.1-fold to harbor point mutations on average. In addition, it showed higher editing efficiency in indels and reported that it was compatible with shorter PBS sequences. To further increase the efficiency, an optimized prime editor called PE3 used an additional sgRNA to direct Cas9 (H840A) nickase to produce a nick in the non-edited DNA strand and increased approximately three times the editing efficiency of PE2. Since a high level of indels could be formed depending on the location of the additional sgRNA, the authors resolved this issue by designing the additional sgRNA to target the edited strand but not the original one. This variant of the PE3 system is called PE3b, as it achieved a 13-fold reduction in the average number of indels in human cell lines compared to PE3 while maintaining editing efficiency [78].

2.1 Improving the PE Efficiency

Since it was first published, the possibilities of the use of PE have advanced and broadened. Prime editing mechanism is a complex process influenced by multiple factors, including prime editor structure, pegRNA design, and cellular determinants [79]. Different groups have been developing new strategies to increase the editing efficiency of prime editing in animal and human cells [80]. For example, it is known that the 3′ extension of a pegRNA is critical for priming reverse transcription and templating the desired edit. Nelson et al. [81] discovered that exonucleases could hinder prime editing efficiency by degrading the 3′ extension of pegRNA [81]. pegRNA optimization by incorporating structured RNA motifs to the 3′ terminus of pegRNAs enhanced stability by preventing degradation of the 3′ extension. This strategy is called engineered pegRNAs (epegRNAs) that improved prime editing efficiency three to fourfold in human cells without increasing off-target editing activity [81].

Another strategy was manipulating the DNA repair pathway to increase PE efficiency and reduce indels [82]. The temporary inhibition of DNA mismatch repair (MMR) by MLH1dn significantly improves the effectiveness of PE and reduces the occurrence of indels in various cell types [82]. Transient co-expression of MLH1dn (a dominant-negative variant of the MMR protein MLH1) with PE2 and PE3 yielded PE4 (PE2 + MLH1dn) and PE5 (PE3 + MLH1dn), respectively [82]. PE4 and PE5 versions enhanced the editing efficiency by sevenfold over PE2 and twofold over PE3, respectively [79, 82]. With further efforts to enhance prime editing efficiency, an improved prime editor architecture, "PEmax," was obtained by optimizing RT codon usage, Cas9 mutations, linker length /composition, and nuclear localization signals (NLS) tags based on the PE2 protein. The combination of PEmax with PE4/PE5 systems (known as PE4max and PE5max, respectively) along with epegRNAs significantly improved editing efficiency [80, 82, 83]. Additional optimization strategies were used in various cell types, including prime editing protein engineering, pegRNA structure, stability improvements, repair mechanism suppression, and two-pegRNA implementation [80].

Most pioneering studies in prime editing have been implemented in animal and human cells, and applications of prime editing in plants are mostly proof of concept and optimization studies (Table 2.2). The first report on prime editing in plants was published by Lin et al. [84]. They obtained a variety of edits, including insertions up to 15 bp in wheat and rice protoplast, by optimizing codon, promoter, and editing conditions. This study was followed by studies demonstrating the applicability of prime editors in various plant species, including tomato [85], potato [86], maize [87], *Arabidopsis* [88], *N. benthamiana* [88], and rice [89, 90]. However, these studies showed that the application of prime editing is limited by the low efficiency and optimization studies required to reach its full potential. Therefore, various approaches have been rapidly developed and applied in plants to overcome the limitations of prime editors [91, 92].

Table 2.2 Selected applications of prime editors in plants

Plant species	Targeted gene(s)	Type of prime editor	Purpose	Delivery technique	Tissue	Reference
Chickpea	*GFP*	Dual-peg	Method optimization	PEG-mediated	Protoplasts	[99]
Cowpea	*GFP*	Dual-peg	Method optimization	PEG-mediated	Protoplasts	[99]
Maize	*ZmALS1* and *ZmALS2*	PE2, PE3, PE3b	Method optimization	Agrobacterium-mediated	Unspecified	[87]
Peanut	*GFP*	Dual-peg	Method optimization	PEG-mediated	Protoplasts	[99]
Physcomitrium patens	*PpAPT*	PPE2 and PPE3	Proof of concept, herbicide resistance	Agrobacterium-mediated	Protoplasts	[86]
Potato	*StALS*	PE2 and PE3	Proof of concept, herbicide resistance	Agrobacterium-mediated	Unspecified	[86]
Rice	*OsALS*, *OsIPA1*, *OsTB1*	PE2	Herbicide resistance, high yield	Agrobacterium-mediated	Calli	[89]
Rice	*GFP*	Sp-PE2	Proof of concept	Agrobacterium-mediated	Calli	[90]
Rice	*OsCDC48*, *OsALS*, *OsDEP1*, *OsEPSPS*, *OsLDMAR*, *OsGAPDH*, *OsAAT*, *TaUbi10*, *TaGW2*, *TaGASR7*, *TaLOX2*, *TaMLO*, *TaDME1*	pPE2, pPE3, pPE3b	Proof of concept, method optimization	Agrobacterium-mediated	Calli	[84]
Rice	*OsPDS*, *OsACC1*, *OsWx*	pPE2, pPE3, pPE3b	Method development	Agrobacterium-mediated	Calli	[100]

(continued)

Table 2.2 (continued)

Plant species	Targeted gene(s)	Type of prime editor	Purpose	Delivery technique	Tissue	Reference
Rice	*OsALS*, *OsACC*, *OsDEP1*	PE-P1, PE-P2	Proof of concept	Agrobacterium-mediated	Calli	[101]
Rice	*hptll*, *OsEPSPS*	PE3	Proof of concept	Agrobacterium-mediated	Calli	[102]
Rice	*ALS*, *APO1*, *SLR1*, *OsSPL14*, *APO2*	Sp-PE2, Sp-PE3, Sa-PE3	Proof of concept	Agrobacterium-mediated	Calli	[90]
Rice	*GFP*	Dual-peg	Method optimization	PEG-mediated	Protoplasts	[99]
Rice	*OsALS*, *OsRDD1-miR*, *OsAAT*, *OsACC*, *OsCDC48*, *OsEPSPS*, *OsGAPDH*, *OsIPA1*, *OsLDMAR*, *OsNRT1.1B*, *OsODEV*, *OsPDS*, *OsROC5*	ePPE	Method development, Herbicide resistance	PEG-mediated, Agrobacterium-mediated	Protoplasts, Calli	[95]
Rice	*OsALS*, *OsACC*, *OsEPSPS*	PE4, PE5, ePE3max ePE5max	Herbicide resistance	PEG-mediated, Agrobacterium-mediated	Protoplasts, Calli	[103]
Rice	*OsPDS*, *OsACC*, *OsALS*, *OsCDC48*	pPE2max enpPE2	Method development, Herbicide resistance	Agrobacterium-mediated	Calli	[104]

Rice	*OsSPL14*, *mhptII*, *OsDHDPS*, *mhptII*, *OsNR2*, *mhptII*	PE3-HS	Proof of concept	Bombardment	Calli	[105]
	OsSPL14 + *OsALS*, *OsDHDPS* + *OsALS*, *OsNR2* + *OsALS*, *OsSPL14* + *OsALS* + *OsDHDPS*	PE3-AS				
	OsSPL14 + *OsALS* + *mhptII*, *OsDHDPS* + *OsALS* + *mhptII*, *OsNR2* + *OsALS* + *mhptII*, *OsSPL14* + *OsALS* + *OsDHDPS* + *mhptII*, *OsSPL14* + *OsALS* + *OsEPSPS* + *mhptII*	PE3-DS				
Rice	*OsROC*, *OsALS*, *OsCDC48*, *OsDEP1*	PPE3	Method optimization	Agrobacterium-mediated	Calli	[96]
Rice	*OsALS*, *OsOSD1*, *OsBADH2*, *OsPDS*, *OsSPP*, *OsAAP6*	PE2	Method development	Agrobacterium-mediated	Calli	[106]
Tomato	*SlALS2*, *SlPDS1*, *SlGAI*	PE1, PE2, and PE3	Method optimization	Agrobacterium-mediated	Unspecified	[85]
Wheat	*TaDME-T1*, *TaDME-T2*, *TaGW2*, *TaLOX2*, *TaNAC2*, *TaSBEIIa*	ePPE	Method development	PEG-mediated	Protoplasts	[95]

Dual-pegRNA strategy employs two pegRNAs in trans to simultaneously encode the same edits, increasing PE efficiency by expanding the size and type of genomic mutations in rice [93]. Lin et al. [93] also optimized the melting temperature (Tm) of the PBS combined with a dual-pegRNA strategy and increased the editing efficiency from 2.9-fold to 17.4-fold in rice protoplasts [93]. Xu et al. [94] reported that changing the C-terminal reverse transcriptase Cas9 nickase fusion with N- terminal fusion improved the editing efficiency at some target sites in rice and maize [94]. In addition to this modification, codon optimization of M-MLV RT by introducing multiple-nucleotide substitutions enhanced editing frequency up to 24.3% and 6.2% in rice and maize, respectively [94]. Zong et al. [95] indicated that engineering the M-MLV reverse transcriptase by deletion of the RT RNase H domain and the addition of a virus-derived protein which is called engineered plant prime editor (ePPE) improved prime editing efficiency by ~1.8–3.4-fold in rice and wheat [95]. Zou et al. [96] optimized prime editing by combining PE3 system and epegRNAs, which include a structured RNA motif (evopreQ1 or mpknot) with an 8 bp linker at the 3′ terminus of the pegRNA [96]. Their PPE3-evopreQ1 and PPE3-mpknot systems improved the prime editing efficiencies in rice protoplast, and PPE3-evopreQ1system showed a more significant increase compared to PPE3-mpknot system [96]. This study also increased PE efficiency by at least 2.8 times by applying an appropriate high-temperature treatment. Although each modification enhances PE efficiency, combining these approaches could result in even more significant efficiency improvements [81].

Different prime editing systems in plants, targeted genes, the purpose of the study, and plant delivery technique are summarized in Table 2.2 and also reviewed in [97, 98].

3 Future Prospects and Limitations

3.1 Base Editors

The precise and efficient conversion of single bases at targeted genomic sites is made possible by the CRISPR/Cas base editing technology, which has found wide applications across various plants, as shown in Table 2.1. Although this technology holds great promise for plant trait development, it needs to be improved in order to overcome several limitations, including off-target activity, editing window length, PAM site compatibility, bystander effect, sequence preferences, and the limited capability in editing only four types of base changes [4, 7, 25]. In recent years, substantial efforts have been dedicated to reducing these limitations and enhancing the specificity of base editors in mammalian cells and plants [2, 7].

Comprehensive whole-genome sequencing studies have revealed that base editors can induce gRNA-dependent and gRNA-independent off-target mutations throughout the entire genome [98, 107]. Several effective strategies have been

reported to reduce gRNA-dependent off-target effects, such as employing alternative Cas9 variants, enlarging the gRNA sequence, and delivering base editors through RNP (ribonucleoprotein) complexes [98, 108, 109]. Moreover, gRNA-independent off-target mutations were observed in mice and rice using cytosine base editors (CBEs) but not adenine base editors (ABEs). This occurrence is likely attributed to the excessive expression of the deaminase, resulting in random mutations throughout the genome, particularly in gene-enriched regions. Effective strategies to mitigate gRNA-independent off-target effects involve employing alternative deaminases instead of rAPOBEC1 or modifying the deaminase domain through engineering [110].

Jin et al. [111] reported unexpected and unpredictable genome-wide off-target mutations induced by CBEs BE3 and high-fidelity BE3 (HF1-BE3) in rice [111]. The study emphasized the need to optimize the cytidine deaminase domain and/or UGI components to minimize the occurrence of off-target mutations. Additionally, improved variants of CBEs, such as YEE-BE3, were suggested as a potential approach for reducing off-target edits in plants [111]. In another study, upon analyzing off-targets of ABE, considering the predicted top off-target sites with 1- or 2-nt mismatches, it was found that the TadA* (modified version of TadA) deaminases displayed negligible off-target activity (0–4.65% frequency). Furthermore, they suggested that TadA variants exhibit minimal off-target effects dependent on sgRNA [71]. Target selection can be restricted in base editing applications because of the limitations of PAM site compatibility and editing window length [98]. In order to surpass these limitations, various Cas orthologs and engineered variants with altered PAM specificities have been employed to expand the scope of base editors [62, 112]. However, although these variants expand the scope of base editors, they can decrease editing efficiency and enhance the target dependence [98]. In addition to these limitations, large genomes of plants with duplicated regions and genes could pose additional obstacles in selecting target genes and plant transformation steps in base editing [98].

3.2 Prime Editors

While prime editing represents a significant advancement in plant genome editing, the technology is still in its early stages, necessitating further research and studies to unlock its capabilities and potential. One significant challenge with prime editing is its relatively low efficiency [84, 97]. The editing efficiency frequencies observed in plants were considerably lower than those reported in mammalian cells, and numerous sites exhibited a lack of editing, particularly in dicot species [113]. It is also reported that editing efficiencies for insertions were lower than for deletions and substitutions [114]. Although it is possible to obtain targeted mutation in stable transgenic lines by prime editing, as shown in rice and tomato, the occurrence of homozygous and biallelic edits is infrequent, highlighting the inefficiency of prime editing in plants [84, 90, 102, 113]. Researchers have devised various strategies to

overcome these limitations, including using engineered prime-editing proteins, enhancements in prime-editing guide RNA design, manipulation of the mismatch repair pathway, and optimization of delivery strategies [92, 97]. These approaches aim to enhance the effectiveness and efficiency of prime editing for more precise and robust genome modifications. Ensuring high efficiency in prime editing relies heavily on the careful design of the pegRNA [78, 93]. Selecting an appropriate combination of the primer binding site (PBS) and reverse transcriptase (RT) template is crucial for optimizing prime editing efficiency. Typically, efficient PBSs range from 8 to 15 nucleotides, while RT templates are between 10 and 20 nucleotides long [78, 93]. Although the specific matrix of optimal PBS and RT template combinations is determined through empirical observations, several factors contribute to selecting the ideal pegRNA design, including GC content, primary sequence motifs, and secondary structures within the pegRNA 3′ extensions [84]. The design of pegRNA is considerably more complex than sgRNA design for other CRISPR-based editing techniques, as it requires adherence to multiple fundamental rules and the various combinations of PBS and RT templates [92, 93]. As a result, its manual design is time-consuming, error-prone, and challenging in high-throughput applications [92]. Several design tools have been developed to overcome this limitation [93, 115, 116].

In conclusion, the development of base editing and prime editing technologies has revolutionized the field of plant genome editing, providing efficient and precise tools for targeted genetic modifications. In addition, the ability to introduce single nucleotide changes without the need for double-stranded DNA breaks has opened new possibilities for crop improvement, disease resistance, and trait engineering. However, despite the significant progress made in this field, challenges still need to be addressed, such as improving editing efficiency and specificity, optimizing delivery methods, and addressing off-target effects. Nevertheless, with continued research and development, base editing, and prime editing hold great promise for advancing the field of plant biotechnology and crop improvement.

References

1. Hess, G.T., et al.: Methods and applications of CRISPR-mediated base editing in eukaryotic genomes. Mol. Cell. **68**(1), 26–43 (2017)
2. Molla, K.A., Yang, Y.: CRISPR/Cas-mediated base editing: technical considerations and practical applications. Trends Biotechnol. **37**(10), 1121–1142 (2019)
3. Molla, K.A., et al.: Precise plant genome editing using base editors and prime editors. Nat. Plants. **7**(9), 1166–1187 (2021)
4. Rees, H.A., Liu, D.R.: Base editing: precision chemistry on the genome and transcriptome of living cells. Nat. Rev. Genet. **19**(12), 770–788 (2018)
5. Komor, A.C., et al.: Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. **533**, 420–424 (2016)
6. Jiang, F., et al.: Structures of a CRISPR-Cas9 R-loop complex primed for DNA cleavage. Science. **351**(6275), 867–871 (2016)

7. Anzalone, A.V., Koblan, L.W., Liu, D.R.: Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors. Nat. Biotechnol. **38**(7), 824–844 (2020)
8. Koblan, L.W., et al.: Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction. Nat. Biotechnol. **36**(9), 843–846 (2018)
9. Li, J., et al.: Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system. Mol. Plant. **10**(3), 526–529 (2017)
10. Lu, Y., Zhu, J.-K.: Precise editing of a target base in the rice genome using a modified CRISPR/Cas9 system. Mol. Plant. **10**(3), 523–525 (2017)
11. Zong, Y., et al.: Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. Nat. Biotechnol. **35**(5), 438–440 (2017)
12. Zhang, R., et al.: Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing. Nat. Plants. **5**(5), 480–485 (2019)
13. Chen, Y., et al.: CRISPR/Cas9-mediated base-editing system efficiently generates gain-of-function mutations in Arabidopsis. Sci. China Life Sci. **60**(5), 520–523 (2017)
14. Xue, C., et al.: Manipulating mRNA splicing by base editing in plants. Sci. China Life Sci. **61**, 1293–1300 (2018)
15. Veillet, F., et al.: Transgene-free genome editing in tomato and potato plants using agrobacterium-mediated delivery of a CRISPR/Cas9 cytidine base editor. Int. J. Mol. Sci. **20**(2), 402 (2019)
16. Veillet, F., et al.: Expanding the CRISPR toolbox in P. patens using SpCas9-NG variant and application for gene and base editing in solanaceae crops. Int. J. Mol. Sci. **21**(3), 1024 (2020)
17. Qin, L., et al.: High-efficient and precise base editing of C•G to T•A in the allotetraploid cotton (Gossypium hirsutum) genome using a modified CRISPR/Cas9 system. Plant Biotechnol. J. **18**(1), 45–56 (2020)
18. Tian, S., et al.: Engineering herbicide-resistant watermelon variety through CRISPR/Cas9-mediated base-editing. Plant Cell Rep. **37**(9), 1353–1356 (2018)
19. Cai, Y., et al.: Target base editing in soybean using a modified CRISPR/Cas9 system. Plant Biotechnol. J. **18**(10), 1996 (2020)
20. Malabarba, J., et al.: New strategies to overcome present CRISPR/Cas9 limitations in apple and pear: efficient dechimerization and base editing. Int. J. Mol. Sci. **22**(1), 319 (2020)
21. Xing, S., et al.: Fine-tuning sugar content in strawberry. Genome Biol. **21**(1), 1–14 (2020)
22. Wu, J., et al.: Engineering herbicide-resistant oilseed rape by CRISPR/Cas9-mediated cytosine base-editing. Plant Biotechnol. J. **18**(9), 1857 (2020)
23. Guyon-Debast, A., et al.: A blueprint for gene function analysis through base editing in the model plant Physcomitrium (Physcomitrella) patens. New Phytol. **230**(3), 1258–1272 (2021)
24. Sretenovic, S., et al.: Exploring C-To-G base editing in rice, tomato, and poplar. Front. Genome Editing. **3**, 24 (2021)
25. Azameti, M.K., Dauda, W.P.: Base editing in plants: applications, challenges, and future prospects. Front. Plant Sci. **12**, 664997 (2021)
26. Jiang, Z., et al.: CRISPR base editing and prime editing: DSB and template-free editing systems for bacteria and plants. Synth. Syst. Biotechnol. **5**(4), 277–292 (2020)
27. Gaudelli, N.M., et al.: Programmable base editing of T to G·C in genomic DNA without DNA cleavage. Nature. **551**(7681), 464–471 (2017)
28. Rees, H.A., et al.: Analysis and minimization of cellular RNA editing by DNA adenine base editors. Sci. Adv. **5**(5), eaax5717 (2019)
29. Kim, H.S., et al.: Adenine base editors catalyze cytosine conversions in human cells. Nat. Biotechnol. **37**(10), 1145–1148 (2019)
30. Hua, K., Tao, X., Zhu, J.K.: Expanding the base editing scope in rice by using Cas9 variants. Plant Biotechnol. J. **17**(2), 499–504 (2019)
31. Li, C., et al.: Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. Genome Biol. **19**, 1–9 (2018)
32. Yan, F., et al.: Highly efficient A·T to G·C base editing by Cas9n-guided tRNA adenosine deaminase in rice. Mol. Plant. **11**(4), 631–634 (2018)

33. Kang, B.-C., et al.: Precision genome engineering through adenine base editing in plants. Nat. Plants. **4**(7), 427–431 (2018)
34. Hua, K., et al.: Simplified adenine base editors improve adenine base editing efficiency in rice. Plant Biotechnol. J. **18**(3), 770–778 (2020)
35. Cortizas, E.M., et al.: UNG protects B cells from AID-induced telomere loss. J. Exp. Med. **213**(11), 2459–2472 (2016)
36. Chen, L., et al.: Programmable C: G to G: C genome editing with CRISPR-Cas9-directed base excision repair proteins. Nat. Commun. **12**(1), 1384 (2021)
37. Kurt, I.C., et al.: CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. Nat. Biotechnol. **39**(1), 41–46 (2021)
38. Zhao, D., et al.: Glycosylase base editors enable C-to-A and C-to-G base changes. Nat. Biotechnol. **39**(1), 35–40 (2021)
39. Tian, Y., et al.: Efficient C-to-G editing in rice using an optimized base editor. Plant Biotechnol. J. **20**(7), 1238 (2022)
40. Zeng, D., et al.: Exploring C-to-G and A-to-Y Base editing in Rice by using new vector tools. Int. J. Mol. Sci. **23**(14), 7990 (2022)
41. Lee, J., et al.: Application of CRISPR-Based C-to-G Base editing in rice protoplasts. Appl. Biol. Chem. **66**(1), 1–5 (2023)
42. Zhang, X., et al.: Dual base editor catalyzes both cytosine and adenine base conversions in human cells. Nat. Biotechnol. **38**(7), 856–860 (2020)
43. Grünewald, J., et al.: A dual-deaminase CRISPR base editor enables concurrent adenine and cytosine editing. Nat. Biotechnol. **38**(7), 861–864 (2020)
44. Sakata, R.C., et al.: Base editors for simultaneous introduction of C-to-T and A-to-G mutations. Nat. Biotechnol. **38**(7), 865–869 (2020)
45. Xie, J., et al.: ACBE, a new base editor for simultaneous C-to-T and A-to-G substitutions in mammalian systems. BMC Biol. **18**(1), 1–14 (2020)
46. Li, C., et al.: Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors. Nat. Biotechnol. **38**(7), 875–882 (2020)
47. Bottero, E., et al.: Generation of a multi-herbicide-tolerant alfalfa by using base editing. Plant Cell Rep. **41**(2), 493–495 (2022)
48. Li, Z., et al.: Gene disruption through base editing-induced messenger RNA missplicing in plants. New Phytol. **222**(2), 1139–1148 (2019)
49. Bastet, A., et al.: Mimicking natural polymorphism in eIF 4E by CRISPR-Cas9 base editing is associated with resistance to potyviruses. Plant Biotechnol. J. **17**(9), 1736–1750 (2019)
50. Nakazato, I., et al.: Targeted base editing in the plastid genome of Arabidopsis thaliana. Nat. Plants. **7**(7), 906–913 (2021)
51. Huang, X., Wang, Y., Wang, N.: Base editors for citrus gene editing. Front. Genome Editing. **4**, 852867 (2022)
52. Wang, G., et al.: Development of an efficient and precise adenine base editor (ABE) with expanded target range in allotetraploid cotton (Gossypium hirsutum). BMC Biol. **20**(1), 45 (2022)
53. Luo, J., et al.: Cytosine base editors (CBEs) for inducing targeted DNA base editing in Nicotiana benthamiana. BMC Plant Biol. **23**(1), 1–10 (2023)
54. Zong, Y., et al.: Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. Nat. Biotechnol. **36**(10), 950–953 (2018)
55. Shimatani, Z., et al.: Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat. Biotechnol. **35**(5), 441–443 (2017)
56. Ren, B., et al.: A CRISPR/Cas9 toolkit for efficient targeted base editing to induce genetic variations in rice. Sci. China Life Sci. **60**(5), 516–519 (2017)
57. Ren, B., et al.: Improved base editor for efficiently inducing genetic variations in rice with CRISPR/Cas9-guided hyperactive hAID mutant. Mol. Plant. **11**(4), 623–626 (2018)
58. Hao, L., et al.: CRISPR/Cas9-mediated adenine base editing in rice genome. Rice Sci. **26**(2), 125–128 (2019)

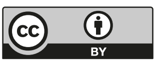
59. Zhong, Z., et al.: Improving plant genome editing with high-fidelity xCas9 and non-canonical PAM-targeting Cas9-NG. Mol. Plant. **12**(7), 1027–1036 (2019)
60. Endo, M., et al.: Genome editing in plants by engineered CRISPR–Cas9 recognizing NG PAM. Nat. Plants. **5**(1), 14–17 (2019)
61. Negishi, K., et al.: An adenine base editor with expanded targeting scope using SpCas9-NGv1 in rice. Plant Biotechnol. J. **17**(8), 1476 (2019)
62. Wang, M., et al.: Targeted base editing in rice with CRISPR/ScCas9 system. Plant Biotechnol. J. **18**(8), 1645–1647 (2020)
63. Liu, X., et al.: A CRISPR-Cas9-mediated domain-specific base-editing screen enables functional assessment of ACCase variants in rice. Plant Biotechnol. J. **18**(9), 1845 (2020)
64. Kuang, Y., et al.: Base-editing-mediated artificial evolution of OsALS1 in planta to develop novel herbicide-tolerant rice germplasms. Mol. Plant. **13**(4), 565–572 (2020)
65. Zhang, C., et al.: Expanding the base editing scope to GA and relaxed NG PAM sites by improved xCas9 system. Plant Biotechnol. J. **18**(4), 884 (2020)
66. Zeng, D., et al.: Engineered Cas9 variant tools expand targeting scope of genome and base editing in rice. Plant Biotechnol. J. **18**(6), 1348 (2020)
67. Zeng, D., et al.: PhieCBEs: plant high-efficiency Cytidine Base editors with expanded target range. Mol. Plant. **13**(12), 1666–1669 (2020)
68. Li, J., et al.: Optimizing plant adenine base editor systems by modifying the transgene selection system. Plant Biotechnol. J. **18**(7), 1495 (2020)
69. Xu, Z., et al.: SpRY greatly expands the genome editing scope in rice with highly flexible PAM recognition. Genome Biol. **22**(1), 6 (2021)
70. Liu, T., et al.: The ScCas9(++) variant expands the CRISPR toolbox for genome editing in plants. J. Integr. Plant Biol. **63**(9), 1611–1619 (2021)
71. Yan, D., et al.: High-efficiency and multiplex adenine base editing in plants using new TadA variants. Mol. Plant. **14**(5), 722–731 (2021)
72. Wei, C., et al.: Efficient generation of homozygous substitutions in rice in one generation utilizing an rABE8e base editor. J. Integr. Plant Biol. **63**(9), 1595–1599 (2021)
73. Li, R., et al.: High-efficiency plastome base editing in rice with TAL cytosine deaminase. Mol. Plant. **14**(9), 1412–1414 (2021)
74. Xu, R., et al.: Development of an efficient plant dual cytosine and adenine editor. J. Integr. Plant Biol. **63**(9), 1600–1605 (2021)
75. Tan, J., et al.: PhieABEs: a PAM-less/free high-efficiency adenine base editor toolbox with wide target scope in plants. Plant Biotechnol. J. **20**(5), 934–943 (2022)
76. Wang, H., et al.: Development of plant cytosine base editors with the Cas12a system. Crop. J. **11**(5), 1451–1457 (2023)
77. Ren, B., et al.: Three novel alleles of OsGS1 developed by base-editing-mediated artificial evolution confer glufosinate tolerance in rice. Crop. J. **11**(2), 661–665 (2023)
78. Anzalone, A.V., et al.: Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. **576**(7785), 149–157 (2019)
79. Doman, J.L., et al.: Designing and executing prime editing experiments in mammalian cells. Nat. Protoc. **17**, 2431–2468 (2022)
80. Huang, Z., Liu, G.: Current advancement in the application of prime editing. Front. Bioeng. Biotechnol. **11**, 1039315 (2023)
81. Nelson, J.W., et al.: Engineered pegRNAs improve prime editing efficiency. Nat. Biotechnol. **40**, 1–9 (2021)
82. Chen, P.J., et al.: Enhanced prime editing systems by manipulating cellular determinants of editing outcomes. Cell. **184**(22), 5635–5652 (2021). e29
83. Chen, P.J., Liu, D.R.: Prime editing for precise and highly versatile genome manipulation. Nat. Rev. Genet. **24**(3) 161–177 (2022)
84. Lin, Q., et al.: Prime genome editing in rice and wheat. Nat. Biotechnol. **38**(5), 582–585 (2020)
85. Lu, Y., et al.: Precise genome modification in tomato using an improved prime editing system. Plant Biotechnol. J. **19**(3), 415 (2021)

86. Perroud, P.F., et al.: Prime editing in the model plant Physcomitrium patens and its potential in the tetraploid potato. Plant Sci. **316**, 111162 (2022)
87. Jiang, Y.Y., et al.: Prime editing efficiently generates W542L and S621I double mutations in two ALS genes in maize. Genome Biol. **21**(1), 257 (2020)
88. Wang, L., et al.: Spelling changes and fluorescent tagging with prime editing vectors for plants. Front. Genome Editing. **3**, 617553 (2021)
89. Butt, H., et al.: Engineering herbicide resistance via prime editing in rice. Plant Biotechnol. J. **18**(12), 2370–2372 (2020)
90. Hua, K., et al.: Precision genome engineering in rice using prime editing system. Plant Biotechnol. J. **18**(11), 2167–2169 (2020)
91. Sretenovic, S., Qi, Y.: Plant prime editing goes prime. Nat. Plants. **8**(1), 20–22 (2022)
92. Hua, K., Han, P., Zhu, J.-K.: Improvement of base editors and prime editors advances precision genome engineering in plants. Plant Physiol. **188**(4), 1795–1810 (2022)
93. Lin, Q., et al.: High-efficiency prime editing with optimized, paired pegRNAs in plants. Nat. Biotechnol. **39**(8), 923–927 (2021)
94. Xu, W., et al.: A design optimized prime editor with expanded scope and capability in plants. Nat. Plants. **8**(1), 45–52 (2022)
95. Zong, Y., et al.: An engineered prime editor with enhanced editing efficiency in plants. Nat. Biotechnol. **40**(9), 1394–1402 (2022)
96. Zou, J., et al.: Improving the efficiency of prime editing with epegRNAs and high-temperature treatment in rice. Sci. China Life Sci. **65**(11), 2328–2331 (2022)
97. Hassan, M.M., et al., Prime editing technology and its prospects for future applications in plant biology research. BioDesign Res. **2020**, 1–14 (2020)
98. Li, Y., et al.: Applications and prospects of CRISPR/Cas9-Mediated Base editing in plant breeding. Curr. Issues Mol. Biol. **45**(2), 918–935 (2023)
99. Biswas, S., et al.: Optimization of prime editing in rice, peanut, chickpea, and cowpea protoplasts by restoration of GFP activity. Int. J. Mol. Sci. **23**(17), 9809 (2022)
100. Xu, R., et al.: Development of plant prime-editing systems for precise genome editing. Plant. Commun. **1**(3), 100043 (2020)
101. Xu, W., et al.: Versatile nucleotides substitution in plant using an improved prime editing system. Mol. Plant. **13**(5), 675–678 (2020)
102. Li, H., et al.: Precise modifications of both exogenous and endogenous genes in rice by prime editing. Mol. Plant. **13**(5), 671–674 (2020)
103. Jiang, Y., et al.: Optimized prime editing efficiently generates glyphosate-resistant rice plants carrying homozygous TAP-IVS mutation in EPSPS. Mol. Plant. **15**(11), 1646–1649 (2022)
104. Li, J., et al.: Development of a highly efficient prime editor 2 system in plants. Genome Biol. **23**(1), 161 (2022)
105. Li, H., et al.: Multiplex precision gene editing by a surrogate prime editor in rice. Mol. Plant. **15**(7), 1077–1080 (2022)
106. Liang, Z., et al.: Addition of the T5 exonuclease increases the prime editing efficiency in plants. J. Genet. Genomics. **50**, 582 (2023)
107. Slaymaker, I.M., et al.: Rationally engineered Cas9 nucleases with improved specificity. Science. **351**(6268), 84–88 (2016)
108. Bharat, S.S., et al.: Base editing in plants: current status and challenges. Crop. J. **8**(3), 384–395 (2020)
109. Rees, H.A., et al.: Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery. Nat. Commun. **8**(1), 15790 (2017)
110. Yu, Y., et al.: Cytosine base editors with minimized unguided DNA and RNA off-target events and high on-target activity. Nat. Commun. **11**(1), 2052 (2020)
111. Jin, S., et al.: Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. Science. **364**(6437), 292–295 (2019)
112. Ren, B., et al.: Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. Mol. Plant. **12**(7), 1015–1026 (2019)

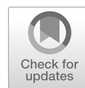
113. Lu, Y., et al.: Precise genome modification in tomato using an improved prime editing system. Plant Biotechnol. J. **19**(3), 415–417 (2020)
114. Wang, L., et al., Spelling changes and fluorescent tagging with prime editing vectors for plants. Front. Genome Editing **3**, 617553 (2021)
115. Siegner, S.M., et al.: PnB designer: a web application to design prime and base editor guide RNAs for animals and plants. BMC Bioinformatics. **22**, 1–12 (2021)
116. Hsu, J.Y., et al.: PrimeDesign software for rapid and simplified design of prime editing guide RNAs. Nat. Commun. **12**(1), 1034 (2021)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.



Chapter 3
Novel Delivery Methods for CRISPR-Based Plant Genome Editing



Barbara Doyle Prestwich, Teodoro Cardi, Allah Bakhsh, Alessandro Nicolia, and Kaushal Kumar Bhati

Abstract Using modern genome editing tools, scientists are increasingly able to engineer animals and plants for better traits and improved downstream outcomes that benefit humans. As part of the CRISPR-Cas system, guide RNA (gRNA) is used to identify the target sequence, while Cas is an endonuclease that performs the nucleotide cleavage. It is imperative that these two components are delivered to the nucleus of the cell in order to ensure an optimal editing process. As a consequence of differences in the cellular structure and biomolecular composition of the outer membrane, plants are not capable of being cloned genetically in the same manner as animal cells. A more optimized method and pipeline must be developed to improve the efficiency of transformations and genome editing for plants. In this book chapter, we highlight traditional and novel delivery methods used for optimal delivery of plant genome editing components. We discuss the potential and limitations of these methods in the light of recent literature and available experimental validations.

B. D. Prestwich
School of Biological Earth and Environmental Science, University College Cork, Cork, Ireland

T. Cardi
CNR-IBBR, Institute of Biosciences and Bioresources, Portici (NA), Italy

A. Bakhsh
Centre of Excellence in Molecular Biology, University of Punjab, Lahore, Pakistan

A. Nicolia
Council for Agricultural Research and Economics, Research centre for Vegetable and Ornamental Crops, Pontecagnano, Italy

K. K. Bhati (✉)
Louvain Institute of Biomolecular Science, University of Louvain, Louvain la Neuve, Belgium

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_3

1 Introduction

CRISPR/Cas systems are a vital component of genome engineering tools for animals and plants due to their ability to analyse gene function and accelerate trait development [1]. Efficient editing, convenient design, cost-effectiveness, and simplicity provide an edge to CRISPR-Cas over other editing tools. The system consists of guide RNA (gRNA), for target sequence recognition, and Cas, a CRISPR-associated protein endonuclease, which exhibits different versions and is sometimes modified and/or linked to other molecules to achieve specific targets. All relevant components must be delivered into the cell nucleus to achieve genome editing or other modifications.

Compared to animal systems, plants have distinctive characteristics which may limit the application of those methods developed for animal cells, thus requiring the development of specific plant-based methods. In this chapter, a number of traditional and novel delivery methods are discussed with reference to their potential and their limitations, with the ultimate aim of increasing efficiency and accuracy, and broadening the application of genotype-independent delivery systems in plants.

2 Biological Delivery Methods

2.1 Bacteria Based Methods

Successful editing outcomes in crops are reliant on the availability of efficient genotype-independent delivery systems which facilitate the introduction of individual genome editing components. Bacterial-mediated delivery of CRISPR/Cas components into plant cells is probably the most popular method in use, and one that incorporates a foreign gene into the plant genome using a binary vector system. The natural ability of *Agrobacterium tumefaciens* to engineer plant cells in the wild, and whose mechanism has been exploited by plant biotechnologists in the lab, means that this bacterial-based delivery system has been used efficiently for transforming both monocot and eudicot plants, notwithstanding the recalcitrant nature of some plant species to *Agrobacterium* infection. To address this recalcitrance with some plant species, Raman et al. [2] developed a strategy whereby a type III secretion system from *Pseudomonas* was expressed in *Agrobacterium* to allow for the efficient delivery of several effectors to suppress the plant response system and aid transformation. They found an increase of up to 400% in transformation efficiency using this approach. With a unique mode of action, which is reliant on an interplay of host and bacterial functions to effect transformation [3], *Agrobacterium* retains the title of most popular delivery system in use today. There are many excellent published reviews on what is known to date on the mechanisms of

Agrobacterium-mediated delivery to plant cells [4–7]. Here we will examine the role *Agrobacterium* plays as an efficient delivery system in the introduction of CRISPR/Cas components.

Cas9 was first discovered in 2005 and the first genome edited plants using CRISPR followed less than a decade later [8]. Since then, plants drawn from over 24 families (including those containing the major food crops) have been edited using CRISPR/Cas9 components [9] in *Agrobacterium* (both *tumefaciens* and *rhizogenes*) where it continues to hold a prominent position as an efficient delivery system including in transient assays where leaf cell agroinfiltration has been used [9]. *Agrobacterium*-mediated transformation (AMT) relies on the plant being inoculated with the bacteria containing the requisite reagents for gene editing. This means a binary plasmid containing a gene-editing cassette inserted between the border sequences on the T-DNA integrates into the plant genome leading to stable transformation as referenced in earlier reviews [6]. In general, AMT relies on tissue culture for the regeneration of a stable transformant. With this comes the possibility of producing unwelcome somaclonal variants. One way to circumvent this is to use AMT with a Floral Dip procedure. Here, transformed seeds can be obtained outside of the tissue culture process. However, this method has had limited success beyond *Arabidopsis thaliana* [10]. Another report of successful transformation outside of a tissue culture system is referenced in the same paper by Laforest and Nadakuduti [10]. Here an improved method of gene-editing efficiency is described whereby developmental regulators are co-delivered with CRISPR components and overexpressed leading to improved regeneration and transformation of soil-grown plants using *Agrobacterium* which had been injected into plants creating de novo meristems. An efficient transformation system using AMT was developed in wheat by Zhang et al. [11]. Earlier transformation studies in wheat were focused on the gene gun which has drawbacks in terms of copy number insertions and gene silencing effects. Generally, with AMT events, single copy insertions are the norm with low levels of gene silencing detected. In the study by Zhang et al. [11], the authors report efficient editing events in wheat. Generally, if DNA-free genome editing is required, alternative methods such as PEG must be employed. One of the drawbacks of Agrobacterium-mediated transformation is the fact that T-DNA is integrated into the genome. And this can be problematic for regulators. A report by Dalla Costa et al. [12] examined two strategies for producing T-DNA free CRISPRed fruit trees using *Agrobacterium*. The first strategy was based on the site specific recombinase Flp/FRT system. This system was reliant on the recognition of a 34-bp long sequence which was excised along with any undesired sequences from an optimised T-DNA vector system. The second strategy focused on the use of synthetic cleavage target sites (CTS) which were engineered adjacent to the left and right border sequences of the vector. These CTS were recognised by the Cas9 cleavage system and removed. Molina-Risco et al. [13] describe an improved method for the AMT and gene editing of tropical japonica rice where *Oryza sativa* is seen as a model monocot species.

2.2 Virus Vector-Based Methods

Plant viruses-based vectors are known for being reliable, efficient tools for transient protein expression and VIGS (Virus induced gene silencing) [14, 15]. More recently, plant viruses are being used as a delivery vehicle for genome editing tools, such as CRISPR/Cas9, to introduce specific mutations in the plant genome. These approaches are collectively known as VIGE (*Viruses induced genome editing*) [16–22]. VIGE allows DNA integration free methods to study gene function, modify plant traits, and develop novel crop features [23, 24]. The transiently introduced recombinant viral clones replicate within the plant, which then spread and infect the cells, delivering the genome editing machinery to the desired target site. This method has the advantage of being able to reach cells in different tissues and organs of the plant, leading to high efficiency genome editing. The overall choice of virus vector depends on the targeted host plant species and the size of the cargo insert that infectious viral replicon could sustainably carry while replicating in the plant cell. The replicon size threshold also limits the recombinant cargo size (nuclease and sgRNA cassette). The past few years have witnessed development of plants-based VIGE approaches [25, 26].

Several known plant viral vectors have been tested for their functional efficiency in delivering the CRISPR/Cas9 cassette for precise genome editing. These recent studies have optimised critical factors for success in the genome editing process such as choice of host species, infection method and replication of recombinant clones and copy number of sgRNAs. The viruses have better replication and transfection efficiency in their specific hosts, and this limits the utilisation of VIGE vectors in a broad host range. However, if used with the right host the VIGE approach expands beyond gene knockout and recent work proved its application in the precise gene replacement using geminivirus replicon of wheat dwarf virus (WDV) [27]. The WDV replicon could carry Cas9, sgRNA and a donor DNA and the editing efficiency was ~12 fold higher than non-viral methods. Up to that point, viral vectors from two RNA viruses viz. Tobacco etch virus (TEV) and potato virus X (PVX) had been used in tandem to express Cas12a and sgRNA respectively [19, 20]. All reported viral vectors have their own limitations and benefits [26]. Broadly, the use of DNA viruses-based vectors could lead to integration of a viral genome in plants, although the benefit is that they provide more cargo space due to the bigger replicon size. On the other hand, RNA viruses ensure integration-free genome editing but have smaller genomes hence the cargo size is limited. In the following sections, we have discussed some of the most successful VIGE viral vectors across model and crop plants.

2.2.1 Tobacco Rattle Virus (TRV)

Tobacco Rattle Virus (TRV) is a positive single-strand plant virus (Bipartite, RNA1 and RNA2) that infects the roots of tobacco plants and has a broad range of host plants. TRV could systematically infect and replicate in different plant cells and tissues. These attributes make TRV an ideal RNA virus candidate to deliver CRISPR/

Cas9 modules. Several recent studies have engineered this virus to express the guide RNA and Cas9 nuclease, which then target specific genes to demonstrate heritable editing. TRV has been successfully tested in *Arabidopsis* (targets: *AtAGL1, AtTT4;* [28]) and Tobacco (*NbPDS, NbAG* and *NbPCNA*; [17]). These methods provide a rapid and efficient way to introduce genetic modifications in plants and have been applied in various crops, including tobacco, to produce plants with improved traits. TRV has been exclusively used for the delivery of sgRNAs to plants expressing Cas9. The TRV vector system is successfully used in model hosts like *N. benthamiana* and Arabidopsis. The targeting and editing efficiency were positively impacted by 3′ end sgRNAs tagging with *Flowering Locus T (FT)* transcript [17].

2.2.2 Barley Stripe Mosaic Virus (BSMV)

Barley Stripe Mosaic Virus (BSMV) vector is a plant virus that has been used as a delivery system for genetic engineering in plants. BSMV is a positive-sense RNA virus with a tripartite genome (alpha, beta and gamma RNA) that infects barley and other cereal crops, causing a mosaic pattern on the leaves. Researchers have utilised the natural replication and movement properties of the virus to deliver transgenes into plant cells, making it an effective tool for plant genetic engineering. BSMV has been used to transiently express sgRNAs and the editing process has been tested successfully in *N. benthamiana*, wheat and maize plants [21, 29, 30]. For wheat, multiple genes (*TaGW2/7, TaUPL3 and TaQ*) were targeted by expressing sgRNA scaffold from the gamma chain of BSMV replicon [29]. These engineered viral particles were then used for infection of Cas9 expressing wheat plants. The authors evaluated that sgRNA fusion with mobile RNAs like *tRNA*, *AtFT*, and *Vern3* did not result in improved editing efficiency. On the contrary, another study on different wheat varieties confirmed the enhanced editing efficiency for BSMV sgRNA fusion with TaFT mRNA [21]. The authors argued that mobile mRNA fusion efficiency could be influenced by choice of wheat genotype and expression level of Cas9 nuclease.

2.2.3 Bean Yellow Dwarf Virus (BeYDV)

Bean yellow dwarf virus (BeYDV) belongs to the plant geminivirus family. It infects legume crops such as beans and peanuts. The virus can replicate and move within the plant along with its satellite replicons, making it an effective tool for delivering bigger transgenes cargos into plant cells. Bigger cargo carrying ability has prompted the use of BeYDV to deliver Cas9 together with sgRNA scaffold. By engineering the virus to contain a specific transgene, the virus can be used to infect the plant, delivering the transgene into the plant genome. These methods have been improvised now to introduce precise genome modifications. So far, BeYDV based CRISPR/Cas9 delivery has been validated in tobacco, potato, and tomato [31–33].

3 Physical and Chemical Methods

3.1 Physical Methods

Efficient delivery of the CRISPR/Cas9 complex into the nucleus of the targeted cell is an essential aspect to make it functional. The various forms of delivery approaches are adopted for instance messenger RNA, ribonucleoprotein (RNP-complex of sgRNA and Cas proteins preassembled *in vitro*), and plasmid DNA (pDNA) [34]. RNPs are usually constituted by a complex of protein (*e.g.*, Cas9, Cas12a) and an RNA, such as the sgRNA-scaffold RNA single strand. Delivery of the complex via RNP avoids drawbacks related to mRNA and pDNA. RNP delivery avoids intracellular transcription and translation and speeds up genome editing. Apart from this, it not only edits efficiently but also decreases the immune response, off-target effects, and insertional mutagenesis, making it a promising method of genome editing [35, 36]. Amongst the options to deliver CRISPR systems into plant cells, biolistic is one which is widely adopted [37, 38].

3.1.1 Gene Gun/Biolistic-Based Delivery

Gene gun based delivery or biolistics is a direct physical delivery method for microprojectiles carrying foreign DNA into plant cells or tissues at high velocity. DNA is coated onto gold or tungsten microprojectiles before gearing up for cell wall penetration of the target plant. Upon entry into the cell, dissociation from the particles takes place to either integrate stably or express transiently in the host genome [39].

To substantially breach the cell wall barrier, the gene gun method is widely adopted in order to deliver foreign DNA into the plant cell. Previously, RNA-guided Cas9 endonuclease was effectively used to modify the genome of various plants. Regardless of the success, particle bombardment of the plasmids containing the Cas9, gRNA, and marker genes often incorporated in the genome resulting in off-site cutting, gene disruption, and plant mosaicism [40]. Moreover, DNA molecules can also integrate at the double-strand break site hence decreasing the efficacy of gene insertion and gene editing. To address these undesirable effects, one suggested solution was to pre-integrate the Cas9 nuclease in plants to deliver the gRNA in the form of RNA molecules. Though successful the process was laborious and resource-demanding to develop and characterise the pre-integrated lines.

There are several documented reports which demonstrate the effective delivery of CRISPR reagents for genome editing using biolistics. They comprise the *in planta* genome editing of wheat via SAM and the production of novel variants of the maize ARGOS8 gene. Shi et al. [41] used gene editing approach to develop new allelic variants of ARGOS8 in maize. ARGOS8 encodes a negative regulator of ethylene responses, that is expressed at low levels in most inbred maize lines.

The group increased ARGOS8 expression by substituting or knocking in GOS promoter replacing native promoter via HDR to drive ARGOS8 expression. The field evaluation of hybrids exhibited increased yield under stress regimes [41]. In wheat, a group of researchers bombarded 210 plants and found 11 transgenic lines with the mutant TaGASR7 allele, and the mutation was transferred to the next generation of three transgenic lines with no observation of the presence of Cas9 and guide RNA [38, 42]. Recent research in wheat showed some promising results regarding transgene-free genome editing by means of transient expression [43].

RNP complexes were delivered by biolistics into maize embryo cells [44]. Cas9-gRNA RNPs were employed to target four different genes: MS26, MS45 (male fertility genes), acetolactate synthase (ALS2), and liguleless 1 (LIG). The results were comparable to DNA plasmids and ranged from 0.21% to 0.69% in all four immature embryo cells of maize [44]. Similar results have been reported for wheat using the same method where 0.18% was obtained for TaGW2-B1 and 0.21% for TaGW2-D1 [45]. The RNP complex is of similar or greater efficacy as compared to the plasmid-mediated editing method and gives transgene-free plants with reduced off-target frequency along with the ability to directly target the genomic region of interest with the concomitant degradation of the RNP complex within hours [38].

In order to decrease uneven bombardments, a double-barrelled gene gun along with software that counts the cells was applied as a technical improvement [10].

In case of RNP transfections, a single nucleotide mismatch between the sgRNA and the target site greatly reduces the off-targeting of homolog sequences. Besides, RNPs were also found to accommodate the hefty heritable inversion of 75.7 Mb in maize chromosome 2, when constructed with gRNAs flanking the junctions of the required inversion [38, 46]. This highly specific engineering of chromosomes is invaluable to crop breeding.

Apart from all the above-mentioned success stories of RNP transport using biolistic, delivery systems for CRISPR/Cas9 continue to be a considerable hindrance concerning its efficient use. For now, an all-purpose delivery system is still lacking. Each method possesses both merits and demerits. Moreover, options are there for the transport of small cargos as compared to massive protein-nucleic acid complexes. Although the gene gun method is capable of transforming a myriad of cell types and tissues in the absence of a binary vector, some obvious limitations include the laborious work of explant preparation and random incorporation of cargo at various sites in the genome. In the case of RNPs, they offer DNA-free gene editing that instantly alters the target site by sidestepping the transcription and translation machinery of the cell and degrades rapidly. Although there is a possibility that the bombarded explant and resultant phenotypes experience uncertain downstream effects [10]. Additionally, particle bombardment has a high consumable and equipment cost along with complicated integration patterns and gives relatively low throughput [39]. Moreover, the costlier method to deliver Cas9/gRNA RNP lacks the control of bombardment sites like the nucleus, plastid and mitochondria [47].

3.1.2 Electroporation

In electroporation, electrical pulses are used to generate transient pores in the plasma membrane in order to allow nucleic acids to enter the cell [48]. These microscopic pores allow not only the micro-, but also the macromolecules to move either inside or outside the cell. An electroporator device performs the electroporation activity that comprises three basic portions: a power supply for the pulse, electroporation cuvettes, and electrodes. According to the simple mechanism of electroporation, water molecules are the first to pierce the lipid bilayer and make unstable hydrophilic pores. Then reorientation of adjacent lipids with their polar heads towards these water molecules takes place due to the increase in transmembrane voltage that in turn lowers the energy required to form an aqueous aperture leading to the formation of metastable hydrophilic pores [48, 49]. Fortunately, these electropores can be recovered and resealed in the case of optimised electric pulses. It has been observed that the duration of the electric field and its intensity are of immense importance concerning the healing of the cells as unsuitable electric currents may lead to cell death [50].

Ensuring uniform electroporation can be difficult to achieve in plant tissue due to the presence of variable cell types and different three-dimensional organisations due to gap junctions. So, this non-homogeneous distribution makes some cells experience a greater degree of electroporation than others. To address this issue, optimization needs to be performed with respect to electrode position, size, shape, and different cells [51].

Some examples of application of electroporation include tobacco protoplasts with cucumber mosaic viral RNA using exponential and square wave electroporation pulses [52]. Similarly, electro-pulsed colt cherry protoplasts demonstrated efficient regeneration capability and a greater number of shoots per callus along with a prolific root system in comparison to non-electro-pulsed ones. Besides, protoplast-derived tissues of *Solanum dulcamara L.,* which is a woody medicinal plant, showed improved morphogenesis when compared to the untreated protoplasts. Apart from this, roots of regenerated shoots were established efficiently with prolific root systems [50]. Recently, electroporation has been used for gene editing with CRISPR technology [53].

3.1.3 Sonication and Pulsed-Laser

Sonication can involve acoustic and ultrasound and has been observed to enhance the growth processes in plants [54]. Moreover, acoustic microstreaming and cavitation are caused by ultrasound and can modify enzyme stability, cell growth, and ultrastructure. It has the potential to discharge DNA from the nucleus, modify the permeability of the cell membrane, cleave the extracellular polymers, enhance cell surface charges, and reduce the stability of the cell. Furthermore, the duration of sound irradiation, frequency, and intensity of sonication are some of the factors to

be considered [54]. During the process, the cell wall interacts first with the sound waves and probably experiences the variation followed by the cell membrane [55]. Ultrasound irradiates the bubbles and then leads to the crumpling of those cavities resulting in the release of a large amount of energy. This activity provokes various physical and chemical modifications like microstreaming in the plant cells and cell suspensions enhancing mass transport [54]. However, high-intensity irradiation can spoil the cell structure and inhibit plant growth [56]. The properties of sound waves that influence sonication include duration, intensity, pressure level, and frequency. Apart from these, the distance between the source and the target plant, sensitivity of the cells, genotype, and species are other factors affecting organogenesis and plant growth [57]. Moreover, ribonucleoproteins comprising the sgRNA and Cas9 protein were inserted into the extracellular vesicles by sonication for gene therapy [58]. Plant species modified by ultrasound *in vitro* include rice, aloe, carrot, commercial squash, gerbera, hazelnut, and red microalga [55, 56, 59]. To further strengthen our understanding of the technique, advanced knowledge about various processes sparked by sonication and more insight into sounds would generate greater yield and better growth [60].

Recently, RNPs have been delivered into tobacco cells via cavitation bubbles generated using a pulsed laser [61]. The resulting shockwave achieves the efficient transfection of walled cells in tissue explants by creating transient membrane pores. Genome-edited plants were produced with an efficiency of 35.2 and 16.5% for phytoene desaturase (PDS) and actin depolymerizing factor (ADF) genes, respectively.

3.1.4 Silicon Carbide Whiskers

Different types of silicon nanoparticles are used for different purposes. Some of them are relatively more difficult to synthesise and process than others and hence their applications are limited. Those that are relatively inexpensive and uncomplicated find more usage and have wider applications. Silicon Carbide (SiC) whiskers/fibres are among those nanoparticles belonging to a wide variety of silicon-based nanoparticles that fit this description. They are needle-like structures attached to a base. They appeared as a tool for the physical delivery of DNA in the 1990s where they were used to create pores in tobacco and maize cells through abrasion allowing the penetration of the exogenous DNA when mixed with the cell suspension [62]. The SiC fibres do not carry the genes themselves, but rather help the foreign DNA slide into the target cells. To date many crops have been transformed using this method. Crops, like wheat and rice that are recalcitrant to the Agrobacterium infection/transfection, have not only shown higher efficacy but more stable transgenic expression with SiC whiskers when compared to the particle bombardment method (cDNA coated microprojectiles) [63]. The transformation has been conducted on cell suspensions, embryos, embryogenic cells, and calli of different plant species such as cotton, maize, tobacco, onion, and rice [64–67].

Several types of SiC whiskers have been used for research on different plant species. These types differ based on their diameter and length. To achieve optimum results through this method some precautions like the pretreatment of the explant with osmotic agents and proper mixing of cells with SiC whiskers etc., have been adopted [68].

Some of the characteristics of SiC whiskers that make them desirable for DNA transfer into plant cells include compatibility with almost all plant species, the ability to help transform a wide range of cells, enhanced regeneration rate of transformants, ease of setup, and quick and inexpensive. The indirect involvement of SiC whiskers in DNA delivery can also be attributed to their positive/desired character because the amount of DNA available for transformation can be controlled. On the contrary, there are some limitations to this method that include their moderate transformation efficiency, the need for tissue culture and regeneration, and the requirement of a sophisticated protocol for successful transformation.

So far, most of the effort has been directed towards showcasing the effectiveness of this approach through the utilisation of reporter genes like GUS and GFP. Although, SiC-mediated plant transformation has been frequently reported, no studies have been conducted to deliver CRISPR/Cas for genome editing experiments in plants using this technology [69]. However, SiC whiskers have been used to edit the genome of other organisms such as algae and viruses through CRISPR/Cas [70, 71].

3.2 Chemical Methods

3.2.1 PEG-Mediated Delivery

Polyethylene glycol (PEG) is a polyether composed of repeated ethylene glycol units [-$(CH_2CH_2O)_n$]. PEG is available with different structures (*e.g.*, branched, star) having different molecular weights (MW) and showing different aggregation states: PEGs below MW 700 are liquid, PEGs comprised between MW 1000 and MW 2000 are soft solids, PEG above MW 2000 are hard crystalline solids. Polyethylene oxide (PEO) is a synonym for PEG, however macromolecules with MW below 20,000 are usually referred as PEG while those with MW above 20,000 are called PEO. PEG is biocompatible, highly soluble in water as well as in organic/inorganic solvents therefore being extremely important in solubilization and permeation [72]. In plant science, PEG properties were initially exploited in cell fusion experiments to obtain somatic hybrids (reviewed in [73]), but PEG is also capable of precipitating DNA molecules and stimulating their efficient cellular uptake by endocytosis [74]. Suitable PEG (usually MW 6000) and divalent cations (Ca^{2+}) concentrations were initially identified to achieve genetic transformation [75, 76] with further improvements in efficiencies by Shillito and coauthors [77]. Besides genetic transformation of plant protoplasts, with stable integration in genomic DNA, it became evident that PEG-mediated transfection allowed episomal transient expression of

the introduced DNA [78]. These fundamental experiments paved the way for thousands of studies that made large use of PEG-mediated transformation/transfection of isolated plant cells by different types of reagents (i.e., DNA, RNA, protein), with the establishment of consolidated platforms such as the Transient Expression in Arabidopsis Mesophyll Protoplast (TEAMP) system [79]. Typically, the concentration of PEG ranges from 12.5% to 20% (final concentration). The interest in using PEG for transfecting plant protoplasts, revamped in recent years because its application in the site directed mutagenesis protocols of various model species and crops (reviewed in [73, 80]).

Indeed, there are several examples of successful PEG-mediated delivery of RNPs in protoplasts of important crops such as potato, tomato, rice, and others (reviewed in [80]). The use of plant protoplasts requires an established regeneration protocol to obtain mutated adult plants, that additionally may show genetic instability and undesired somaclonal variation [36]. To bypass these issues, in pioneering work, Toda et al. [81] reported the direct delivery of RNPs (Cas9 based) in rice zygotes by PEG+Ca^{2+} mediated transfection thus achieving somatic mutagenesis with high frequencies (up to 64%).

3.2.2 Lipofection

The delivery of RNPs in plant cells need further development and lipofection could represent a promising delivery method. Briefly, lipofection (*i.e.*, lipid transfection, liposome-based transfection) takes advantage of tiny lipid vesicular structure called liposomes that can be multilamellar or unilamellar and neutral, positively, or negatively charged [74]. Liposomes are easily produced, and they can form lipoplexes by encapsulating DNA, RNA, or proteins; lipoplexes will further release their content into the cells upon endocytosis or fusion with membranes. Lipofection has been extensively used in mammalian cell transfection, while, in plants, following the initial demonstration of exogenous DNA transfer in isolated plant protoplasts by liposomes [82], lipofection by cationic lipids (positively charged liposomes) has been used exclusively for DNA or RNA transfer through the negatively charged plant cell membranes (protoplasts) with limited examples [83]. Recently, sweet orange genome edited plants have been obtained by delivering lipid-based nanostructures, produced with the cationic lipid-based transfection agent Lipofectamine™, in protoplasts. In detail, lipid particles encapsulated the CRISPR/Cas9 DNA construct, protecting it from endosomal and enzymatic degradation [84]. While in a first attempt, Liu et al. [83] tested the delivery of RNPs (Cas9 based) in tobacco protoplasts isolated from BY-2 cells, by using two different reagents (Lipofectamine™ 3000, Lipofectamine™ RNAiMAX) with a transfection efficiency of 66% and a mutation frequency of 6%, but without regenerating plants. RNP delivery in plant cells by lipofection is still in its infancy, but it is considered an easy and inexpensive method for cell transfection that will probably largely benefit in the future by the technological improvements achieved in other research fields.

4 Emerging Technologies

Apart from a few applications, current approaches to precisely modify higher plant genomes rely on *de novo* regeneration from tissues or cell-derived calli. The lack of genotype-independent protocols and/or the induction of somaclonal variation during the *in vitro* growth phase call for the development of genotype-independent, simple, and economic *in planta* procedures. The latter are particularly necessary in view of routinely implementing editing approaches in breeding programs. Some emerging technologies aiming to overcome present limitations of available procedures have recently emerged.

4.1 In planta de novo *Induction of Meristems*

Two methods to edit higher plant genomes have been recently developed in *Nicotiana benthamiana* and validated in tomato, potato, and grape [85]. Both rely on the co-delivery of plasmids with the gene of interest and genes encoding Developmental Regulators (*Wus2*, *STM* and others) to somatic tissues of germinating seedlings or mature plants grown *in vivo*. When specific sgRNAs were tested in transgenic Cas9$^+$ plants, edited shoots, able to transmit the induced mutations to the progeny, were regenerated from *de novo* formed meristems. Nevertheless, to obtain shoot formation, while reducing negative pleiotropic effects on their developments, the method requires a careful combination of different Developmental Regulators in genotypes tested. Further, the co-transfer of the nuclease remains to be demonstrated.

4.2 Editing During Haploid Induction

In several crops, either maternal or paternal haploids by chromosome elimination of one parent after fertilisation can be induced by interspecific crosses, knock-out of specific genes (e.g., *MATL* in maize or rice) or the manipulation of the gene encoding the centromere-specific histone CENH3 protein [86]. Capitalising on this information, site-directed mutagenesis in maize, *Arabidopsis*, and wheat has been achieved through the expression of editing reagents in the zygote, prior to elimination of chromosomes derived from the haploid-inducer parent and ploidy doubling in derived plants [86–88]. This method is attractive because it allows the production of DNA-free edited plants in just two generations but requires the availability of genotypes able to stably express editing reagents in the gametes and to induce chromosome elimination in the zygotes.

4.3 Editing Through Grafting

A breakthrough approach has been demonstrated recently by Yang and colleagues [89]. The authors produced transgenic *Arabidopsis* plants expressing a modified version of Cas9 and sgRNAs to which a tRNA sequence had been added. The latter allowed *in planta* long-distance movement of guide RNAs and nuclease transcripts from rootstock to scion, where translation occurred, producing edits visible both in the mother plant and derived progenies. Interestingly, the same was observed in interspecific graft combinations involving *Arabidopsis* and *Brassica rapa*, enlarging the potential scope of the technology to all species where graft compatibility occurs. It is, however, necessary to produce transgenic rootstocks for each editing target, although alternative ways to deliver and express mobile editing reagents in the rootstock could be attempted.

4.4 Nanotechnologies

Innovative nanoparticle-based methods *for in vivo* delivery of CRISPR/Cas have been recently developed in animal cells [90, 91]. Due to the presence of the cell wall, which shows a Size Exclusion Limit (SEL) of 5–20 nm and other differences with animals (f.i., the absence of a true circulatory system), research for similar alternative methods in plant cells lags behind. Nevertheless, the field is quite dynamic and nanomaterials (e.g., metallic/magnetic, silicon-based, carbon-based, lipid-based, polymeric, DNA nanostructures, peptide-based), showing at least one dimension below 100 nm and adjustable physico-chemical properties, could be adapted to deliver various cargoes precisely and in a controlled way to different plant tissues and without genotype-dependency, as reviewed elsewhere [92–96]. The possibility to directly transfer proteins or multiple biomolecules would allow the exploitation of RNPs without using laborious protoplast or biolistic-based approaches. The nanoparticle-mediated delivery of proteins in plants, however, presents difficulties not strictly related to their size [97].

Magnetic nanoparticles (MNP) have been tested, with results not always reproducible, to deliver plasmid DNA in pollen of cotton and other species using a magnetic field (magnetofection) [98–100]. In maize, the pre-treatment of pollen grains to open aperture was critical to obtain positive results. Single-walled carbon nanotubes (SWCNT) show high aspect and surface area-to-volume ratios. Functionalized with polyethyleneimine (PEI), chitosan or imidazolium, they have been assessed to deliver GFP/YFP plasmid DNA in leaves, chloroplasts, and pollen of various species, respectively [101–103], obtaining protein expression without gene integration. Intriguingly, in case of chloroplast expression, the delivery system was designed to release the loading DNA based on the stroma pH. Carbon dots (CD) with a 5–10 nm

spherical shape showed internalisation and transient plasmid DNA expression in roots, leaves, and embryogenic callus cells [104], while the delivery of Cas9 and gRNA plasmid DNA, leading to somatic editing of *SPO11*, has been reported by [105] after spraying plasmid coated CDs on wheat leaves. Rosette nanotubes (RNTs) derive from the self-assembly of complementary guanine and cytosine motifs, in solution self-organised in rosettes which eventually form biocompatible hollow nanotubes with an internal and external diameter of 1.1 and 3.4 nm, respectively. They could represent a less toxic alternative to SWCNTs and have been used to express mCherry plasmid DNA in wheat microspores [106].

RNA molecules (siRNA, dsRNA) have also been delivered to leaves and pollen by using SWCNTs, gold nanoparticles of different shape and size, DNA nanostructures with different characteristics, LDH clay nanosheets, inducing gene silencing and virus protection, when a viral gene was targeted [107–111]. Interestingly, with DNA nanostructures as carriers, RNA interference was induced at the transcript or protein level depending on the DNA nanostructure shape and the siRNA attachment locus [108], while, among gold particles, only nanorods entered the cells, but silencing was obtained only with the non-internalised spheres colocalized with the cell wall [109].

The co-delivery of a protein and plasmid DNA has been demonstrated in onion, tobacco and teosinte cells using gold functionalized Mesoporous Silica Nanoparticles (MSN) and the biolistic method [112]. A similar approach allowed the delivery of a functional CRE recombinase in maize embryos, determining the excision of DNA sequences flanked with *loxP* [113]. Recently, preliminary results of RNP delivery in wheat pollen have been reported by using nanoassemblies (5–10 nm in diameter) with polycation linear homopolymer PDMAEMA [114].

Cell penetrating peptides (CPP) are natural (protein derived) or artificially designed molecules generally between 5 and 30 amino acids, with the ability to be translocated through the cell membrane either directly or *via* endocytosis. Based on physicochemical properties they are classified as cationic, hydrophobic or amphipathic. They can bind to various kinds of molecular cargoes, either covalently or non-covalently, allowing their transfection in various recipient cells to transiently modify gene expression and metabolic pathways [115, 116]. In relation to genome editing, the feasibility of delivering proteins of varying sizes, including ADH (150 kDa) or GUS (272 kDa), has been demonstrated in triticale microspores as well as in intact *Arabidopsis* leaves [117, 118]. The transfer of the *GUS* linear plasmid DNA or of multiple biomolecules at the same time has been also shown [117, 119]. Proof of genome editing of the *IPK1* gene, albeit at low frequency, has been obtained in wheat microspores and derived haploid embryos by delivering ZFNs complexed with two different CPPs. Edited plants, however, could not be regenerated [115, 120]. Polyion complex vesicles synthesised by mixing two oligolysine peptides and displaying a CPP (CPP-PICsome) have been recently shown to be able to encapsulate Cas9 RNP complexes [121]. When introduced into *Arabidopsis* calli by vacuum and compression, editing of the target Phytoene Desaturase gene *PDS3* was obtained, although mutation rate was around 0.007%.

With reference to the use of nanotechnologies for genetic engineering and genome editing, quite a lot of research developed in the last years trying to adapt concepts and technologies derived from investigation in the human field to plants, although in most cases only at the proof-of-concept level. Nucleic acids or proteins could be successfully delivered to plant cells, constituting the basis to develop methods for delivering editing reagents. Nevertheless, moving from one system to another, e.g., from small plasmids encoding reporter genes to large constructs encoding Cas encoding genes, is not straightforward, while, concerning DNA binding conditions on Nanoparticless, the trade-off between cell wall trafficking and accessibility of the transcription machinery can be a challenge [122]. The size and other characteristics of Cas molecules generally used are an obstacle for the fast development of plant-based nanotechnological approaches, but the continuous discovery of new nucleases, some of them hypercompact, open new perspectives [123]. The recent results with CPP [121] are also promising. Nevertheless, the possibility to transiently edit *in planta* the germline, without relying on undifferentiated growth *in vitro* and *de novo* regeneration, and passing only the induced mutations to the progeny, remains a desirable objective not yet achieved.

5 Conclusions and Perspectives

Plant genome editing has been successfully demonstrated and applied to add or delete gene(s) in crop plants for functional genomic studies. The technology has gained momentum recently because of simplicity, efficiency, low cost and ability to target multiple genes. The variation in mutation efficiency in plants is associated with a number of different factors. Most importantly, the mode of delivery of the CRISPR/Cas9 components is of crucial importance for genome modification in plants. Plants have a complex genome structure due to common occurrence of polyploidy and other genomic re-arrangements. Thus, the delivery of CRISPR/Cas components to plant cells is still a challenge for researchers to attain high editing efficiency. There are many CRISPR delivery methods currently in use and development (Fig. 3.1). The various delivery methods such as biological (*Agrobacterium*-mediated, virus vector based), physical and chemical methods (PEG or biolistic based) are being applied to obtain efficient genome editing efficiency. Each delivery method has advantages and disadvantages. These methods deliver CRISPR systems to cells with the aid of chemicals or devices that make cells more amenable to delivery. Besides that, scientists are developing new ways and means for the delivery of CRISPR components to plant cells with the aim of establishing highly efficient and genotype-independent delivery systems for genome editing (summarised in Table 3.1). We assume the novel delivery methods those provide the opportunity for generating transgene-free genome edited plants will be most preferred in the future. These transgene free methods will boost acceptance of CRISPR/Cas technologies in food and agriculture.

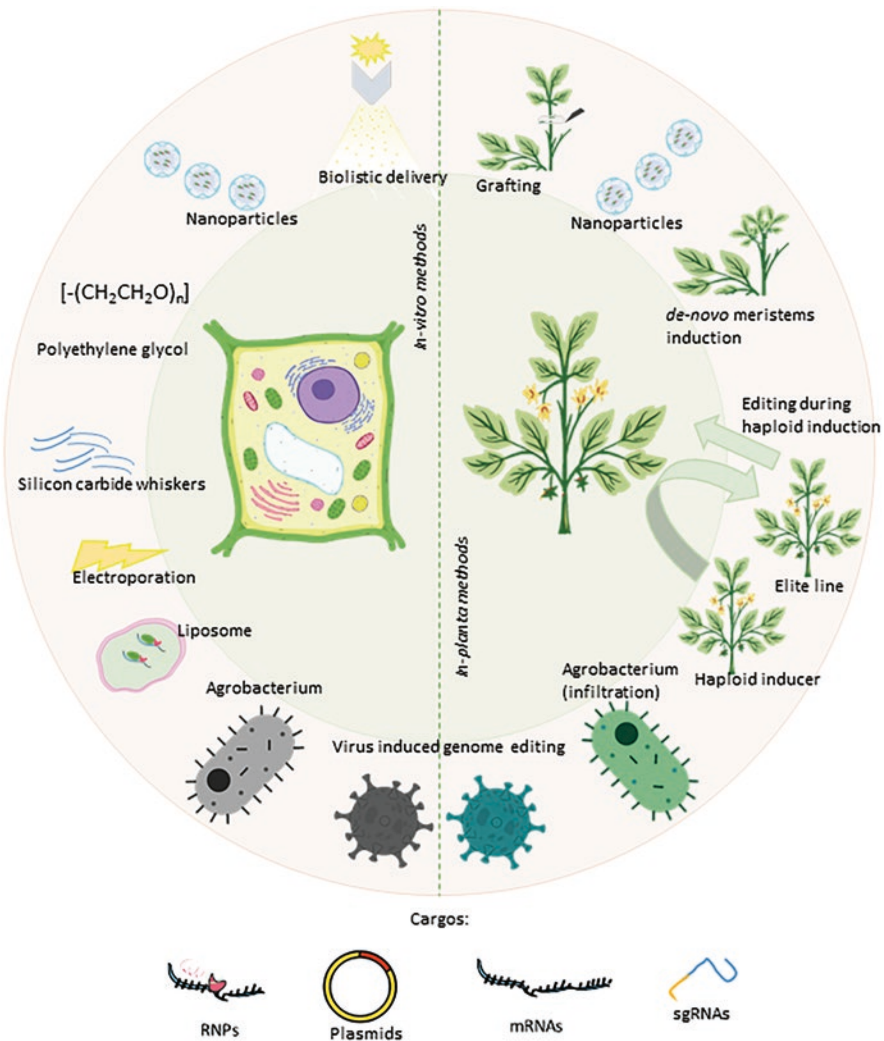


Fig. 3.1 Different delivery methods for components and T-DNA constructs to achieve genome editing in plant tissues. The left part of the scheme represents the delivery methods for in-vitro genome editing. While the right part of the infographic shows the in-planta methods for the delivery of genome editing tools, those potentially could give edits in early generations without integration of T-DNA. Additionally, the cargos for delivery of the different genome editing components are presented at the bottom

Table 3.1 Novel delivery methods for CRISPR-based nucleases for plant genome editing

	Target tissue	Advantages	Disadvantages	Comments	Selected references
Biological-					
Agrobacterium tumefaciens & *rhizogenes*	In vitro: Seed, meristem, leaf, petiole, internode or *In planta*: Floral Dip, de novo meristems	Used for both monocot and eudicot species in >24 plant families including major food crops	T-DNA integration into host genome using binary vector system. Potential of somaclonal variation when using *in vitro* methods	Improved transformation frequency observed by suppressing plant defence response system.	[2–7, 9, 10, 13]
Virus-Induced Genome Editing (VIGE) e.g Barley Stripe Mosaic Virus Bean Yellow Dwarf Virus	Various	Can access cells in different tissues and organs of monocots and eudicots. RNA-based viruses ensure integration free editing.	Replicon size dictates recombinant cargo size. DNA-based vectors could lead to integration.	Choice of vector depends on target host plant.	[16–22, 29]

(continued)

Table 3.1 (continued)

	Target tissue	Advantages	Disadvantages	Comments	Selected references
Physical-					
Biolistics	Various, including embryos & shoot apical meristems.	Used for monocot & eudicots.	Random and multiple integration patterns observed. High consumable costs.	Stable or transient expression achieved. Modifications include the use of a 'double-barrelled gene gun'.	[38–40, 42, 44]
Electroporation	Various, including protoplasts	Used for monocots & dicots.	Achieving uniform electroporation can be difficult in some tissue types.	Properties that influence electroporation include duration, intensity, pressure and frequency.	[48, 49]
Sonication-acoustic/ ultrasound	Seed	Used for monocots & dicots.	Still in the early stages of development.	No reports as yet with respect to successful genome editing in plants. Has been successful in algae and viruses.	[49, 58]
Nanoparticles: Silicon Carbide Whiskers	Cell suspensions, embryos, embryogenic calli	Used for monocots & eudicots. Compatible with most plant species. Quick and inexpensive.	Moderate transformation efficiency. Requires in vitro regeneration process and sophisticated protocols.		[70, 71, 124]

Chemical-					
Polyethylene glycol (PEG)	Protoplasts, Zygotes	Used for monocot and eudicots. Direct delivery permitting DNA-free mutagenesis. High frequency editing in zygotes using direct delivery of RNPs in rice has been reported.	Protoplasts require an established regeneration protocol which can be difficult for some plant species. May be subject to levels of unwanted somaclonal variation.	Offers flexibility in terms of the different types of reagents that can be introduced.	[36, 73, 80, 81]
Lipofection	Protoplasts	Considered a relatively easy and inexpensive method.	More work needed to prove its merit in plants particularly in relation to regeneration of plant material.	Based on the release of contents into the cell upon endocytosis. Still in its infancy.	[83, 84]
Emerging technologies-					
In Planta induction of Meristems	Somatic tissue of seedlings or mature plants	Avoids in vitro regeneration protocols thus no somaclonal variation.	Further work needed to refine the co-delivery of plasmids with gene of interest and developmental regulators.	Technique validated in tomato, potato and grape.	[85]
Editing during haploid induction	Zygote	Allows for DNA-free edited plants in two generations.	Relies on genotypes with ability to stably express editing agents in gametes and to induce chromosome elimination in zygotes.	To date achieved in Arabidopsis, maize and wheat.	[86–88]

(continued)

Table 3.1 (continued)

	Target tissue	Advantages	Disadvantages	Comments	Selected references
Editing through grafting	Rootstock and scions	To date has been demonstrated in arabidopsis and brassica.	Necessary to produce transgenic rootstock for each editing target.	In planta long distance movement of gRNAs and nuclease transcripts observed with edits produced in both mother and progeny.	[89]
Nanotechnologies	Roots, leaves, embryogenic calli, pollen	Genotype independent, bypasses in vitro culture including effects of somaclonal variation, DNA-free editing	Low mutation rate thus far. Still at proof of concept stage for many of the nanotechnology methods.	Genome editing has been obtained albeit at low levels thus far.	[97, 101–103, 105]

References

1. Makarova, K.S., Wolf, Y.I., Iranzo, J., Shmakov, S.A., Alkhnbashi, O.S., Brouns, S.J.J., Charpentier, E., Cheng, D., Haft, D.H., Horvath, P., Moineau, S., Mojica, F.J.M., Scott, D., Shah, S.A., Siksnys, V., Terns, M.P., Venclovas, Č., White, M.F., Yakunin, A.F., Yan, W., Zhang, F., Garrett, R.A., Backofen, R., van der Oost, J., Barrangou, R., Koonin, E.V.: Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. Nat. Rev. Microbiol. **18**, 67–83 (2020)
2. Raman, V., Rojas, C.M., Vasudevan, B., Dunning, K., Kolape, J., Oh, S., Yun, J., Yang, L., Li, G., Pant, B.D., Jiang, Q., Mysore, K.S.: Agrobacterium expressing a type III secretion system delivers Pseudomonas effectors into plant cells to enhance transformation. Nat. Commun. **13**, 2581 (2022)
3. Tiwari, M., Mishra, A.K., Chakrabarty, D.: Agrobacterium-mediated gene transfer: recent advancements and layered immunity in plants. Planta. **256**, 37 (2022)
4. Gelvin, S.B.: *Agrobacterium* -mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. Rev. **67**, 16–37 (2003)
5. Jones, H.D., Doherty, A., Wu, H.: Review of methodologies and a protocol for the agrobacterium-mediated transformation of wheat. Plant Methods. **1**, 5 (2005)
6. Hwang, H.-H., Yu, M., Lai, E.-M.: *Agrobacterium* -mediated plant transformation: biology and applications. Arabidopsis Book. **15**, e0186 (2017)
7. Thompson, M.G., Moore, W.M., Hummel, N.F.C., Pearson, A.N., Barnum, C.R., Scheller, H.V., Shih, P.M.: *Agrobacterium tumefaciens*: a bacterium primed for synthetic biology. BioDesign Res. **2020** (2020)
8. Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M., Sheen, J.: Multiplex and homologous recombination–mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat. Biotechnol. **31**, 688–691 (2013)
9. Shan, S., Soltis, P.S., Soltis, D.E., Yang, B.: Considerations in adapting CRISPR/Cas9 in nongenetic model plant systems. Appl. Plant Sci. **8**, e11314 (2020)
10. Laforest, L.C., Nadakuduti, S.S.: Advances in delivery mechanisms of CRISPR gene-editing reagents in plants. Front. Genome Editing. **4**, 1 (2022)
11. Zhang, Z., Hua, L., Gupta, A., Tricoli, D., Edwards, K.J., Yang, B., Li, W.: Development of an Agrobacterium-delivered CRISPR/Cas9 system for wheat genome editing. Plant Biotechnol. J. **17**, 1623–1635 (2019)
12. Dalla Costa, L., Piazza, S., Pompili, V., Salvagnin, U., Cestaro, A., Moffa, L., Vittani, L., Moser, C., Malnoy, M.: Strategies to produce T-DNA free CRISPRed fruit trees via Agrobacterium tumefaciens stable gene transfer. Sci. Rep. **10**, 20155 (2020)
13. Molina-Risco, M., Ibarra, O., Faion-Molina, M., Kim, B., Septiningsih, E.M., Thomson, M.J.: Optimizing agrobacterium-mediated transformation and CRISPR-Cas9 gene editing in the tropical japonica Rice variety presidio. Int. J. Mol. Sci. **22**, 10909 (2021)
14. Rössner, C., Lotz, D., Becker, A.: VIGS goes viral: how VIGS transforms our understanding of plant science. Annu. Rev. Plant Biol. **73**, 703–728 (2022)
15. Wang, M., Gao, S., Zeng, W., Yang, Y., Ma, J., Wang, Y.: Plant virology delivers diverse toolsets for biotechnology. Viruses. **12**, 1338 (2020)
16. Ali, Z., Abul-faraj, A., Li, L., Ghosh, N., Piatek, M., Mahjoub, A., Aouida, M., Piatek, A., Baltes, N.J., Voytas, D.F., Dinesh-Kumar, S., Mahfouz, M.M.: Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol. Plant. **13**, 1–4 (2015)
17. Ellison, E.E., Nagalakshmi, U., Gamo, M.E., Huang, P.-j., Dinesh-Kumar, S., Voytas, D.F.: Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. Nat. Plants. **6**, 620–624 (2020)
18. Luo, Y., Na, R., Nowak, J.S., Qiu, Y., Lu, Q.S., Yang, C., Marsolais, F., Tian, L.: Development of a Csy4-processed guide RNA delivery system with soybean-infecting virus ALSV for genome editing. BMC Plant Biol. **21**, 419 (2021)

19. Uranga, M., Aragonés, V., Selma, S., Vázquez-Vilar, M., Orzáez, D., Daròs, J.: Efficient Cas9 multiplex editing using unspaced sgRNA arrays engineering in a *Potato virus X* vector. Plant J. **106**, 555–565 (2021)
20. Uranga, M., Vazquez-Vilar, M., Orzáez, D., Daròs, J.-A.: CRISPR-Cas12a genome editing at the whole-plant level using two compatible RNA virus vectors. CRISPR J. **4**, 761–769 (2021)
21. Li, T., Hu, J., Sun, Y., Li, B., Zhang, D., Li, W., Liu, J., Li, D., Gao, C., Zhang, Y., Wang, Y.: Highly efficient heritable genome editing in wheat using an RNA virus and bypassing tissue culture. Mol. Plant. **14**, 1787–1798 (2021)
22. Oh, Y., Kim, H., Kim, S.-G.: Virus-induced plant genome editing. Curr. Opin. Plant Biol. **60**, 101992 (2021)
23. Chen, H., Su, Z., Tian, B., Liu, Y., Pang, Y., Kavetskyi, V., Trick, H.N., Bai, G.: Development and optimization of a barley stripe mosaic virus-mediated gene editing system to improve Fusarium head blight resistance in wheat. Plant Biotechnol. J. **20**, 1018–1020 (2022)
24. Senthil-Kumar, M., Mysore, K.S.: Tobacco rattle virus–based virus-induced gene silencing in Nicotiana benthamiana. Nat. Protoc. **9**, 1549–1562 (2014)
25. Uranga, M., Aragonés, V., Daròs, J.-A., Pasin, F.: Heritable CRISPR-Cas9 editing of plant genomes using RNA virus vectors. STAR Protoc. **4**, 102091 (2023)
26. Zhang, C., Liu, S., Li, X., Zhang, R., Li, J.: Virus-induced gene editing and its applications in plants. Int. J. Mol. Sci. **23**, 10202 (2022)
27. Gil-Humanes, J., Wang, Y., Liang, Z., Shan, Q., Ozuna, C.V., Sánchez-León, S., Baltes, N.J., Starker, C., Barro, F., Gao, C., Voytas, D.F.: High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. Plant J. **89**, 1251–1262 (2017)
28. Ali, Z., Eid, A., Ali, S., Mahfouz, M.M.: Pea early-browning virus-mediated genome editing via the CRISPR/Cas9 system in Nicotiana benthamiana and Arabidopsis. Virus Res. **244**, 333–337 (2018)
29. Wang, W., Yu, Z., He, F., Bai, G., Trick, H.N., Akhunova, A., Akhunov, E.: Multiplexed promoter and gene editing in wheat using a virus-based guide RNA delivery system. Plant Biotechnol. J. **20**, 2332–2341 (2022)
30. Hu, J., Li, S., Li, Z., Li, H., Song, W., Zhao, H., Lai, J., Xia, L., Li, D., Zhang, Y.: A barley stripe mosaic virus-based guide RNA delivery system for targeted mutagenesis in wheat and maize. Mol. Plant Pathol. **20**, 1463–1474 (2019)
31. Baltes, N.J., Gil-Humanes, J., Čermák, T., Atkins, P.A., Voytas, D.F.: DNA replicons for plant genome engineering. Plant Cell. **26**, 151–163 (2014)
32. Butler, N.M., Baltes, N.J., Voytas, D.F., Douches, D.S.: Geminivirus-mediated genome editing in potato (Solanum tuberosum L.) using sequence-specific nucleases. Front. Plant Sci. **7**, 1–13 (2016)
33. Čermák, T., Baltes, N.J., Čegan, R., Zhang, Y., Voytas, D.F.: High-frequency, precise modification of the tomato genome. Genome Biol. **16**, 232 (2015)
34. Wang, M., Glass, Z.A., Xu, Q.: Non-viral delivery of genome-editing nucleases for gene therapy. Gene Ther. **24**, 144–150 (2017)
35. Zhang, S., Shen, J., Li, D., Cheng, Y.: Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. Theranostics. **11**, 614–648 (2021)
36. Zhang, Y., Iaffaldano, B., Qi, Y.: CRISPR ribonucleoprotein-mediated genetic engineering in plants. Plant Commun. **2**, 100168 (2021)
37. Miller, K., Eggenberger, A.L., Lee, K., Liu, F., Kang, M., Drent, M., Ruba, A., Kirscht, T., Wang, K., Jiang, S.: An improved biolistic delivery and analysis method for evaluation of DNA and CRISPR-Cas delivery efficacy in plant tissue. Sci. Rep. **11**, 7695 (2021)
38. Ghogare, R., Ludwig, Y., Bueno, G.M., Slamet-Loedin, I.H., Dhingra, A.: Genome editing reagent delivery in plants. Transgenic Res. **30**, 321–335 (2021)
39. Ozyigit, I.I., Yucebilgili Kurtoglu, K.: Particle bombardment technology and its applications in plants. Mol. Biol. Rep. **47**, 9831–9847 (2020)
40. Kanchiswamy, C.N.: DNA-free genome editing methods for targeted crop improvement. Plant Cell Rep. **35**, 1469–1474 (2016)

41. Shi, J., Gao, H., Wang, H., Lafitte, H.R., Archibald, R.L., Yang, M., Hakimi, S.M., Mo, H., Habben, J.E.: ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol. J. **15**, 207–216 (2017)
42. Hamada, H., Liu, Y., Nagira, Y., Miki, R., Taoka, N., Imai, R.: Biolistic-delivery-based transient CRISPR/Cas9 expression enables in planta genome editing in wheat. Sci. Rep. **8**, 14422 (2018)
43. Zhang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K., Qiu, J.-L., Gao, C.: Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat. Commun. **7**, 12617 (2016)
44. Svitashev, S., Schwartz, C., Lenderts, B., Young, J.K., Mark Cigan, A.: Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat. Commun. **7**, 13274 (2016)
45. Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., Liu, J., Zhang, H., Liu, C., Ran, Y., Gao, C.: Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat. Commun. **8**, 14261 (2017)
46. Schwartz, C., Lenderts, B., Feigenbutz, L., Barone, P., Llaca, V., Fengler, K., Svitashev, S.: CRISPR–Cas9-mediated 75.5-Mb inversion in maize. Nat. Plants. **6**, 1427–1431 (2020)
47. Sandhya, D., Jogam, P., Allini, V.R., Abbagani, S., Alok, A.: The present and potential future methods for delivering CRISPR/Cas9 components in plants. J. Genet. Eng. Biotechnol. **18**, 25 (2020)
48. Kumar, P., Nagarajan, A., Uchil, P.D.: Electroporation. Cold Spring Harb. Protoc. 2019, pdb.top096271 (2019)
49. Kotnik, T., Rems, L., Tarek, M., Miklavčič, D.: Membrane electroporation and electropermeabilization: mechanisms and models. Annu. Rev. Biophys. **48**, 63–91 (2019)
50. Ozyigit, I.I.: Gene transfer to plants by electroporation: methods and applications. Mol. Biol. Rep. **47**, 3195–3210 (2020)
51. Dymek, K., Rems, L., Zorec, B., Dejmek, P., Galindo, F.G., Miklavčič, D.: Modeling electroporation of the non-treated and vacuum impregnated heterogeneous tissue of spinach leaves. Innov. Food Sci. Emerg. Technol. **29**, 55–64 (2015)
52. Van Wert, S.L., Saunders, J.A.: Electrofusion and electroporation of plants. Plant Physiol. **99**, 365–367 (1992)
53. Lee, M.H., Lee, J., Choi, S.A., Kim, Y.-S., Koo, O., Choi, S.H., Ahn, W.S., Jie, E.Y., Kim, S.W.: Efficient genome editing using CRISPR–Cas9 RNP delivery into cabbage protoplasts via electro-transfection. Plant Biotechnol. Rep. **14**, 695–702 (2020)
54. Rokhina, E.V., Lens, P., Virkutyte, J.: Low-frequency ultrasound in biotechnology: state of the art. Trends Biotechnol. **27**, 298–306 (2009)
55. Chen, B., Huang, J., Wang, J., Huang, L.: Ultrasound effects on the antioxidative defense systems of Porphyridium cruentum. Colloids Surf. B: Biointerfaces. **61**, 88–92 (2008)
56. Liu, Y., Takatsuki, H., Yoshikoshi, A., Wang, B., Sakanishi, A.: Effects of ultrasound on the growth and vacuolar H+-ATPase activity of aloe arborescens callus cells. Colloids Surf. B: Biointerfaces. **32**, 105–116 (2003)
57. George, E.F., Hall, M.A., de Klerk, G.J.: Plant propagation by tissue culture, 3rd edn, p. 1. Springer, Dordrecht (2007)
58. Horodecka, K., Düchler, M.: CRISPR/Cas9: principle, applications, and delivery through extracellular vesicles. Int. J. Mol. Sci. **22**, 6072 (2021)
59. Safari, M., Ghanati, F., Behmanesh, M., Hajnorouzi, A., Nahidian, B., Mina, G.: Enhancement of antioxidant enzymes activity and expression of CAT and PAL genes in hazel (Corylus avellana L.) cells in response to low-intensity ultrasound. Acta Physiol. Plant. **35**, 2847–2855 (2013)
60. Teixeira da Silva, J.A., Dobránszki, J.: Sonication and ultrasound: impact on plant growth and development. Plant Cell Tissue Organ Cult. **117**, 131–143 (2014)
61. Augustine, S.M., Cherian, A.V., Seiling, K., Di Fiore, S., Raven, N., Commandeur, U., Schillberg, S.: Targeted mutagenesis in *Nicotiana tabacum ADF* gene using shockwave-

mediated ribonucleoprotein delivery increases osmotic stress tolerance. Physiol. Plant. **173**, 993–1007 (2021)
62. Kaeppler, H.F., Gu, W., Somers, D.A., Rines, H.W., Cockburn, A.F.: Silicon carbide fiber-mediated DNA delivery into plant cells. Plant Cell Rep. **9**, 415–418 (1990)
63. Kaeppler, H.F., Somers, D.A., Rines, H.W., Cockburn, A.F.: Silicon carbide fiber-mediated stable transformation of plant cells. Theor. Appl. Genet. **84**, 560–566 (1992)
64. Asad, S., Mukhtar, Z., Nazir, F., Hashmi, J.A., Mansoor, S., Zafar, Y., Arshad, M.: Silicon carbide whisker-mediated embryogenic callus transformation of cotton (Gossypium hirsutum L.) and regeneration of salt tolerant plants. Mol. Biotechnol. **40**, 161–169 (2008)
65. Brisibe, E.A., Gajdosova, A., Olesen, A., Andersen, S.B.: Cytodifferentiation and transformation of embryogenic callus lines derived from anther culture of wheat. J. Exp. Bot. **51**, 187–196 (2000)
66. Matsushita, J., Otani, M., Wakita, Y., Tanaka, O., Shimada, T.: Transgenic plant regeneration through silicon carbide whisker-mediated transformation of Rice (Oryza sativa L.). Breed. Sci. **49**, 21–26 (1999)
67. Petolino, J.F., Hopkins, N.L., Kosegi, B.D., Skokut, M.: Whisker-mediated transformation of embryogenic callus of maize. Plant Cell Rep. **19**, 781–786 (2000)
68. Asad, S., Arshad, M.: Silicon carbide whisker-mediated plant transformation. In: Gerhardt, R. (ed.) Properties and applications of silicon carbide. INTECH (2011)
69. Mujtaba, M., Wang, D., Carvalho, L.B., Oliveira, J.L., Espirito Santo Pereira, A. do, Sharif, R., Jogaiah, S., Paidi, M.K., Wang, L., Ali, Q., Fraceto, L.F.: Nanocarrier-mediated delivery of miRNA, RNAi, and CRISPR-Cas for plant protection: current trends and future directions. ACS Agric. Sci. Technol. **1**, 417–435 (2021)
70. Chen, J.E., Barbrook, A.C., Cui, G., Howe, C.J., Aranda, M.: The genetic intractability of Symbiodinium microadriaticum to standard algal transformation methods. PLoS One. **14**, e0211936 (2019)
71. Noel, E.A., Weeks, D.P., Van Etten, J.L.: Pursuit of chlorovirus genetic transformation and CRISPR/Cas9-mediated gene editing. PLoS One. **16**, e0252696 (2021)
72. Zarrintaj, P., Saeb, M.R., Jafari, S.H., Mozafari, M.: Application of compatibilized polymer blends in biomedical fields. In: Ajitha, A., Sabu, T. (eds.) Compatibilization of polymer blends. Elsevier, Amsterdam (2020)
73. Yue, J.-J., Yuan, J.-L., Wu, F.-H., Yuan, Y.-H., Cheng, Q.-W., Hsu, C.-T., Lin, C.-S.: Protoplasts: from isolation to CRISPR/Cas genome editing application. Front. Genome Editing. **3**, 19 (2021)
74. Ortiz-Matamoros, M.F., Villanueva, M.A., Islas-Flores, T.: Genetic transformation of cell-walled plant and algae cells: delivering DNA through the cell wall. Brief Funct. Genomics. **17**, 26–33 (2018)
75. Krens, F.A., Molendijk, L., Wullems, G.J., Schilperoort, R.A.: In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature. **296**, 72–74 (1982)
76. Paszkowski, J., Shillito, R.D., Saul, M., Mandák, V., Hohn, T., Hohn, B., Potrykus, I.: Direct gene transfer to plants. EMBO J. **3**, 2717–2722 (1984)
77. Shillito, R.D., Saul, M.W., Paszkowski, J., Müller, M., Potrykus, I.: High efficiency direct gene transfer to plants. Nat. Biotechnol. **3**, 1099–1103 (1985)
78. Töpfer, R., Pröls, M., Schell, J., Steinbiß, H.-H.: Transient gene expression in tobacco protoplasts: II. Comparison of the reporter gene systems for CAT, NPT II, and GUS. Plant Cell Rep. **7**, 225–228 (1988)
79. Yoo, S.-D., Cho, Y.-H., Sheen, J.: Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. **2**, 1565–1572 (2007)
80. Rustgi, S., Naveed, S., Windham, J., Zhang, H., Demirer, G.S.: Plant biomacromolecule delivery methods in the 21st century. Front. Genome Editing. **4**, 51 (2022)
81. Toda, E., Koiso, N., Takebayashi, A., Ichikawa, M., Kiba, T., Osakabe, K., Osakabe, Y., Sakakibara, H., Kato, N., Okamoto, T.: An efficient DNA- and selectable-marker-free genome-editing system using zygotes in rice. Nat. Plants. **5**, 363–368 (2019)

82. Lurquin, P.F.: Entrapment of plasmid DNA by liposomes and their interactions with plant protoplasts. Nucleic Acids Res. **6**, 3773–3784 (1979)
83. Liu, W., Rudis, M.R., Cheplick, M.H., Millwood, R.J., Yang, J.-P., Ondzighi-Assoume, C.A., Montgomery, G.A., Burris, K.P., Mazarei, M., Chesnut, J.D., Stewart, C.N.: Lipofection-mediated genome editing using DNA-free delivery of the Cas9/gRNA ribonucleoprotein into plant cells. Plant Cell Rep. **39**, 245–257 (2020)
84. Mahmoud, L.M., Kaur, P., Stanton, D., Grosser, J.W., Dutt, M.: A cationic lipid mediated CRISPR/Cas9 technique for the production of stable genome edited citrus plants. Plant Methods. **18**, 33 (2022)
85. Maher, M.F., Nasti, R.A., Vollbrecht, M., Starker, C.G., Clark, M.D., Voytas, D.F.: Plant gene editing through de novo induction of meristems. Nat. Biotechnol. **38**, 84–89 (2020)
86. Kelliher, T., Starr, D., Su, X., Tang, G., Chen, Z., Carter, J., Wittich, P.E., Dong, S., Green, J., Burch, E., McCuiston, J., Gu, W., Sun, Y., Strebe, T., Roberts, J., Bate, N.J., Que, Q.: One-step genome editing of elite crop germplasm during haploid induction. Nat. Biotechnol. **37**, 287–292 (2019)
87. Wang, B., Zhu, L., Zhao, B., Zhao, Y., Xie, Y., Zheng, Z., Li, Y., Sun, J., Wang, H.: Development of a haploid-inducer mediated genome editing system for accelerating maize breeding. Mol. Plant. **12**, 597–602 (2019)
88. Budhagatapalli, N., Halbach, T., Hiekel, S., Büchner, H., Müller, A.E., Kumlehn, J.: Site-directed mutagenesis in bread and durum wheat via pollination by cas9/guide RNA-transgenic maize used as haploidy inducer. Plant Biotechnol. J. **18**, 2376–2378 (2020)
89. Yang, L., Machin, F., Wang, S., Saplaoura, E., Kragler, F.: Heritable transgene-free genome editing in plants by grafting of wild-type shoots to transgenic donor rootstocks. Nat. Biotechnol. **41**, 1–10 (2023)
90. Duan, L., Ouyang, K., Xu, X., Xu, L., Wen, C., Zhou, X., Qin, Z., Xu, Z., Sun, W., Liang, Y.: Nanoparticle delivery of CRISPR/Cas9 for genome editing. Front. Genet. **12**, 788 (2021)
91. Khirallah, J., Eimbinder, M., Li, Y., Xu, Q.: Clinical progress in genome-editing technology and in vivo delivery techniques. Trends Genet. **39**, 208–216 (2023)
92. Cunningham, F.J., Goh, N.S., Demirer, G.S., Matos, J.L., Landry, M.P.: Nanoparticle-mediated delivery towards advancing plant genetic engineering. Trends Biotechnol. **36**, 882–897 (2018)
93. Wang, P., Zhao, F.J., Kopittke, P.M.: Engineering crops without genome integration using nanotechnology. Trends Plant Sci. **24**, 574–577 (2019)
94. Wang, J.W., Grandio, E.G., Newkirk, G.M., Demirer, G.S., Butrus, S., Giraldo, J.P., Landry, M.P.: Nanoparticle-mediated genetic engineering of plants. Mol. Plant. **12**, 1037–1040 (2019)
95. Demirer, G.S., Silva, T.N., Jackson, C.T., Thomas, J.B., Ehrhardt, D.W., Rhee, S.Y., Mortimer, J.C., Landry, M.P.: Nanotechnology to advance CRISPR–Cas genetic engineering of plants. Nat. Nanotechnol. **16**, 243–250 (2021)
96. Savage, N.: Improving crop resilience with nanoparticles. Nature. **608**, S16–S17 (2022)
97. Wang, J.W., Cunningham, F.J., Goh, N.S., Boozarpour, N.N., Pham, M., Landry, M.P.: Nanoparticles for protein delivery in planta. Curr. Opin. Plant Biol. **60**, 102052 (2021)
98. Zhao, X., Meng, Z., Wang, Y., Chen, W., Sun, C., Cui, B., Cui, J., Yu, M., Zeng, Z., Guo, S., Luo, D., Cheng, J.Q., Zhang, R., Cui, H.: Pollen magnetofection for genetic modification with magnetic nanoparticles as gene carriers. Nat. Plants. **3**, 956–964 (2017)
99. Vejlupkova, Z., Warman, C., Sharma, R., Scheller, H.V., Mortimer, J.C., Fowler, J.E.: No evidence for transient transformation via pollen magnetofection in several monocot species. Nat. Plants. **6**, 1323–1324 (2020)
100. Wang, Z., Zhang, Z., Zheng, D., Zhang, T., Li, X., Zhang, C., Yu, R., Wei, J., Wu, Z.: Efficient and genotype independent maize transformation using pollen transfected by DNA-coated magnetic nanoparticles. J. Integr. Plant Biol. **64**, 1145–1156 (2022)
101. Demirer, G.S., Zhang, H., Matos, J.L., Goh, N.S., Cunningham, F.J., Sung, Y., Chang, R., Aditham, A.J., Chio, L., Cho, M.-J., Staskawicz, B., Landry, M.P.: High aspect ratio nanomaterials enable delivery of functional genetic material without DNA integration in mature plants. Nat. Nanotechnol. **14**, 456–464 (2019)

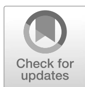
102. Kwak, S.-Y., Lew, T.T.S., Sweeney, C.J., Koman, V.B., Wong, M.H., Bohmert-Tatarev, K., Snell, K.D., Seo, J.S., Chua, N.-H., Strano, M.S.: Chloroplast-selective gene delivery and expression in planta using chitosan-complexed single-walled carbon nanotube carriers. Nat. Nanotechnol. **14**, 447–455 (2019)
103. Lew, T.T.S., Park, M., Wang, Y., Gordiichuk, P., Yeap, W.-C., Mohd Rais, S.K., Kulaveerasingam, H., Strano, M.S.: Nanocarriers for transgene expression in pollen as a plant biotechnology tool. ACS Mater. Lett. **2**, 1057–1066 (2020)
104. Wang, B., Huang, J., Zhang, M., Wang, Y., Wang, H., Ma, Y., Zhao, X., Wang, X., Liu, C., Huang, H., Liu, Y., Lu, F., Yu, H., Shao, M., Kang, Z.: Carbon dots enable efficient delivery of functional DNA in plants. ACS Appl. Bio. Mater. **3**, 8857–8864 (2020)
105. Doyle, C., Higginbottom, K., Swift, T.A., Winfield, M., Bellas, C., Benito-Alifonso, D., Fletcher, T., Galan, M.C., Edwards, K., Whitney, H.M.: A simple method for spray-on gene editing in planta. bioRxiv, 805036 (2019)
106. Cho, J.-Y., Bhowmik, P., Polowick, P.L., Dodard, S.G., El-Bakkari, M., Nowak, G., Fenniri, H., Hemraz, U.D.: Cellular delivery of plasmid DNA into wheat microspores using rosette nanotubes. ACS Omega. **5**, 24422–24433 (2020)
107. Mitter, N., Worrall, E.A., Robinson, K.E., Li, P., Jain, R.G., Taochy, C., Fletcher, S.J., Carroll, B.J., Lu, G.Q., Xu, Z.P.: Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nat. Plants. **3**, 16207 (2017)
108. Zhang, H., Demirer, G.S., Zhang, H., Ye, T., Goh, N.S., Aditham, A.J., Cunningham, F.J., Fan, C., Landry, M.P.: DNA nanostructures coordinate gene silencing in mature plants. Proc. Natl. Acad. Sci. U. S. A. **116**, 7543–7548 (2019)
109. Zhang, H., Goh, N.S., Wang, J.W., Pinals, R.L., González-Grandío, E., Demirer, G.S., Butrus, S., Fakra, S.C., Del Rio Flores, A., Zhai, R., Zhao, B., Park, S.-J., Landry, M.P.: Nanoparticle cellular internalization is not required for RNA delivery to mature plant leaves. Nat. Nanotechnol. **17**, 197–205 (2022)
110. Demirer, G.S., Zhang, H., Goh, N.S., Pinals, R.L., Chang, R., Landry, M.P.: Carbon nanocarriers deliver siRNA to intact plant cells for efficient gene knockdown. Sci. Adv. **6**, eaaz0495 (2020)
111. Yong, J., Zhang, R., Bi, S., Li, P., Sun, L., Mitter, N., Carroll, B.J., Xu, Z.P.: Sheet-like clay nanoparticles deliver RNA into developing pollen to efficiently silence a target gene. Plant Physiol. **187**, 886–899 (2021)
112. Martin-Ortigosa, S., Valenstein, J.S., Lin, V.S.-Y., Trewyn, B.G., Wang, K.: Gold functionalized mesoporous silica nanoparticle mediated protein and DNA codelivery to plant cells via the biolistic method. Adv. Funct. Mater. **22**, 3576–3582 (2012)
113. Martin-Ortigosa, S., Peterson, D.J., Valenstein, J.S., Lin, V.S.-Y., Trewyn, B.G., Lyznik, L.A., Wang, K.: Mesoporous silica nanoparticle mediated intracellular Cre protein delivery for maize genome editing via loxP sites excision. Plant Physiol. **164**, 537 (2013)
114. Gogoi, N., Kanwal, M., Norman, M., Downs, J., Ahmad, N., Mago, R., Bariana, H., Müllner, M., Bansal, U., Jones, B.: Wheat pollen uptake of CRISPR/Cas9 RNP-PDMAEMA nanoassemblies results in targeted loss of gene function in progeny. bioRxiv 2022.06.02.494465 (2022)
115. Soliman, A., Laurie, J., Bilichak, A., Ziemienowicz, A.: Cell Penetrating Peptides. Methods in Molecular Biology, vol 2383. Humana, New York, NY (2022)
116. Wu, H., Zhang, K., Zhang, Z., Wang, J., Jia, P., Cong, L., Li, J., Duan, Y., Ke, F., Zhang, F., Liu, Z., Lu, F., Wang, Y., Li, Z., Chang, M., Zou, J., Zhu, K.: Cell-penetrating peptide: a powerful delivery tool for DNA-free crop genome editing. Plant Sci. **324**, 111436 (2022)
117. Chugh, A., Amundsen, E., Eudes, F.: Translocation of cell-penetrating peptides and delivery of their cargoes in triticale microspores. Plant Cell Rep. **28**, 801–810 (2009)
118. Ng, K.K., Motoda, Y., Watanabe, S., Sofiman Othman, A., Kigawa, T., Kodama, Y., Numata, K.: Intracellular delivery of proteins via fusion peptides in intact plants. PLoS One. **11**, e0154081 (2016)

119. Thagun, C., Motoda, Y., Kigawa, T., Kodama, Y., Numata, K.: Simultaneous introduction of multiple biomacromolecules into plant cells using a cell-penetrating peptide nanocarrier. Nanoscale. **12**, 18844–18856 (2020)
120. Bilichak, A., Sastry-Dent, L., Sriram, S., Simpson, M., Samuel, P., Webb, S., Jiang, F., Eudes, F.: Genome editing in wheat microspores and haploid embryos mediated by delivery of ZFN proteins and cell-penetrating peptide complexes. Plant Biotechnol. J. **18**, 1307–1316 (2020)
121. Odahara, M., Watanabe, K., Kawasaki, R., Tsuchiya, K., Tateishi, A., Motoda, Y., Kigawa, T., Kodama, Y., Numata, K.: Nanoscale Polyion complex vesicles for delivery of cargo proteins and Cas9 ribonucleoprotein complexes to plant cells. ACS Appl. Nano. Mater. **4**, 5630–5635 (2021)
122. Ali, Z., Serag, M.F., Demirer, G.S., Torre, B., Di Fabrizio, E., Landry, M.P., Habuchi, S., Mahfouz, M.: DNA-carbon nanotube binding mode determines the efficiency of carbon nanotube-mediated DNA delivery to intact plants. ACS Appl. Nano. Mater. **5**, 4663–4676 (2022)
123. Liu, S., Sretenovic, S., Fan, T., Cheng, Y., Li, G., Qi, A., Tang, X., Xu, Y., Guo, W., Zhong, Z., He, Y., Liang, Y., Han, Q., Zheng, X., Gu, X., Qi, Y., Zhang, Y.: Hypercompact CRISPR–Cas12j2 (CasΦ) enables genome editing, gene activation, and epigenome editing in plants. Plant Commun. **3**, 100453 (2022)
124. Rastogi, A., Tripathi, D.K., Yadav, S., Chauhan, D.K., Živčák, M., Ghorbanpour, M., El-Sheery, N.I., Brestic, M.: Application of silicon nanoparticles in agriculture. 3 Biotech. **9**, 90 (2019)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 4
Balancing Trait Improvement with Tradeoff Side-Effects Using Genome Editing Technology



Julia Johanna Wind

Abstract Improving traits by breeding involves compromising between desired traits and possible undesired side effects. Often encountered examples include pathogen resistance versus yield, shelf life time versus fruit quality, and seed number versus seed weight. Genome editing can be used to reduce the effect of some of these tradeoffs. Different genetic reasons underlying a tradeoff require different approaches: important to note is whether a detrimental effect is caused by a unique gene, or several analogs/ homologs, because the strategy needs to be adopted accordingly. Unique genes, for example, can be substituted by analogs, and homologs have the advantage that, amongst the available options, the gene causing the fewest pleiotropic effects can be altered in its activity. When the detrimental effect of a tradeoff is caused by two genetically linked genes, this can lead to linkage drag. To break this type of tradeoff genome editing can be used to force a crossover event. Overcoming a tradeoff can generate a new one, but can nevertheless result in an improved crop variety.

1 Different Genetic Reasons Underlying a Tradeoff

The first plant breeding technique was the selection and propagation of plants with improved heritable traits, which were based on the cumulative net positive outcome of genetic changes. In more modern breeding, Quantitative Trait Loci (QTL) studies have led to the discovery of alleles responsible for the improvement of traits. Molecular breeding was then developed to cross such alleles into the crop variety of interest, thereby increasing speed and accuracy of the breeding process. Studying the effects that these alleles had on a trait made apparent how common pleiotropic effects are due to the introduction of an allele from a wild donor to a receiving elite line: often the improvement of one trait goes hand in hand with negative effects on other traits. The association between breeding values of linked traits can be positive

J. J. Wind (✉)
Keygene N.V., Wageningen, Gelderland, Netherlands
e-mail: julia.wind@keygene.com

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_4

or negative so that if one trait changes, other traits will change as well. A positive value indicates a win-win situation. For example, shorter plants have been shown to be easier for the mechanical harvest and also lead to a yield gain because plants put more energy into seed production. A negative value indicates a tradeoff: for example, an average increase in seed number often leads to a decrease in average seed weight. Genome wide association study (GWAS) experiments are very effective in indicating which genes or alleles are important for a given trait. Trait correlations are often caused by a set of genes that form the connection between two traits. However, some alleles have been discovered which cause breaks in trait associations. They can be the key to overcoming tradeoffs. However, such findings are rare and can often not be translated to crop plants. Transgenic approaches can be used to introduce these alleles. Alternative approaches to influence trait associations include selection of novel alleles from mutant populations or targeted mutagenesis with genome editing. Mutant populations have been created by random mutagenesis with a mutagen such as ethyl methanesulfonate (EMS), and such populations are very expensive to make because they require thousands of chemically treated individuals that are grown for multiple generations. Therefore, targeted mutagenesis is attractive as it can make targeted mutations in the gene or promoter of interest, so that only a few plants need to be edited instead of a large population. A few types of editing are discussed here: editing to create genetic variation including knockouts, editing to alter expression of target genes and cutting DNA to induce crossovers in recombination cold spots. These techniques are discussed in the context of a few well-known tradeoffs.

2 Unique Genes

During plant evolution, two important whole genome duplications have occurred. First, in the ancestral seed plant whole genome duplication has led to the divergence of the seed plants. Second, whole genome duplication in the ancestral angiosperm plant has led to the angiosperm radiation. These whole genome duplications lead to gene duplication. Changes accumulate in the gene copies over time, because the alleles are under different selective pressure. This can result in divergence of gene function. It was estimated that 65% of genes have at least one duplicated copy. Genes are often part of gene families, but this does not mean that they can always be substituted by a homolog or an ortholog: this is clear when a knockout or an allele of the gene results in a phenotype. Such genes can be important regulators of plant growth and development, and often have pleiotropic effects when they are modified. Therefore, reducing the negative effects of a tradeoff is most difficult for these types of genes. Most of the time, finding another gene that affects the trait in a similar way, but with fewer pleiotropic effects would be preferable. Three examples of tradeoffs caused by unique genes are discussed in the next sections.

2.1 Flowering Time vs. Yield

FLOWERING LOCUS T (FT) is a well-known and often studied flowering time regulator that is unique for its central role in the induction of flowering in most plant species [1]. Knockouts of FT exhibit a late flowering phenotype, and overexpression reduces time before flowering. FT also plays a role in control of growth, heterosis, tuberization and the regulation of stomatal opening in a variety of plants [1]. Therefore, FT can have pleiotropic effects on plant growth and development. Natural variation for flowering time in canola is frequently associated with FT alleles and comes with a biomass tradeoff [2]. Swinnen and colleagues have summarized examples from literature of genetic variation in cis-regulatory elements in genes that underly initial domestication of various crops [3]. A large part of these alleles are in the FT promoter, suggesting that altered expression pattern of this gene is of major importance in the domestication of crops, very likely due to its effect on flowering time and flowering synchrony. Pleiotropic effects are also evident in other flowering time regulators, such as TERMINAL FLOWER 1 (TFL1). *Brassica napus* knockouts for BnaC03.TFL1 does not only have reduced flowering time but also reduced plant height, as well as reduced branch number, number of siliques, and seeds per silique [4]. Variation in the FT promoter through genome editing technologies could result in more genetic variation aimed at reducing pleiotropy. Changing expression of FT is also likely to improve synchronous flowering as a positively correlated trait, but it might be hard to limit the negatively correlated traits that result from early maturity, because of the link between early flowering and early maturity. If earlier flowering time is needed, it could be preferred to find genes that can speed the overall growth so that the plant matures faster. A knockout in cpn60β4 in *Arabidopsis thaliana* was shown to accelerate plant development [5]. Using CRISPR, cpn60β4 orthologs could be knocked out in the species for which accelerated flowering is needed, as this gene is conserved in angiosperms [5]. This might lead to a similar effect on reducing flowering time without early maturity.

2.2 Fruit Shelf Life vs. Lycopene Content

The Food and Agriculture Organization from the United Nations has calculated that tomato has become the third most grown vegetable worldwide in the last decade and is therefore considered a very valuable crop [6]. Two properties are indispensable for the success of tomato: firmness and high lycopene content. Delayed ripening improves firmness and reduces the damage during the shipping of tomatoes as well as storability, while high lycopene improves the attractiveness for consumers, the value for the processing industry, as well as its nutritional value. Transcription factors that are important in improving shelf life are NON-RIPENING (NAC-NOR) and RIPENING-INHIBITOR (MADS-RIN). Both proteins are not knockouts but

alleles causing a phenotype: NOR is a partially mistranslated but functional protein and RIN is a fusion between two proteins. They both cause physiological, transcriptional and hormonal changes in ripening tomato fruits. Both of these regulators delay fruit ripening in similar but not identical ways. Crossing the RIN mutation to eight different wild type tomato lines clearly demonstrated that the improvement of shelf life negatively correlates with reddening of the tomato in the F1 progeny, and it was shown that the reduced reddening is due to a twofold reduction of lycopene. Conclusively, RIN and NOR mutations can improve shelf life at the cost of lycopene production. However, transgenic lines aiming to improve astaxanthin content surprisingly accumulated high levels of lycopene and also displayed an improvement in shelf life [7]. The authors explain the phenotype as the result of the extended duration of lycopene synthesis, so lycopene can accumulate, whereas the lack of β-carotene or its metabolic products possibly reduced the feedback inhibition. This phenotype demonstrates that firmness and lycopene content are not necessarily always correlated. The genes that were overexpressed originated from marine bacteria from the *Brevundimonas* genus and these genes are not present in tomato. However, the enzyme that forms β-carotene or its metabolic products are present in the tomato genome and hence could be knocked out. Alternatively, reducing the expression with an RNAi construct of a fruit-specific expressed pectate lyase (Solyc03g111690) reduced the softening of the tomato fruit, showing that it is possible to bypass the overall climacteric ripening program [8]. This could be easily reproduced with a genome edit aiming to knockout or knockdown this gene. A third example that firmness and carotenoid levels are not always correlated is the phenotype of the *hp1* and *hp1-w* mutants. These plants produce tomatoes with delayed ripening, higher levels of carotenoid and other phytonutrients due to altered light transduction [9]. However, the authors point out that this results in undesirable whole plant phenotypic changes and therefore they suggest that the knockdown of the gene responsible for the phenotype, DNA damage-binding protein 1 (*DDB1*), should be occurring only during fruit ripening. This demonstrates that the tradeoff of high lycopene content vs. delayed ripening can be resolved by taking one of several possible approaches that could bypass the tradeoffs: either by rerouting the lycopene pathway, directly targeting the enzymes involved in softening, or by altering the light signaling pathway specifically in the tomato.

2.3 Seed Number vs. Seed Weight

For many crops, an increase in seed number leads to a reduction in seed weight. Grain Weight 2 (TaGW2-A1), is a well-characterized gene in wheat that has a positive effect on seed weight but reduces the number of seeds. The introduction of the wild emmer allele *GNI-A1* into wheat was demonstrated to break this correlation because this allele could improve seed weight without affecting seed number, as reviewed by [10]. It was demonstrated that a single amino acid substitution is responsible for this (G182R). Alternatively, overexpressing an α-expansin gene in

wheat led to transgenic lines with a higher seed weight, no significant change in seed number, and hence an increase in grain yield of 12.3% [10]. These two examples show that an increase in seed weight does not always lead to a reduced seed number. The opposite was also demonstrated. In rice, elevated *NOG1* expression was shown to increase the number of grains per plant without affecting the grain weight, and this was due to a natural variation of a 12 bp insertion in the promoter [10]. Crossing an allele that improves seed weight to another allele that improves seed number would be a good strategy to improve total yield. The above examples are in wheat and rice, and thus analogs/homologs should be found in other grain crops of interest. A list of candidates that could have a comparable function could be made, and these genes can be edited, either by SNP introduction (to GNI-A1 homologs) or through promoter editing (for α-expansin or NOG1 expression). This could be done simultaneously with genome editing technologies aimed at multiplexing of editing tools. Together these examples show that the genetic link of two traits causing a tradeoff can be broken through introducing/creating/knocking out alleles that do not affect both traits. Different tools can be used to achieve this effect. The detailed molecular studies of tradeoffs allow for the use of targeted mutagenesis. Hence, genome editing can be very helpful when multiple alleles need to be modified or when natural variants are not available in the available germplasm.

3 Making Use of Expression Diversity in Orthologs/Homologs

As mentioned before, many genes are part of gene families. This essentially means that a mutation in such a gene, if is not dominant or dominant-negative, does not lead to a phenotype because the function is compensated by a homolog with a similar function. For breeders this means that for many genes, single knock-outs are not effective for improving traits. However, if a gene that is important for a trait, is part of a gene family, and causes additional negative effects on other traits, there is an opportunity to substitute such a gene with a homolog.

3.1 Fruit Size vs. Inflorescence Branching

Using genome editing technology to modify promoters can be aimed at introducing single nucleotide polymorphisms (SNPs), small and larger deletions and even rearranging the promoter randomly though multiplex editing. Generating a population of plants with variations in the promoter sequence of a single gene can be used for fine-tuning the desired effect. A series of deletions in the promoter of CLAVATA3 in tomato (SlCLV3) was linked to altered expression and it was shown that the altered expression coincided with an altered level of locule number and thus fruit size, though not predictably [11]. Reducing SlCLV3 activity also promoted inflorescence

branching [12], which can lead to excessive number of fruits, dampening the effect that reduced SlCLV3 expression can have on fruits size due to disturbed source to sink transport. It was shown that a null mutant for SlCLV3 in tomato is compensated by elevated expression of the ortholog SlCLE9 [12]. The authors also show that SlCLE9 has a similar function as SlCLV3, though the SlCLE9 knockout has a weak phenotype with only a subtle effect on locule number. This means that an edited promoter population for either SlCLV3 or SlCLE9 could be a valid strategy to obtain a tomato plant with a high number of locules and regular inflorescence branching.

3.2 Shorter Plants vs. Plant Morphology

Mutants in the gibberellic acid biosynthesis and catabolism pathway often result in a dwarf or elongated phenotype respectively, due to modified gibberellic acid levels. However, this often comes with pleiotropic effects such as increased tillering in rice, increased culm bending in sorghum, and in one case can result in complete inhibition of flowering in rice. Tomato *internode elongated-1* (*EI-1*) is a splice-site mutation in the SlGA2ox7 gene. SlGA2ox7 is a gibberellin 2-oxidase that catalyzes the breakdown of certain bioactive gibberellins and *EI-1* results in a dwarf phenotype. *EI-1* leads to an increase in bioactive gibberellins in stems and petioles [13]. However, since SlGA2ox7 is more highly expressed in hypocotyls and internodes than in petioles, the elongated internode mutation phenotype is stronger here. Due to this organ specific expression, *EI-1* exhibits a reduced elongation specific effect only, while its ortholog Solyc10g005360 has a different expression pattern and is therefore expected to have more pleiotropic effects, including in the leaves. Consequently, the former gene would be a preferred candidate for genome editing as higher expression could lead to shorter plants without affecting the morphology of the leaves. Similarly, in pea the mutant *Le-1* has a mutation in a gibberellin 3-oxidase, which results in a shoot specific phenotype, while the roots are unaffected. In summary, to shorten the height of a crop species, unwanted pleiotropic phenotypes could be avoided by learning about the expression patterns in all genes that are affecting this phenotype, and choosing a gene that has a tissue specific expression.

3.3 Vitamin C vs. Growth

Ascorbate peroxidases (APX) catalyze the H_2O_2-dependent oxidation of ascorbic acid (vitamin C) in plants, and can therefore reduce ascorbic acid levels. In Arabidopsis, a knocked out major cytosolic isoform of APX led to severe growth retardation. In contrast, in tomato there are nine homologs that encode for APX enzymes that catalyze the breakdown of ascorbate, with one being highest expressed

in red ripe tomato fruits. Specific mutation of *SlAPX4* by genome editing led to an increase of ascorbic acid in fruit with no detected growth impairment [14]. Hence, this approach might be easily copied to other crops for the biofortification of fruits, in case their genomes contain a family of *APX* homologs.

3.4 Blast Resistance vs. Yield

Rice blast is a disease caused by the fungal pathogen *Magnaporthe oryzae* and is a huge problem in rice cultivation. Resistance to this disease has a tradeoff with yield. *Pigm* is a locus that has been used in breeding for durable and broad-spectrum resistance to rice blast. The locus consists of 13 homologs coding for NLR receptors and two of these, *PigmR* and *PigmS* are separated by just two genes. While *PigmR* confers blast resistance but at a yield cost, *PigmS* attenuates the blast resistance, and therefore counteracts the yield cost by promoting seed set [15]. Interestingly, increased expression by transgenic overexpression of both loci, overcomes this tradeoff. Hence, the *Pigm* locus effect on yield could be improved by editing their promoters for improved expression of both.

4 Overcoming Linkage Drag

Sometimes a tradeoff is caused by two genes that are in close proximity to each other on a chromosome. Such genes might genetically be linked if recombination between the genes is low or absent. This would make separation of these genes difficult or even impossible. If one of the two genes has a positive effect on a trait, but the other one has a negative effect on the same or another important trait, this presents a tradeoff known as linkage drag. Linkage drag examples include virus resistance vs. yield in tobacco, viral resistance vs. bacterial resistance in tomato, abiotic stress resistance vs. yield and quality traits in sunflower, and heading date vs. root biomass in wheat. In a series of near isogenic lines, the precise site was determined where recombination could break the tradeoff between viral resistance and bacterial resistance in tomato [16]. Recombination of the *I-3* gene out of the donor *Solanum pennelli* introgression at the end of the chromosome would remove the unknown gene from *S. pennelli* that causes bacterial spot susceptibility. The technology to induce precise crossover events is still in development, but some progress has been made. Controlling the recombination event has been shown in yeast. In plants linkage drag could be broken by swopping chromosome arms between linked loci [17]. The authors also demonstrated how two chromosome arms were exchanged in the *ALS2* locus for the *Solanum pennellii* and *Solanum lycopersicum cv. M82*. This shows that performing controlled recombination to remove a gene with a deleterious effect while maintaining the novel introgressed allele in plants is a promising new breeding tool.

5 Concluding Remarks and Future Perspectives

Applying genome editing technology to reduce tradeoff side-effects has potential as a tool in plant breeding. Especially knockout editing or promoter editing has been proposed due to their broad applicability. More precise edits can result in altered protein function, and to obtain this, different editing technologies are being developed [18]. Due to the nature of DNA editing enzymes and the properties of chromosomes in alive cells, not every nucleotide can be edited in such a controlled manner. Also, off-target effects are a problem for genome editing success. Solving this remains a challenge for the future. It should be considered that not all crops can be edited yet. For example, incomplete sequenced genomes, difficulty in editing itself, resistance to regeneration or obligatory outcrossing are hurdles to overcome. Some tradeoffs were broken through an allele that was discovered with a QTL experiment. Other tradeoffs can be broken through editing (promoters) of genes, especially if tradeoff-breaking alleles have not been discovered in the crop of interest. Both approaches can be useful additions to the breeders' toolbox. Breaking tradeoffs is not easy. In fact, breaking one tradeoff could create a novel one. An example is the GNP1^{TQ} allele in rice which has broken the seeds number vs. seed weight tradeoff. When this allele was introduced in the Lemont background, this did not result in a higher yield because the variety could not meet the increased sink capacity needed for the filling of the grains [10]. So next, the grain number vs. sink capacity tradeoff needs to be addressed. In the end, by balancing tradeoff side-effects with trait improvement, new crop varieties can be bred that meet novel breeding demands.

Acknowledgements Thanks to Dr. Nathalie Dollé-van Orsouw, Dr. Matthias Berens, Dr. Paul Bundock and Dr. Thea van der Wijk for the critical reading of the manuscript.

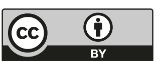
References

1. Pin, P.A., Nilsson, O.: The multifaceted roles of FLOWERING LOCUS T in plant development. Plant Cell Environ. **35**, 1742–1755 (2012)
2. Raman, H., et al.: GWAS hints at pleiotropic roles for FLOWERING LOCUS T in flowering time and yield-related traits in canola. BMC Genomics. **20**, 1–18 (2019)
3. Swinnen, G., Goossens, A., Pauwels, L.: Lessons from domestication: targeting cis-regulatory elements for crop improvement. Trends Plant Sci. **21**, 506–515 (2016)
4. Sriboon, S., et al.: Knock-out of TERMINAL FLOWER 1 genes altered flowering time and plant architecture in Brassica napus. BMC Genet. **21**, 1–13 (2020)
5. Tiwari, L.D., Grover, A.: Cpn60β4 protein regulates growth and developmental cycling and has bearing on flowering time in Arabidopsis thaliana plants. Plant Sci. **286**, 78–88 (2019)
6. FAOSTAT. Agricultural production statistics 2000–2020. 1–15 (2022)
7. Enfissi, E.M.A., et al.: The road to astaxanthin production in tomato fruit reveals plastid and metabolic adaptation resulting in an unintended high lycopene genotype with delayed over-ripening properties. Plant Biotechnol. J. **17**, 1501–1513 (2019)
8. Uluisik, S., et al.: Genetic improvement of tomato by targeted control of fruit softening. Nat. Biotechnol. **34**, 950–952 (2016)

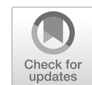
9. Wang, A., et al.: The tomato HIGH PIGMENT1/DAMAGED DNA BINDING PROTEIN 1 gene contributes to regulation of fruit ripening. Hortic. Res. **6**, 1–10 (2019)
10. Dwivedi, S.L., Reynolds, M.P., Ortiz, R.: Mitigating tradeoffs in plant breeding. iScience. **24**, 1–22 (2021)
11. Rodríguez-Leal, D., Lemmon, Z.H., Man, J., Bartlett, M.E., Lippman, Z.B.: Engineering quantitative trait variation for crop improvement by genome editing. Cell. **171**, 470–480 (2017)
12. Rodriguez-Leal, D., et al.: Evolution of buffering in a genetic circuit controlling plant stem cell proliferation. Nat. Genet. **51**, 786–792 (2019)
13. Schrager-Lavelle, A., et al.: The role of a class III gibberellin 2-oxidase in tomato internode elongation. Plant J. **97**, 603–615 (2019)
14. Do, J.H., et al.: Development of a genome-edited tomato with high ascorbate content during later stage of fruit ripening through mutation of SlAPX4. Front. Plant Sci. **13**, 1–11 (2022)
15. Deng, Y., et al.: Epigenetic regulation of antagonistic receptors confers rice blast resistance with yield balance. Science (1979). **355**, 962–965 (2017)
16. Li, J., Chitwood, J., Menda, N., Mueller, L., Hutton, S.F.: Linkage between the I-3 gene for resistance to Fusarium wilt race 3 and increased sensitivity to bacterial spot in tomato. Theor. Appl. Genet. **131**, 145–155 (2018)
17. Bundock, P., Stuurman, J.: Method for removing genetic linkage in a plant. 1–55 (2014)
18. Liu, G., Lin, Q., Jin, S., Gao, C.: The CRISPR-Cas toolbox and gene editing technologies. Mol. Cell. **82**, 333–347 (2022)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.



Chapter 5
CRISPR/Cas Mutation Screening: From Mutant Allele Detection to Prediction of Protein Coding Potential



Elke Vereecke, Katrijn Van Laere , and Tom Ruttink

Abstract CRISPR/Cas is a gene-editing technique that allows for the precise and specific introduction of a mutation into a DNA sequence. The outcome of a mutation on encoded protein depends on the type of mutation (deletion, insertion and/or substitution) and the position of the mutation in the DNA sequence. It can be predicted by using screening methods that are able to identify a mutation at nucleotide level. Here, several screening methods are discussed with a difference in complexity, resolution and scalability and the results are interpretated by taken into account the central dogma of the molecular biology. Two modules of the SMAP package, SMAP *haplotype-window* and SMAP *effect-prediction,* are proposed and implemented in a high-throughput screening workflow that allows for the automated and streamlined screening of CRISPR experiments.

1 Precision Gene Editing: Design Guided by Gene Structural Features

Gene editing, *e.g.,* by CRISPR/Cas, is widely used for plant functional genomics research and has huge potential for targeted improvement of desired heritable traits in crops [1]. The precision and specificity of CRISPR/Cas allows for dedicated

E. Vereecke (✉)
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Melle, Belgium

VIB Center for Plant Systems Biology, Ghent, Belgium
e-mail: elke.vereecke@ilvo.vlaanderen.be

K. Van Laere
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Melle, Belgium
e-mail: katrijn.vanlaere@ilvo.vlaanderen.be

T. Ruttink
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Melle, Belgium

Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
e-mail: tom.ruttink@ilvo.vlaanderen.be

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_5

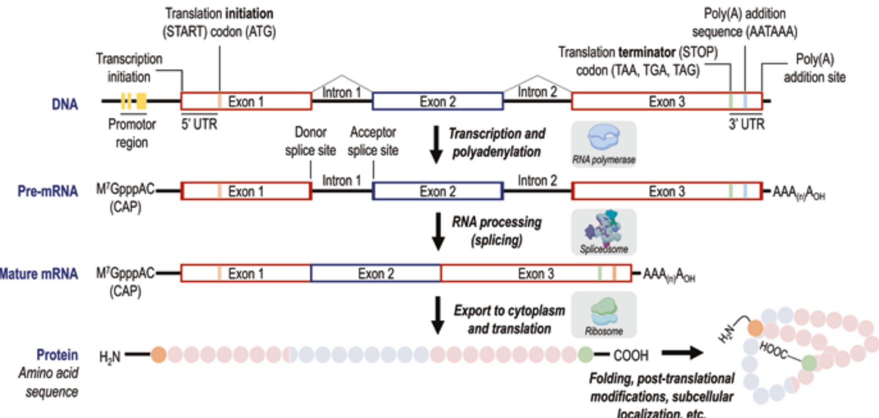


Fig. 5.1 The central dogma of molecular biology posits that the genetic information encoded in a genic DNA sequence is transcribed into messenger RNA (mRNA) and then translated into protein. Transcription factors bind to regulatory elements in the promoter of a gene and the flanking genomic DNA sequence is transcribed into pre-messenger RNA (pre-mRNA) by an RNA polymerase. This pre-mRNA molecule matures through the removal of introns resulting in the juxtaposition of exons to form mature mRNA. The mature mRNA is translated into a protein by ribosomes that start from the translation initiation (START) codon and follow the open reading frame (ORF) until the first translation termination (STOP) codon. Transcription and translation are tightly interrelated dynamic processes that are regulated at different levels and rely on various structural features in a gene [2]. Therefore, mutations in a DNA sequence may affect any of these processes and gRNAs and CRISPR/Cas DNA modifiers may be designed to create specific changes in the expression, structure, stability, activity, and/or function of a protein

screening for induced mutant alleles at target sites. Combinations of mutant alleles detected by molecular screens on the one hand, with the central dogma of molecular biology that explains the relationship between the primary DNA sequence, gene structural features, and expression of the encoded protein (Fig. 5.1) on the other, allows prediction of the effect of the mutation on protein functionality. Thus, a comprehensive gene editing screening workflow to characterize the generated mutants ideally spans the entire path between "gene structure based" CRISPR/Cas and gRNA design, molecular detection of the mutant alleles, and prediction of the effect of the mutation on the encoded protein sequence, hence capturing the actual outcome of gene editing. Here, we review the different types of mutations that can be introduced by variants of CRISPR/Cas gene editing, highlight several molecular screening and detection techniques and place them in this overarching perspective.

2 Types of Mutations Introduced by CRISPR/Cas Mediated Gene Editing

In its basic form, CRISPR/Cas mediated gene editing introduces a double-stranded DNA break at a specific genomic position defined by the gRNA. Subsequent non-perfect repair via non-homologous end-joining (NHEJ) or via homology-directed

repair (HDR) results in the introduction of mutations in the target DNA sequence [3]. Mutations induced by CRISPR/Cas occur within a short range flanking the protospacer adjacent motif (PAM) site (*e.g.*, 3–4 bp upstream of the PAM site for Cas9). NHEJ creates allelic series of mutations, typically in the range of short deletions and insertions (one up to tens of nucleotides), or few substitutions. Potential target sites, cleavage efficiencies, and induced scarring patterns may be predicted based on the gRNA sequence using machine learning, but accurate models for plants require further training on large-scale data sets [4, 5]. CRISPR/Cas is widely used to create targeted gene knockouts in several plant species. For example, Wang et al. [6] used CRISPR/Cas to knock out the susceptibility genes of the *mildew-resistance locus* (*MLO*) in wheat, generating wheat that is resistant to powdery mildew [6]. Mutations in a gene that result in a non-functional protein encoded by that gene, as described for the mutations in the *MLO* genes, are called loss-of-function (LOF) mutations and can occur when the ORF downstream of the mutation is disrupted (out-of-frame indel or frameshift) [7].

The CRISPR/gRNA complex may also be used as location-specific vehicle to deliver DNA sequence modifiers to a given location and modify the primary sequence (like base-editing or prime-editing) or epigenetic state [8, 9]. In base-editing, a cytidine deaminase (C:G-to-T:A) or adenosine deaminase (A:T-to-G:C) is linked to the CRISPR/gRNA complex and is used for the conversion of a single nucleotide at a specific position [8]. Base-editing can be used to create a specific point mutation, which may result in a single amino acid change in the protein sequence, a premature STOP codon or alter a splicing acceptor or donor site [10]. For example, in tomato and potato, base-editing was successfully used to convert a cytidine into a thymine in the *acetolactate synthase* (*ALS*) gene, conferring resistance to herbicides [11]. Mutations in a gene that result in an enhanced activity or functionality of the protein encoded by that gene, as described for the mutation in the *ALS* gene, are called gain-of-function (GOF) mutations.

While base-editing can only be used for two types of nucleotide substitution, prime-editing can introduce all kinds of predefined mutations, including the deletion, insertion, and/or substitution of specific nucleotides [12]. A prime-editing system consists of a Cas enzyme with nickase activity, reverse transcriptase, and prime-editing guide RNA (pegRNA) with a primer binding site for the specification of the genomic target site and an RNA template that encodes the desired edit [13]. It was already successfully used in rice for the insertion of a fragment up to 15 bp in the OsCDC48-T1 gene [12] and for the triple amino acid substitution in the *EPSPS* gene in rice to confer a higher level of glyphosate resistance [14].

CRISPR/Cas and its variants are also able to target DNA sequences at gene regulatory sites, *e.g.,* transcription factor binding sites, splicing sites, and translation initiation and/or termination codons, thus changing gene structural features or coding potential. This will affect the different processes driving transcription, mRNA maturation, and translation (Fig. 5.1). In addition, the epigenetic state can be modulated by fusing the CRISPR protein with an epigenetic modifier that can affect the methylation state at DNA level or affect the methylation and/or acetylation state at nucleosome level (histone modification) [15, 16]. For example, Gallego-Bartolomé et al. [17] were able to reactivate the transcription of the *FWA* gene by

demethylation of the *FWA* promotor using a dead Cas9 fused to the human demethylase TET2cd [17].

In short, CRISPR/Cas and its variants can be used to introduce a range of mutations into a DNA sequence that have different effects on the encoded protein. LOF or GOF mutations can be generated to study the role of certain proteins in biological processes, to confer resistance to pathogens or herbicides, to divert the metabolic flux of biosynthesis pathways towards valuable compounds, etc.

3 Screening Methods: Complexity, Resolution, and Scalability

Different screening methods are available to detect the outcome of CRISPR/Cas gene editing, to identify which plant material contains a desired gene edited sequence, and to evaluate the mutation efficiency. Screening methods may apply different detection methods (physical properties of an amplified allele vs sequencing-based), comprise targeted or untargeted screening (local sequencing of the predicted edited site (*e.g.,* amplification or capture of the gRNA binding site and flanking regions), or global sequencing (*e.g.,* WGS, RNA-Seq)), and with different levels of throughput and automation (via locus and/or sample multiplexing).

Simply put, any standard molecular detection technique that can discriminate DNA sequence variants (alleles) can also be used to detect CRISPR-induced mutations (Fig. 5.2). PCR-amplification of the target region, coupled to a detection method such as high-resolution melting (HRM) [18], fluorescent probe binding (qPCR or ddPCR [19]), or amplicon length polymorphism (agarose gel-electrophoresis, capillary fragment analysis, or mismatch detection assay [20] (a variant of Cleaved Amplified Polymorphic Sequences (CAPS) markers), or IDAA [20]) can be used to identify mutated alleles (Fig. 5.2). In addition, Kompetitive Allele-Specific PCR (KASP [21]) or primer–extension assays [22] may be used to screen for expected SNPs. These techniques are cheap, easy to implement, and allow for quick routine screening of gene edited mutant collections [23]. However, they only indirectly show the presence of a mutation, and not the actual, exact mutant DNA sequence at the nucleotide level, a prerequisite to interpret the effect of the mutation on the encoded protein.

Amplification and sequencing of target loci of mutants provides information on the specific nucleotides that are deleted, inserted and/or substituted (Fig. 5.2). Sanger (dideoxy-) sequencing generates electropherograms allowing for the determination of the DNA sequence and the identification of mutations [23, 24]. The interpretation of the electropherogram can be challenging, as multiple nucleotides can be called at the same position due to heterozygous insertions, deletions and/or substitutions. Therefore, several computational tools have been developed to deconvolute the electropherograms, such as Tracking of Insertions and Deletions (TIDE) [25], CRISP-ID [26], Deconvolution of Complex DNA Repair (DECODR) [27],

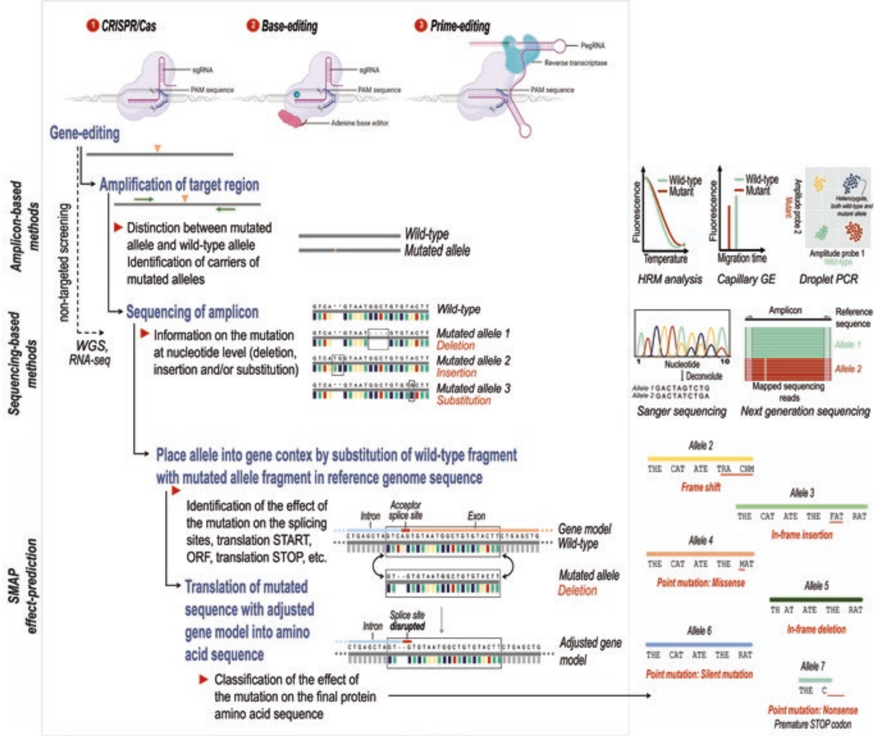


Fig. 5.2 A targeted mutation screening workflow. Mutations can be introduced into a DNA sequence using CRISPR/Cas and the CRISPR/Cas-based variants base-editing and prime-editing. Screening methods are needed to evaluate if a mutation has occurred. In this screening workflow, the different steps needed to: (1) identify if a mutation occurred (differential detection based on physical attributes of amplicons); (2) identify which mutation (deletion, insertion, substitution) occurred at nucleotide level (sequencing-based methods); and (3) evaluate the effect of the mutation on encoded protein sequence (Fig. 5.1) are illustrated together with the expected outcome of the different steps

and Inference of CRISPR Edits (ICE) [28]. These tools utilize distinct algorithms to analyze electropherograms from a wild-type and a gene edited sample, generating a list with predicted mutated sequences [24]. The sensitivity of Sanger sequencing for alternative alleles in a heterozygous or otherwise mixed sample is about 15% [29]. Consequently, low-efficiency editing is likely to be overlooked. Furthermore, these methods are typically performed with a separate amplification and detection reaction for each sample and each locus (simplex), limiting the scalability for mutation screens to large collections at multiple target loci.

Next Generation Sequencing NGS allows for massive parallel sequencing and analysis of heterogeneous samples and substantially lowers the per-sample and per-locus costs in high-throughput mutation screens [3]. Because of its deep read coverage, NGS sensitivity for alternative alleles is 0.1–1% and thus enables screening of bulk samples (*e.g.,* protoplasts after transfection), and efficient 1D, 2D or 3D

pooling schemes [28]. In addition, multiplex amplicon sequencing combined with incorporation of sample-specific barcodes during library preparation facilitates parallel sequencing at hundreds of loci, in hundreds of samples per sequencing run. NGS yields targeted resequencing data that can be analyzed via bioinformatics tools such as CRISPResso2 [30] and SMAP *haplotype-window* [31] (Fig. 5.3). In SMAP *haplotype-window*, sequencing reads are mapped to a reference and the entire read sequence spanning the region between borders (typically the amplicon primer binding sites) is considered as an allele [31]. All the unique alleles are sorted and counted for the calculation of relative allele frequency per locus per sample. A region of interest (ROI) can be defined to focus the analysis on mutations introduced by the gene editing technique in a narrow nucleotide window and ignore additional sequence variants at distance from the edit site. Every allele is compared to the reference in its entirety, allowing for the detection of any combination of insertion, deletion, and/or substitution. SMAP *haplotype-window* will generate an integrated genotype call table with all the observed alleles per locus per sample. Since it is agnostic to the length of the deletion, insertion, or substitution, it can detect any mutation resulting from an edit in the primary DNA sequence in a given window, as long as the amplicon or read length spans the mutated allele. SMAP *haplotype-window* can also process probe-capture enriched, WGS, and RNA-Seq read data from global resequencing screens, for a given list of target loci. PacBio sequencing [32] and nanopore MinION sequencing [33] can be used to detect long-range insertions and deletions, as well as epigenetic DNA modifications introduced by CRISPR/Cas.

4 Mutation Screening in a Broader Perspective: From Nucleotide to Protein

The current repertoire of CRISPR/Cas DNA modifiers combined with gRNA specificity, generates a huge array of design possibilities, especially when based on the principles that predict how protein sequences may be altered by editing the genomic nucleotide sequence. A mutation screening workflow that draws on clever CRISPR design, in turn, should be able to consider detected mutated alleles in their respective gene context and classify the mutated alleles based on predefined desired alleles (*e.g.* a unique base-edit) or on percentage protein sequence similarity to the original wild-type allele [7].

The SMAP *effect-prediction* module from the SMAP package estimates the novel encoded protein sequence and is the final step in the mutation screening workflow (Fig. 5.3) [31]. SMAP *haplotype-window* generates a list of all observed haplotypes per locus per sample, which is directly used as input for SMAP *effect-prediction*, together with all positional information on gene structural features. SMAP *effect-prediction* replaces a segment of the original reference gene sequence by the observed mutated sequence and evaluates all the splicing sites, the translation initiation codon, open reading frame, and translation termination codon [31]. After translation of the most likely ORF in the mutated allele, the amino acid

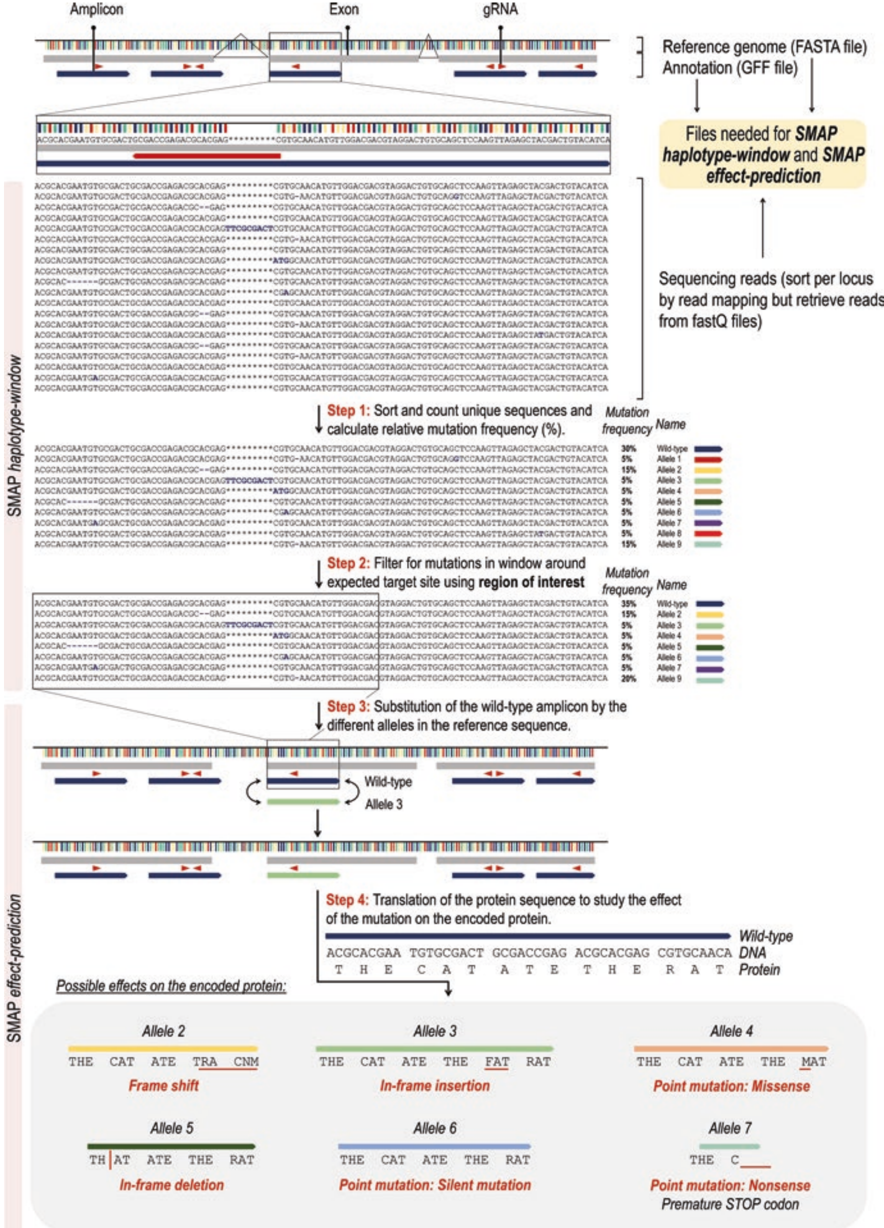


Fig. 5.3 SMAP *haplotype-window* and SMAP *effect-prediction* can be used to analyze highly multiplex amplicon sequencing data and to estimate the novel encoded protein sequence. SMAP *haplotype-window* is a module of the SMAP package that is used to analyze the sequencing reads obtained from NGS. It maps the sequencing reads (in this figure illustrated for a bulk sample) to the reference genome, groups all the alleles with the same mutations, determines a ROI and calculates the mutation frequency. SMAP *effect-prediction* is used to provide biological interpretation of the different mutations that were introduced by substituting the wild-type allele with the mutant allele(s) in the reference genome and translating the novel genic sequence. Mutation types include a frameshift mutation, in-frame indel, missense mutation, deletion, silent mutation and nonsense mutation

sequence is aligned to the original reference protein and the percentage protein sequence similarity is estimated as quantitative score for the remaining protein functionality. Proteins may no longer resemble the original reference protein (frameshift mutation or nonsense mutation), proteins can be identical to the original reference protein (silent mutation), etc. (Fig. 5.3). A threshold value can be set for the percentage sequence similarity to the original reference protein still needed for a protein to perform its function. DNA mutations that result in a protein with a lower percentage of similarity as a given threshold value can be defined as a loss-of-function mutation [34].

5 Conclusions

CRISPR/Cas and CRISPR/Cas variants are widely used to introduce mutations into a DNA sequence. However, mutations can have different effects on the function of a gene and its encoded protein. Here, we describe a molecular screening workflow that focuses on the path from CRISPR/Cas and gRNA design, through screening for mutant alleles, and prediction of the effect of the DNA sequence mutation on the encoded protein, all implemented in modules of the SMAP package. By using SMAP *haplotype-window* and SMAP *effect-prediction,* the detected mutated alleles are placed in their respective gene context, and the mutated alleles can be classified based on percentage of protein sequence similarity to the original wild-type allele. This high-throughput screening workflow allows for the automated and streamlined screening of multiplex CRISPR experiments, in large mutant collections (locus and/or sample multiplexing) and enables fast and easy interpretation of the effect of the mutant alleles on the protein sequence, and automated routine identification of carriers of desired alleles.

References

1. Mao, Y., Botella, J.R., Liu, Y., Zhu, J.-K.: Gene editing in plants: progress and challenges. Natl. Sci. Rev. **6**, 421–437 (2019)
2. Roos, D., de Boer, M.: Mutations in cis that affect mRNA synthesis, processing and translation. Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. **1867**, 1–21 (2021)
3. Bell, C.C., Magor, G.W., Gillinder, K.R., Perkins, A.C.: A high-throughput screening strategy for detecting CRISPR-Cas9 induced mutations using next-generation sequencing. BMC Genomics. **15**, 1–7 (2014)
4. Abadi, S., Yan, W.X., Amar, D., Mayrose, I.: A machine learning approach for predicting CRISPR-Cas9 cleavage efficiencies and patterns underlying its mechanism of action. PLoS Comput. Biol. **13**, 1–24 (2017)
5. Allen, F., et al.: Predicting the mutations generated by repair of Cas9-induced double-strand breaks. Nat. Biotechnol. **37**, 64–82 (2019)
6. Wang, Y., et al.: Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. **32**, 947–951 (2014)

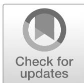
7. Gerasimavicius, L., Livesey, B.J., Marsh, J.A.: Loss-of-function, gain-of-function and dominant-negative mutations have profoundly different effects on protein structure. Nat. Commun. **13**, 1–15 (2022)
8. Azameti, M.K., Dauda, W.P.: Base editing in plants: applications, challenges, and future prospects. Front. Plant Sci. **12**, 1–13 (2021)
9. Li, J., et al.: Development of a highly efficient prime editor 2 system in plants. Genome Biol. **23**, 1–9 (2022)
10. Kluesner, M.G., et al.: CRISPR-Cas9 cytidine and adenosine base editing of splice-sites mediates highly-efficient disruption of proteins in primary and immortalized cells. Nat. Commun. **12**, 1–12 (2021)
11. Veillet, F., et al.: Transgene-free genome editing in tomato and potato plants using agrobacterium-mediated delivery of a CRISPR/Cas9 cytidine base editor. Int. J. Mol. Sci. **20**, 1–10 (2019)
12. Lin, Q., et al.: Prime genome editing in rice and wheat. Nat. Biotechnol. **38**, 582–585 (2020)
13. Jin, S., Lin, Q., Gao, Q., Gao, C.: Optimized prime editing in monocot plants using PlantPegDesigner and engineered plant prime editors (ePPEs). Nat. Protoc. **18**, 831–853 (2023)
14. Li, H., Li, J., Chen, J., Yan, L., Xia, L.: Precise modifications of both exogenous and endogenous genes in Rice by prime editing. Mol. Plant. **13**, 671–674 (2020)
15. Xie, N., Zhou, Y., Sun, Q., Tang, B.: Novel epigenetic techniques provided by the CRISPR/Cas9 system. Stem Cells Int. **2018**, 1–12 (2018)
16. Dubois, A., Roudier, F.: Deciphering plant chromatin regulation via CRISPR/dCas9-based epigenome engineering. Epigenomes. **5**, 1–16 (2021)
17. Gallego-Bartolomé, J., et al.: Targeted DNA demethylation of the arabidopsis genome using the human TET1 catalytic domain. Proc. Natl. Acad. Sci. U.S.A. **115**, E2125–E2134 (2018)
18. Li, R., et al.: Rapid and sensitive screening and identification of CRISPR/Cas9 edited rice plants using quantitative real-time PCR coupled with high resolution melting analysis. Food Control. **112**, 1–6 (2020)
19. Peng, C., et al.: Accurate detection and evaluation of the gene-editing frequency in plants using droplet digital PCR. Front. Plant Sci. **11**, 1–8 (2020)
20. Sentmanat, M.F., Peters, S.T., Florian, C.P., Connelly, J.P., Pruett-Miller, S.M.: A survey of validation strategies for CRISPR-Cas9 editing. Sci. Rep. **8**, 1–8 (2018)
21. Kalendar, R., Shustov, A.V., Akhmetollayev, I., Kairov, U.: Designing allele-specific competitive-extension PCR-based assays for high-throughput genotyping and gene characterization. Front. Mol. Biosci. **9**, 1–13 (2022)
22. Pereiro, I., et al.: Arrayed primer extension technology simplifies mutation detection in Bardet-Biedl and Alström syndrome. Eur. J. Hum. Genet. **19**, 485–488 (2011)
23. Bennett, E.P., et al.: INDEL detection, the 'Achilles heel' of precise genome editing: a survey of methods for accurate profiling of gene editing induced indels. Nucleic Acids Res. **48**, 11958–11981 (2020)
24. Carrington, B., Sood, R.: A comprehensive review of Indel detection methods for identification of Zebrafish knockout mutants generated by genome-editing nucleases. Genes (Basel). **13**, 1–16 (2022)
25. Brinkman, E.K., Chen, T., Amendola, M., van Steensel, B.: Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. **42**, e168–e168 (2014)
26. Dehairs, J., Talebi, A., Cherifi, Y., Swinnen, J.V.: CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing. Sci. Rep. **6**, 28973 (2016)
27. Bloh, K., et al.: Deconvolution of complex DNA repair (DECODR): establishing a novel deconvolution algorithm for comprehensive analysis of CRISPR-edited sanger sequencing data. CRISPR J. **4**, 120–131 (2021)
28. Conant, D., et al.: Inference of CRISPR edits from sanger trace data. CRISPR J. **5**, 123–130 (2022)
29. Hagemann, I.S.: Overview of technical aspects and chemistries of next-generation sequencing. In: Clinical Genomics, pp. 3–19. Elsevier Inc. (2015). https://doi.org/10.1016/B978-0-12-404748-8.00001-0

30. Clement, K., et al.: CRISPResso2 provides accurate and rapid genome editing analysis. Nat. Biotechnol. **37**, 220–224 (2019)
31. Schaumont, D. et al.: Stack Mapping Anchor Points (SMAP): a versatile suite of tools for read-backed haplotyping. bioRxiv 2022.03.10.483555 (2022)
32. Rhoads, A., Au, K.F.: PacBio sequencing and its applications. Genomics Proteomics Bioinformatics. **13**, 278–289 (2015)
33. Feng, Y., Zhang, Y., Ying, C., Wang, D., Du, C.: Nanopore-based fourth-generation DNA sequencing technology. Genomics Proteomics Bioinformatics. **13**, 4–16 (2015)
34. Develtere, W., et al.: SMAP design: a multiplex PCR amplicon and gRNA design tool to screen for natural and CRISPR-induced genetic variation. Nucleic Acids Res. **51**, e37–e37 (2023)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 6
Methods and Techniques to Select Efficient Guides for CRISPR-Mediated Genome Editing in Plants



Fabio D'Orso, Valentina Forte, Simona Baima, Marco Possenti, Daniela Palma, and Giorgio Morelli

Abstract CRISPR technology is revolutionizing genomic engineering by enabling scientists to precisely modify plant DNA, thus representing a powerful tool for plant breeding.

This chapter provides a summary of the approaches and constraints of CRISPR-mediated genome editing in plants, with a focus on the critical prerequisite of efficient CRISPR reagents for successful gene editing in plants.

While computational tools have tremendously improved our ability to design specific guides, their limitations make guide effectiveness prediction unreliable, especially for plants. Therefore, it is strongly recommended to validate CRISPR reagents before investing time and resources in the challenging process of plant transformation.

A number of *in vitro* and *in planta* assays coupled with analytical methods have been proposed to assess the editing performances. Each approach has its own strengths and weaknesses, so the choice of the most suitable system depends on the specific plant species and the type and depth of the genotypic data required.

In many cases, the hairy root assay can provide a good compromise between rapidity, reliability and cost-effectiveness for assessing editing performance in numerous plant species.

Keywords CRISPR · Genome editing · Guide design · Guide efficiency · Computational tools · Agroinfiltration · Protoplasts · Hairy roots · Genotyping

F. D'Orso (✉) · V. Forte · S. Baima · M. Possenti · D. Palma · G. Morelli
CREA – Research Centre for Genomics and Bioinformatics, Rome, Italy
e-mail: fabio.dorso@crea.gov.it

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_6

1 Introduction

With the introduction of precision genome editing using Clustered Regularly Interspaced Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) technology, we have entered a new era of genetic engineering. CRISPR technology has allowed straightforward, cost-effective and efficient gene editing compared with technologies as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). CRISPR represents a new perspective for plant breeding and a powerful alternative for genetic engineering to speed up the introduction of improved traits by precise and predictable modifications (deletions, substitutions, insertions) directly in an elite background [38]. Major challenge for the application of genome editing in crop breeding is generating plants without introducing a transgene, and this has led to new challenges for the regulation and social acceptance of genetically modified organisms (GMO). Furthermore, CRISPR technology can produce novel plants transgene-free indistinguishable from natural variants or generated by conventional breeding techniques [42]. Nowadays it has emerged as a powerful tool for applications in medicine, agriculture, and basic studies of gene function. In plants, since the first demonstration of CRISPR in DNA editing in 2013 there has been much progress in basic plant science and crop improvement with applications for biotic and abiotic tolerance, yield performance improvement, biofortification and enhancement of plant quality [118] ranging from model plant, like *Arabidopsis thaliana*, to food crops [38].

In addition, CRISPR technology can accelerate crop domestication, a labour-intensive process involving alteration of a plant from its wild state to a new form that can serve human needs. Recently, CRISPR was used to domesticate wild tomato, *Solanum pimpinellifolium*, which is remarkably stress tolerant but is defective in terms of fruit production [77]. In one study, six loci that are important for yield and productivity were targeted, and the engineered lines displayed increased fruit size, fruit number and fruit lycopene accumulation [157].

In prokaryotes, CRISPR/Cas are a family of DNA sequences found in bacteria and archaea genomes and as adaptive immune system that naturally protects cells from DNA virus infections [38]. As biotechnological tool, the CRISPR/Cas9 system is comprised of the Cas9 endonuclease and a synthetic single guide RNA (sgRNA), which combines functions of CRISPR RNA (cRNA) and trans-activating CRISPR RNA (tracrRNA) to direct the Cas9 protein to the DNA target sequence preceding the protospacer-associated motif (PAM) [153].

Various strategies and different types of CRISPR/Cas systems, as genetic manipulation tools, have been attempted to generate and study the impact of functional mutations in crop improvement [92].

Furthermore, the requirement for a specific PAM sequence is a major factor restricting the selection of target sequences. For this reason, different engineered SpCas9 or orthologous Cas9 nucleases derived from different organisms, able to recognize different PAM sequences, have been used for genome editing [28].

The desired genetic modification is initiated by inducing double stranded breaks (DSBs) into a target sequence by using nucleases, and it is subsequently attained by

DNA repair through Non-Homologous End Joining (NHEJ) or homology-directed repair (HDR) [36].

The events of genome editing in plant according to the NHEJ mechanism described in the literature, are mainly represented by gene knock-out [117]. The rate of knock-in is much lower [41]. In fact, NHEJ plays a major role in the repair of DSBs through insertions and deletions (indels) at the junction, which change the nucleotide sequence information surrounding the repair region [140]. These indels may cause frameshifts and lead to knock-out of the corresponding gene [122].

HDR-mediated genome editing, also known as gene targeting (GT), is the approach to introduce precise insertion (knock-in) using information from an exogenously supplied DNA donor template to repair the break. The information is copied from the donor template to the chromosome, achieving the desired DNA sequence modification [22]. GT in higher plants was extremely difficult for decades. One of the obstacles in achieving HDR has been the ability to deliver sufficient donor templates to the plant cell to repair the DSB [51]. Development of approaches to improve GT efficiencies in plants are in progress; however, there is still no universal and efficient method for increasing the knock-in frequency. Currently the best approach for precise modification of plant genomes is geminivirus-mediated Gene targeting, but also other non HDR-mediated approaches like Base Editing (BE) and Prime Editing (PE) are promising [44].

Irrespective to the nuclease or editing system, a major concern of CRISPR/Cas technology is the guide efficiency and specificity for a given gene [74]. Moreover, despite the huge repertoire of CRISPR-based molecular tools and their great potential for improving food and nutrition security, genome editing application in agriculture is still slowed down by some limitations. One of the main bottlenecks is the ability to regenerate transformed plants. This process is very time consuming and labour intensive; in many cases leading to very low transformation frequency especially for those species and genotypes which are recalcitrant to plant regeneration or suffer poor transformability. Due to this reason, it becomes essential to rely on highly efficient editing systems and, consequently, execute a flawless experimental design. This should include a careful choice of the most suitable editing system and the validation of the CRISPR reagents before undertaking the stable transformation. In particular, the evaluation of the guide efficiency is of utmost importance to ensure a successful CRISPR experiment.

2 Approaches and Constraints of Genome Editing Using CRISPR Technology

2.1 CRISPR-Based Editors

Many CRISPR-based genome editing tools have been developed to facilitate efficient plant genome engineering. Thanks to its flexibility, efficiency and low cost, CRISPR/Cas technology has been widely used in plants for fundamental and applied research.

CRISPR/Cas systems are divided into two distinct classes according to their structures and functions: class 1 systems (including types I, III, and IV) that use multiprotein complexes to destroy foreign nucleic acids, and class 2 systems (including types II, V, and VI) that use single proteins [93].

The CRISPR/Cas9 system (type II) is the most frequently used. It is composed of a Cas9 nuclease and an engineered single guide RNA (sgRNA). The sgRNA comprises a scaffold sequence necessary for Cas binding and a specific DNA sequence designed to be complementary to the target DNA site, followed by a short DNA sequence acting as a binding signal for Cas9 (Protospacer Adjacent Motif – PAM). Every Cas9 requires a specific PAM to recognize and cleave the target DNA: for example the widely used Cas9 from *Streptococcus pyogenes* (SpCas9) recognizes an 5′-NGG-3′ PAM sequence [134]. Cas9 contains two nuclease domains, HNH and RuvC, which break the double-stranded DNA (dsDNA) site primarily near the PAM in the target DNA. The resulting DSB is then usually repaired by the NHEJ repair pathway that is the most active repair mechanism; this usually leads to nucleotide insertions or deletions (indels) at the cleavage position. This system enables targeted mutations to be introduced into genomes with high efficiency, but the resulting mutations can vary and are not easy to predict.

Although SpCas9 is very efficient, its specific PAM requirement, target specificity and the large protein size limit its applications. To overcome this limitation, several Cas9 orthologs and variants have been studied for their suitability in genome editing, exhibiting diverse preferences for PAM sequences, and varying in their molecular weights. These include *Staphylococcus aureus* (SaCas9), *Neisseria meningitides* (NmeCas9), *Streptococcus thermophilus* (StCas9), *Campylobacter jejuni* (CjeCas9) and others [88]. The PAM sequences recognized by these Cas9s are relatively complex, which limits the widespread use of these nucleases in genome editing. However, some engineered Cas9 variants with an increased PAM compatibility have been reported, such as VQR-Cas9 (NGA PAM), VRER- Cas9 (NGCG PAM), EQR-Cas9 (NGAG PAM), xCas9 (NG, GAA and GTA PAM), SpCas9-NG (NG PAM), SpG, and SpRY, a near-PAMless SpCas9 variant (NRN>NYN PAMs) [88]. These variants allow the targeting of simple, non-canonical PAM sites, thus expanding the range of targetable sequences.

In addition to the Cas9 proteins, class 2 type V CRISPR-Cas systems involving Cas12a (or Cpf1) and Cas12b (or C2c1) have been adopted for modifying genomes at AT-rich PAM sequences [10], and presently AsCpf1, LbCpf1, and FnCpf1 are the most common types of Cpf1 used in genome editing. Another interesting alternative Cas nuclease recently identified is the type I CRISPR/Cas10 that causes long-range deletions [143]. An applicative example can be found in Osakabe et al. [111] where a 7.2 kb deletion has been obtained in tomato.

During CRISPR experiment design, a major concern is the guide efficiency and specificity. In fact, although this system can be programmed to virtually cleave any sequence preceding the PAM site sequence, it does not always succeed to target all the predicted sites [91]. Multiple guides designed with different target sequences determine the rate at which simultaneous modifications can be introduced in the genome and therefore the ability to perform comprehensive genome engineering at

corresponding specific sites [30]. This feature is especially important to edit multiple loci simultaneously in the same individual.

CRISPR system has also been used to achieve a precise change in the plant genome by different approaches like homology-directed DNA repair (HDR)-mediated GT [44], BE [50, 82] and PE [6, 44, 82].

GT is an HDR-mediated targeted gene replacement that requires the presence of a DNA donor template containing the desired sequence delivered with the guide and Cas9. GT enables specific nucleotide changes ranging from a single nucleotide change to large insertions or deletions. However, GT is inefficient because HDR occurs with extremely low frequency in plants, limiting the widespread use of this process for gene modification.

Different strategies have been applied to improve GT efficiencies, such as increasing the copy number of the repair template using geminivirus replicons or releasing the template from a T-DNA or manipulating the DNA repair pathway to improve HDR frequency [44], but despite these attempts, efficiencies are still low.

Unlike HDR, base editors (BE) do not require the formation of DSBs and a repair template. In general, a base editor is composed of an impaired nuclease, nickase (nCas9) or dead (dCas9) fused with a deaminase that can convert a nucleotide into a different one. According to the action types of deaminases, the BE systems are classified as the cytosine BE (CBEs convert C to T), the adenine BE (ABEs convert A to G) and dual-base editors. In these years several base editors have been developed using Cas protein variants with different PAM requirements (SpCas9, SaCas9, SaKKH-Cas9, VQR-Cas9, SpRY, SpCas9-NG), testing several deaminases to improve editing efficiency (APOBEC, BE3, AID, CDA1, A3A, ABE7.10, ABEmax, ABE8e, ABE9) and engineering their connection to Cas9 to alter position and width of the editing window [50, 102]. Currently, efficient editing from C to T and A to G have been achieved with the BE, but not all BE systems work equally in plants and the technology has still some limits. BE is limited by the targeting scope of Cas, it can only work in a narrow activity window and it has a low accuracy when multiple target nucleotides of the deaminase are present within the activity window. In addition, the purity of the cytosine base editing (CBE) product depends on the uracil N-glycosylase inhibitor (UGI).

Prime editing (PE) is a precise genome editing technology capable of introducing a predefined change in a genome without the need for a DSB. PE can achieve a variety of edits, including all 12 types of base substitution, small indels, and replacements. The PE system consists of a Cas9 nickase (nCas9) fused with a reverse transcriptase (RT) enzyme Moloney Murine Leukemia Virus RT (M-MLV RT), and a PE gRNA (pegRNA), an engineered standard sgRNA targeting the specific genome sites that specifies the target site and encodes the desired edit [6]. The nCas9/pegRNA complex binds to the desired target region and nicks one strand of the targeted DNA, providing a primer to initiate the production of edited DNA, while the pegRNA act as a template for reverse transcription.

Different versions of PEs have been developed in plants, such as PE1, PE2, PE3 e PE3b [102]. Unfortunately, the editing efficiency of PE is low. Different strategies have been used to increase PE efficiency, such as the use of alternative promoters for

the expression of Cas9 and RT, as well as the codon optimization of their coding sequences and the fusion with nuclear localization signals [50]. Furthermore, a recent study in rice has shown that designing primer binding site with a melting temperature of 30 °C and the use of two pegRNAs in trans encoding the same edits enhanced the editing efficiency [86]. However, the editing efficiency of PE is still low and further improvement is needed to broaden its application in plants, by optimizing key parameters such as RT enzyme type, experimental condition, and pegRNA design [50].

2.2 Plant Transformation and Regeneration Bottlenecks

To initiate a CRISPR-mediated genome editing experiment, CRISPR reagents are delivered into plant cells through various methods such as agrobacterium-based delivery [2], particle bombardment [112], or protoplast-based delivery [152]. Regardless of the delivery method, plant cells must undergo tissue culture procedures after transformation or transfection to obtain fully edited plants. The process of organogenesis involves three phases: cell dedifferentiation, cell reprogramming, and the development of new apical meristems (root apical meristem or shoot apical meristem) [11, 73]. These steps are challenging, time-consuming, and labor-intensive.

The success of the regeneration process depends on the ability of explant cells to overcome their programmed cell type. Once reprogramming and regeneration are activated, cells acquire a new fate, leading to the generation of new meristems and organs. However, the process is not linear and can encounter obstacles in each phase, affecting organ or plant development.

Factors such as the ratio of auxins and cytokinins [128], carbon sources, salts, vitamins ([25, 151], hormones [40], and the type of explant used [33] can influence the success of regeneration. Epigenetic factors [90] and other intrinsic factors like hormones, hormone receptors, transcription factors, and hormone signal transducers [11, 73] also play a role in guiding cell fate during the regeneration process.

Certain plant species, like *Nicotiana* and tomato [37, 48], can be regenerated *in vitro* with relatively high efficiency, while others like pepper [97] or fruit trees [95, 100, 137] exhibit strong recalcitrance to regeneration. One strategy to enhance regenerative capacity is the expression of key transcription factors involved in meristem organization and development, such as BABY BOOM [14, 131], WUSCHEL [13, 18, 58], and SHOOT MERISTEMLESS [18, 156].

The regenerative capacity of each species can be a limiting factor when using genome editing technologies. These processes can act as bottlenecks in the gene editing pipeline, particularly for plants that are difficult to culture and regenerate *in vitro*. Therefore, it is crucial to test and validate the efficiency of the CRISPR guides used for mutagenesis to increase the likelihood of successful mutagenesis events and reduce the number of plants needed for analysis after transformation and regeneration.

3 *In Silico* Designing of a Successful CRISPR Experiment

3.1 Features Affecting CRISPR-Mediated Editing

When designing a CRISPR experiment, the main issue to be taken into account for successful gene editing is the optimal trade-off between efficiency and specificity of CRISPR machinery. The ultimate goal is to maximize the on-target mutation rate and to avoid off-target mutations, which can occur when unintended genomic sites are cleaved due to sequence homology with the target site(s).

The specificity issue is less problematic in plant breeding than in clinical research because unwanted mutations can be segregated away from the on-target mutation(s) by crossing mutants with wild-type plants. However, the crossing procedure can be laborious, time-consuming, or even impossible for perennial plants and asexually propagated crops. Consequently, as general rule, it is important to choose the RNA guides with highest specificity scores (minimum off-target risk) [35, 103]. Being tightly dependent on the sequence homology, the off-target risk is normally predictable by *in silico* analysis and nowadays many bioinformatic tools are freely available. While SpCas9 tolerates single-base mismatches in the PAM-distal region, the proximal region is much more sensitive, even single mismatches can inhibit the cleavage [47, 62]. Then only guides whose off-sites have at least 1–2 mismatches within the PAM-proximal region should be considered highly specific.

On the contrary, mutagenesis efficiency is another bottleneck for genome editing in plants. In fact, low mutation frequency coupled with poor regeneration performance may jeopardize the success of the experiment leading to no edited plants.

Several aspects can affect a CRISPR-mediated editing experiment and concern intrinsic features of the nucleases and, most importantly, those of the RNA guides, which are different depending on the DNA sequence to be mutagenized.

When designing a CRISPR experiment, the first step is deciding which type of nuclease to use. In plants, the codon-optimized versions of SpCas9 from *Streptococcus pyogenes* are the most used nucleases. Normally its PAM (NGG) is well distributed in the genome [12], therefore, unless specific requirements are needed, NGG-PAM is usually suitable for generating loss-of-function mutants. While for T-rich regions, Cpf1 has found a wide application in plant genome editing [10]. However, in some cases it is necessary to edit specific genomic regions for which SpCas9 or Cpf1 cannot be used, e.g. in Gene-Targeting, Base Editing or Prime Editing experiments. Having a wide repertoire of gene editing tools at one's disposal guarantees a better chance of obtaining the desired mutation. To this end, many efforts have been made to engineer SpCas9 to expand its ability to recognize different [70] or more flexible PAMs [144]. Furthermore, Cas9 orthologues with different PAM preferences have been identified and used for GE [23, 46, 104, 120, 133].

Considering the huge genetic variability in microorganisms, it is expected to see an increasing number of Cas nucleases available for genome editing in the near future. To this end, Ciciani et al. [27] developed a computational pipeline to identify and isolate sequence-tailored Cas9 nucleases expanding the genome editing toolbox to respond to possibly any PAM requirement.

3.2 Selection of the Gene Region for Mutagenesis

The availability/distribution of PAMs within the gene of interest identifies potential target sites for mutagenesis. However, not all positions within the gene are equivalent. The choice of the sequence target depends on the specific aim of the editing. In certain cases, such as the removal or disruption of a specific regulatory element on the promoter or intron, or when modifying a particular sequence by Base Editing, Prime Editing or Gene-Targeting, there may be limited flexibility for selecting the target sequence. To knock-out gene function, it is generally recommended to target the coding sequence relatively close to the N-terminus to generate a premature termination codon (PTC). However, targeting of regions too close to the initial ATG might be impractical due to a lack of PAMs. Even if it were possible, there is a risk that other ATG codons downstream of the mutation could act as a translation re-initiation site, leading to N-terminally truncated proteins with partial activity [129]. If the functional or structural domain(s) of the protein are already characterized, the guide can be designed to target those specific domains, rendering the protein non-functional [125]. However, designing guides on a single exon might not guarantee a successful knockout if alternative splicing eliminates the mutated exon and produces a partially functional protein [129]. Other strategies include designing guides that bind on the exon-intron junctions to disrupt the splicing site and generate mis-processed mRNA, or attempting to delete the entire gene by designing target sequences upstream and downstream of the coding sequence. However, the latter approach may occur at a low frequency due to the length of the gene. Point mutations on the promoter and untranslated regions (UTRs) may have just a little effect on the expression and stability of the mRNA, respectively. For instance, it has been observed in mammalian cells that sgRNAs targeting the 5′ and 3′ UTRs were highly ineffective [34]. To ensure gene disruption, the best strategy is to employ multiple guides targeting different positions along the gene, leading to multiple mutations or deletions of varying size.

In addition to the selection of the nuclease and the position on target gene, another critical aspect in a CRISPR experiment is the guide's ability to form the complex with the nuclease and to trigger the cut on the target site. Several papers have investigated the characteristic of an ideal guide for optimal mutation induction, primarily based on SpCas9 system. It has been observed that nucleotide composition, GC content and secondary structure play a pivotal role in determining guide efficiency.

Wang et al. [145] found that the nucleotide composition at the PAM proximal region was an important factor determining cutting efficiency, in particular the purines (G/A) are preferred in the last 4 bases of the guide, while the pyrimidines are disfavored. These data have been substantially confirmed in other papers; in particular, there is a strong evidence about the preference of a G in the first position before the PAM [34, 103, 148] and the disfavoring of the T at the last 4 bases of the spacer [34, 148]. In plants, Liang et al. [83] did not find a specific relationship between spacer nucleotide composition and guide efficiency. This finding suggests

that there might be a distinction between animals and plants in this regard. However, it is worth noting that further analysis is required as the authors used fewer guides in their study.

CG content has a great impact on sgRNA efficacy [34, 91]. Doench et al. [34] found that sgRNAs with low (35%) or high (75%) CG content were less active in mammalian cells. Similarly, in plants Liang et al. [83] showed that for the 97% of the efficient guides, the CG content spanned between 30% and 80%; then this is the range to be taken into consideration when designing a guide.

In addition to base composition, secondary structures of sgRNA can affect the ability to form the ribonucleoproteic complex and/or its activity. Alterations in the canonical secondary structure of the sgRNA, can impede the interaction between the sgRNA scaffold and the Cas9 or the binding of the sgRNA seed sequence with the target DNA. Hairpin formation in the spacer region of the guide can prevent the recognition of the target DNA and so its cleavage [91]. Liang et al. [83] evaluated the secondary structure of a population of effective sgRNAs and found some common features which allowed to determine some criteria to design the guides. Based on this analysis, it was suggested to check the secondary structure and select guides with an overall intact tetra loop structure (especially for the loops 2, 3 and RAR), with no more than 12 spacer bases pairing with other bases of the sgRNA and no more than 7 consecutive base pairs. Moreover, the spacer sequence should have a low level of self-pairing with no more than 6 base pairs.

3.3 Computational Tools for Guide Activity Prediction

Conventionally, computational methods for efficient and specific guide designing can be classified into three groups: (i) alignment-based methods; suitable guides are designed based only on the alignment, and retrieving on the genome by locating the PAM sequence; (ii) hypothesis-driven; guide activity is predicted by using empirically rules (GC content or nucleotide composition at position 20); (iii) machine and deep learning-based; guides are scored by using training models which consider several features [71, 98, 149].

The most reliable predictions come from the hypothesis-driven and learning-based methods [146, 150, 154] because they are driven by previously described features [149]. However, learning-based models are considered the current cutting edge for *in silico* guide efficacy prediction. The development of reliable models requires large datasets of guides and their respective cut performance determined experimentally. Many algorithms have been developed to design guide suitable for SpCas9 like Azimuth 2.0, CRISPRpred, TUSCAN, CRISPRscan, sgDesigner and many others reviewed in Konstantakos et al. [71]. Furthermore, some computational algorithms emerged also for Cpf1, deepCpf1 [66] and SaCas9 [106]. To date, many web-based bioinformatic platforms are available for guide design and estimation of on- and off-target activity that rely on one or more above-mentioned computational methods [43].

Considering the wide availability of software tools for guide design, choosing the most appropriate is challenging. There are several features which should be taken into consideration such as the kind of input that the program allows, type of nucleases they support, algorithms used to predict on- and off-target activity, availability of genome of interest. The latter is a crucial point as a large number of software tools do not operate with guide design for plants. The most used and recommended tools for plants are: CRISPOR, CRISPR-P, RGEN Cas designer, or CHOPCHOP [43]. Among them, CRISPOR is one of the most complete and reliable. It supports hundreds of species and tens of different nucleases, giving a wide coverage in terms of organisms and molecular tools. Also, it integrates multiple scoring models for sgRNA efficiency prediction for SpCas9, in particular it uses CRISPRscan algorithm [103] and Azimuth 2.0 algorithm [35], considered a state-of-the-art model [71], but also deepCpf1 and Namj's models for Cpf1 and SaCas9, respectively. Furthermore, CRISPOR predicts CRISPR/Cas outcomes in terms of probability to obtain out-of-frame deletions based on the microhomology around the target site [7] or to obtain frameshift due to any type of insertion or deletions [24]. About the specificity, CRISPOR includes scores, such as MIT [47] and CFD [35], for off-targeting prediction and gives support for off-sites identification in the genome and for primer designing.

Albeit machine learning-based guide design algorithms represent exceptionally useful tool, they have some limitations. Most of computational models have been built by using datasets regarding guide performances of SpCas9. With the exception of SaCas9 and Cpf1, other cas nucleases remain completely deprived of reliable models for an optimal guide design. Furthermore, it is important to emphasize that the efficiency prediction reliability may depend on the cell type or on species [80], and that many algorithms have been developed with human or animal datasets. Therefore, it is not obvious that they work equally well for plants. Indeed, Naim et al. [105] examined the prediction performances of several on-line tools and they did not find a statistical correlation between software rankings and *in vivo* effectiveness measured in several plants. This suggests that current algorithms based on rules designed for guides in animals do not perform well for plants.

Consequently, further efforts are required to improve *in silico* guide design for plant genome editing. With this in mind, after a preliminary *in silico* evaluation of guide efficiency by using the most suitable tools, it is advisable to experimentally test their performance before starting with a plant stable transformation.

About prime editing, in addition to usual features of sgRNAs which mediate the binding on the target sequence, other aspects must be evaluated during pegRNA design. In fact, it has two more components affecting the editing efficiency, the RT template which guides the DNA repair and a PBS which anneals to the nicked target DNA strand [6]. It has been recommended that the length range should be between 9 and 15 nt for PBS and 10–15 for RT templates [68]. Moreover, Lin et al. [86] found that, in rice, the optimal melting temperature of PBS should be 30 °C.

Many computational tools have been developed for pegRNA design, Easy-Prime [78], PrimeDesign [48], pegFinder [26], PnB Designer [127], PINE-CONE [132], PE-Analyzer [53], peg-IT [4], PlantPegDesigner [61, 86]. Unfortunately, very few

are suitable for plants. The most advanced one is PlantPegDesign [61, 86] which was developed on the basis of rice experimental data and takes into consideration a series of known parameters such as optimal Tm, the exclusion of the first C in the 3′ extension of the RT template, length of RT, GC content of the PBS and the PE window. This tool is promising, but at the moment it has been used to predict efficient pegRNAs only in rice. More study would be necessary to investigate if PlantPegDesign algorithm parameters are suitable for other plants or if they need to be reevaluated and adjusted accordingly.

At present, the application of prime editing in plants presents significant challenges. Therefore, it would be appropriate to experimentally validate pegRNAs before starting stable transformation in order to save time and resources.

4 Experimental Approaches for CRISPR Reagent Validation

Stable transformation and plant regeneration are tedious, time-consuming and cost intensive. Moreover, for certain species, especially crops, they still pose a challenge [1]. Having efficient CRISPR reagents, particularly guides, is a crucial aspect to realistically obtain edited plants. Since reliable computational software, based on plant specific training datasets is lacking, the evaluation and selection of the most efficient guides should be carried out experimentally. Over time, a number of procedures have been proposed to validate the effectiveness of CRISPR reagents in plants (Fig. 6.1).

4.1 Endonuclease Cleavage In Vitro *Assay*

An easy way to test guide efficiency is through the endonuclease cleavage *in vitro* assay. It has been demonstrated that CRISPR reagents can successfully work *in vitro*, and protocols have been developed to produce and purify recombinant cas nucleases by heterologous expression. Additionally, guides can be prepared by *in vitro* transcription followed by ribonucleoprotein complex assembly and cleavage activity assays [3, 101]. This approach has been used to assess the effectiveness of the CRISPR system with the specific guide [60, 65, 67, 94]. Such a system is very simple and rapid, and the availability of commercial purified nucleases and *in vitro* transcription kits make even easier and attractive its application for guide evaluation. However, the reliability of this system is undermined by several aspects. The efficiency of T7-mediated *in vitro* transcription can be affected by the nucleotide composition of the initially transcribed region [31]. Consequently, the altered amount of guides transcript can affect the efficiency of the cleavage leading to potentially misleading results. Moreover, *in vitro* cleavage assay cannot simulate the *in vivo* expression level of nuclease and guide, which is one of the well-known factors affecting the mutagenesis rate. It has been shown that low concentrations of

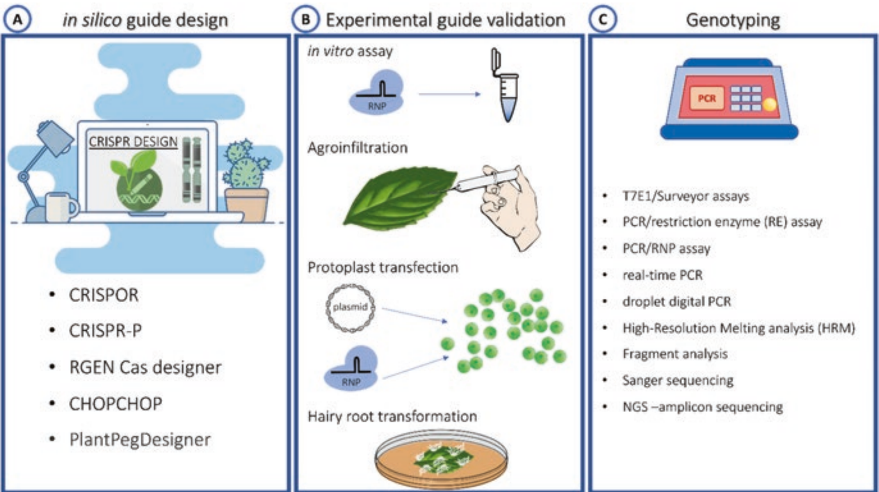


Fig. 6.1 Outline of CRISPR-mediated genome editing design experiments (**a**) Schematic representation of the first step in the CRISPR-mediated genome editing experiments, involving the use of bioinformatic tools to design specific and efficient guides suitable for plant genomes. The selected guides meeting the desired specificity and efficiency criteria undergo experimental validation as shown in (**b**). (**b**) Experimental validation methods for the selected guides, including *in vitro* cleavage assay as a pre-screening system to reduce the number of guides for *in planta* validation. Depending on the species and desired genotyping depth, different assays such as agroinfiltration, protoplasts, or hairy roots can be employed. (**c**) Analytical methods used for genotyping in genome editing experiments. Various techniques can be selected based on the specific requirements of the experiment. (Image sources: Some images have been obtained from the freely available collection on the pixabay.com website)

SpCas9 reduce on-target cleavage activity [47]. In addition, the *in vitro* assay does not replicate the accessibility to the genomic target sequence and the *in vivo* biochemical conditions for the folding of guide secondary structures. Recently, Sagarbarria and Caraan [123] found a discrepancy between *in vitro* and *in vivo* evaluation of sgRNA efficiency, as guides that appeared to be functional *in vitro* did not lead to successful mutagenesis in stable transformed plants of *S. melongena*.

Lastly, the *in vitro* approach, while can provide insights into cut efficiency, has limitations in predicting the types of mutations induced by the repair systems after the DSB. It also does not offer information regarding the ability of Base or Prime editors to introduce the desired edits, nor does it provide any information on the risk of off-targets in the genome.

For these reasons, the *in vitro* approach cannot be considered truly predictive of mutagenesis efficiency and should be taken into account only as pre-screening method. To better simulate physiological conditions and obtain reliable estimates of the mutagenesis rates, *in vivo* systems should be preferred.

4.2 Agroinfiltration Assay

Transient expression systems constitute a good compromise between ease and reliability for testing the efficacy of CRISPR reagents before proceeding with stable transformation. Acting directly on the genome, these systems provide more detailed and realistic information about the *in vivo* cleavage and mutagenesis efficiency. Additionally, they offer the advantage of being rapid to execute, as they do not involve time-consuming tissue culture.

Among the *in vivo* approaches used with plants, agroinfiltration is a rapid method that involves the use of special strains of *Agrobacterium tumefaciens*. These modified bacteria, that have inserted the CRISPR machinery genes into the T-DNA, are infiltrated into the intercellular space of plant tissues by syringe or vacuum infiltration. This process leads to the transient expression of nuclease and guide. Agroinfiltration can be performed in various parts of the plant, such as fruit [110], petal [52], whole seedling [75] and pollen [32]. However, the most commonly used organ for agroinfiltration is the leaf, for which numerous of protocols have been optimized (reviewed in [63]) and high level of expression and high transformation efficiency have been reached [130]. The main advantage of this technique is its rapidity, with high expression levels of transgenes typically achieved within a few days [63]. For this reason, transient agroinfiltration has been widely used as a preliminary experiment to test the effectiveness of constructs expressing guides and cas nucleases for *in vivo* targeting of specific genes through CRISPR-mediated genome editing.

In some cases, this system has been used as proof-of-concept study for assessing whether the CRISPR machinery was active in different species of interest [16, 59, 75, 107, 136], or for testing new cloning approaches to assemble CRISPR constructs [64, 141]. Moreover, several studies have reported the use of agroinfiltration as a technique to experimentally validate constructs before proceeding with stable transformation. Baltes et al. [8] tested the activity of Cas9 and sgRNAs in *Nicotiana benthamiana* to target BeYDV replicons. Zhang et al. [155] used the agroinfiltration to verify the mutagenesis efficiency of hundreds of guides in tomato and *N. benthamiana* to edit 63 immunity associated genes.

Overall, the agroinfiltration method has several limitations. The transformation efficiency may depend on many factors, including the biological compatibility and tissue accessibility of the plant species (and genotypes) with *A. tumefaciens* [147]. For example, comparing some CRISPR constructs harboring guides which recognize the identical target sites in both species, Zhang et al. [155] found a much lower mutagenesis in tomato than *N. benthamiana,* concluding that agroinfiltration system in tomato leaves can give misleading results underestimating the guides efficiency.

To overcome the incompatibility, the recalcitrance or the inaccessibility which make agroinfiltration problematic for some plant species, the guide efficiency could be determined by co-transforming the CRISPR system (Cas9 and guide) with its target DNA in *N. benthamiana*. This method was developed by Khan et al. [64] as a proof of concept that an exogenous gene (YFP) transiently co-transformed in

N. benthamiana together with CRISPR apparatus can be successfully mutated. This system could allow to assess virtually any guide for its target DNA from any species.

Another limitation of agroinfiltration is the inability to accurately estimate the mutagenesis efficiency due to the presence of genomic DNA from non-transformed cells. This can introduce variability in the results, as there is no selection of cells that have integrated the T-DNA. In particular, Li et al. [75] showed that the mutagenesis efficiency appeared to be higher when using protoplast transfection compared to foliar agroinfiltration in both *A. thaliana* and *N. benthamiana*. They proposed that this difference might be attributed to a higher gene transfer efficiency in protoplasts which leads to a different dilution ratio between transformed and untransformed genomic DNA.

4.3 Protoplast Assay

Protoplasts, plant cells deprived of cell wall, are very useful biological and biotechnological tools for both basic and applied plant science [152]. They are mainly, but not exclusively, obtained from leaf through mannitol-mediated plasmolysis and exposure of mesophyll cells to cell-wall-digesting enzymes (macerozyme and cellulase). Due to the high transfection efficiency and rapidity, protoplasts have become an excellent system to evaluate the effectiveness of CRISPR vectors in plants before attempting to transform an entire organism [152]. PEG-mediated protoplast transfection has been employed in a multitude of plant species to check the efficacy of designed CRISPR tools, not only in model plants like *Arabidopsis* [60], *N. benthamiana* [60] or *N. tabacum* [85], but also in several crops, including both dicotyledonous [88, 103] and monocotyledonous plants [15, 85].

Once the protocols for protoplast isolation and transfection are established, the protoplast platform to validate CRISPR constructs is simple, reliable, and not expensive. The yield of viable protoplasts is normally high, and this allows several tests to be carried out from the same preparation, e.g. to evaluate many CRISPR constructs or to examine different experimental conditions [9]. Moreover, protoplast transfection is suitable for both RNPs and plasmids, making it possible to assess not only the effectiveness of the cas-guide complex activity, but also different plasmid architectures, and to make a comparison between RNPs and plasmids. Jiang et al. [60] showed that RNPs are more efficient than plasmids, suggesting that, despite production and purification of cas nuclease and guides may be more challenging (or costly, if purchased) than extracting plasmid from bacteria, RNPs may offer better mutagenesis performance, providing a greater guarantee of successful editing of target genes. Another important feature of protoplast platform is its versatility. Many studies have focused on the evaluation of mutagenesis rate caused mainly by SpCas9 (and its variants or orthologs) and Cpf1 to give random mutations on target sites. Furthermore, it has been proven that protoplasts can be useful to detect also precise mutagenesis events, like those determined by HDR-mediated Gene-Targeting, Prime Editing [60] and Base Editing [39].

Although the plant protoplast platform is robust and widely applicable in multiple plant species, it does have certain limitations. Protoplasts, despite their high transfection efficiency, require specific expertise for their careful handling, from isolation to transfection. The transfection of protoplast generates thousands of independent events, which can be genotyped using various systems. For precise and detailed information about the mutations generated by the CRISPR machinery, amplicon sequencing by NGS is the preferred technology, but it requires specific expertise that may not always be readily available, unless an external service is used. Furthermore, even with NGS technology, there is still some uncertainty regarding the exact mutation rate and composition. This is because it is not possible to differentiate between transfected and non-transfected protoplasts from the total DNA extracted, unless a transfection marker, such as a GFP reporter, is employed.

4.4 Hairy Root Assay

A possible way to overcome the weaknesses of agroinfiltration and protoplast transfection can be given by *Agrobacterium rhizogenes* mediated hairy roots generation.

The hairy root system is a rapid and convenient approach for obtaining stably transformed roots. It is based on the natural ability of *Agrobacterium rhizogenes* (*Rhizobium rhizogenes*) to infect injured parts of the plant, triggering root organogenesis from the wounded sites and giving origin to the well-known "hairy root disease". The combination of hairy root approach and CRISPR/Cas techniques represents an excellent platform for an easy, rapid, accurate and cost-effective evaluation of CRISPR reagents, with the added value of a possible functional analysis of genes of interest in roots.

Currently, in the literature there are several articles reporting the use of hairy root transformation to deliver CRISPR vectors in a multitude of plant species such as tomato [56, 81, 121], potato [19], cucumber [108], soybean ([20];), *Brassica napus* [57], peanut [126], papaya [45], *Populus* [139], rubber dandelion [54], *Brassica carinata* [69], *Salvia miltiorrhiza* [76], *Medicago truncatula* [99].

The main advantage of the hairy root assay counts on the fact that transformed roots can serve as a simulation of stable whole plant transformation. Each root can be considered an independent transformation event, and the transformation efficiency is very high, as escapes can be avoided by antibiotic-mediated selection. These characteristics facilitate straightforward and rapid genotyping, enabling the calculation of the mutagenesis rate as the proportion of roots harboring at least one edited allele among the total number of transformed roots.

The drawbacks of this system include the need for some tissue culture procedures and a waiting time between the infection of the explant and formation of the hairy roots, which can range from 10 days to several weeks depending on the plant species. However, these drawbacks are offset by the simplicity and speed of genotyping using PCR followed by Sanger sequencing. The availability of commercial PCR kits with engineered DNA polymerase that are resistant to PCR inhibitors

present in plant extracts allows for direct genotyping of root fragments without the need for genomic DNA purification, using minimal amounts of plant material. This enables the amplification of numerous samples in a short period of time.

Many plant species, mostly dicotyledonous, are *A. rhizogenes* susceptible, consequently from these plants it is easy to obtain hairy roots transformed with T-DNA harboring CRISPR machinery. This characteristic makes the hairy root system a promising method of choice for evaluating guide efficiency or new CRISPR systems in a rapid and reliable way. Furthermore, it has been shown that tomato hairy roots exhibit morphological similarity to normal adventitious roots, making them a valuable tool for studying the function of genes in this organ [121]. In cases where target genes are completely inactivated, hairy roots can provide insights into the potential phenotypic changes that may arise in roots after stable transformation with CRISPR constructs.

A typical hairy root protocol workflow, established in our lab to evaluate CRISPR approaches in tomato, is described in the Fig. 6.2. With some adaptations, this protocol can serve as a roadmap for designing and validating genome editing experiment in other plant species.

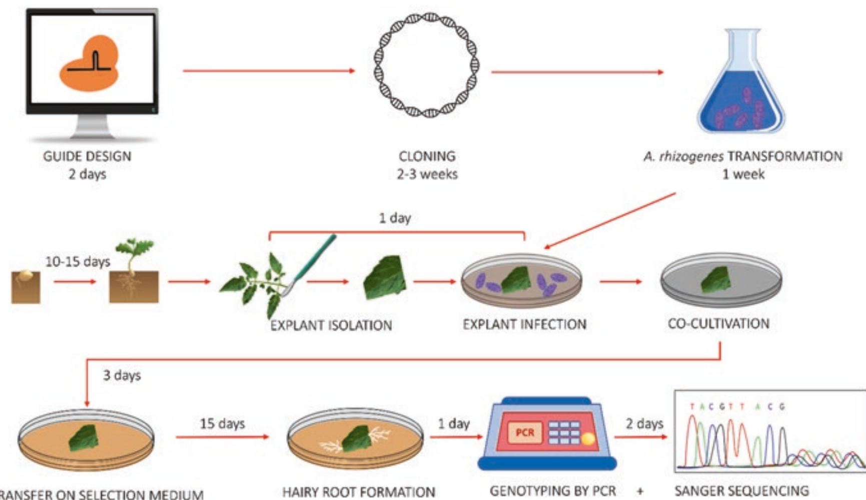


Fig. 6.2 Workflow of CRISPR construct assembly and hairy root generation from tomato explants. The workflow illustrates the steps involved in the generation of CRISPR constructs and the induction of hairy roots in tomato explants. First, computational tools are used to design the guides required for the CRISPR experiment. The guide expression cassette is then produced and assembled with the nuclease and antibiotic resistance expression cassettes. These components are combined to form the final constructs. The CRISPR constructs are cloned into *Agrobacterium rhizogenes* cells, which are used to infect cotyledon and/or leaf explants from 10–15 day old tomato plantlets. The infected explants undergo a co-cultivation process with *Agrobacterium rhizogenes* for 3 days. Subsequently, the explants are transferred to a selective medium and incubated. After approximately 2 weeks, the first hairy roots start to develop and can be collected. The collected hairy roots are then subjected to direct genotyping using PCR followed by Sanger sequencing to analyze the desired genomic modifications. (Image sources: Some images have been obtained from the freely available collection on the pixabay.com website)

5 Analytical Techniques to Estimate the Editing Efficiency

Genotyping is a pivotal step to assess the editing efficiency of CRISPR constructs or RNPs. Effective screening methods are necessary before undertaking the time-consuming transformation and regeneration procedures to validate the efficacy of genome-editing reagents (guide and nuclease) in order to increase the efficiency of genome editing. For this purpose, many experimental methods and bioinformatic tools have been developed to detect and analyze indels generated after CRISPR-mediated targeting.

The choice of analytical technique depends mainly on the type of information required in terms of mutagenesis occurrence, precise identification of indels and the accurate frequency of each type of mutation. Some editing approaches, such as Base Editing, Prime Editing and HDR-mediated knock-in, are not very efficient and the desired mutations may occur at very low frequencies. In these cases, the detection and quantification of induced mutations are very challenging and require specific methods for large-scale mutation detection. Conversely, when gene inactivation with typical out-of-frame mutations is desired, frequencies are normally higher, partly because a wide range of indels can result in a knockout effect. In these cases, techniques that can provide qualitative (or semi-quantitative) information about mutagenesis occurrence are preferred. Such techniques should be simple and cost-effective, should not require special equipment, and should enable the detection of a mutated allele in a background of wild-type alleles even when mutations are obtained at a very low frequency or in a complex polyploid plant genome.

Although sequencing will ultimately be required to confirm and identify the exact sequence of the mutant DNA, the availability of powerful high-throughput strategies during the screening stage can significantly reduce the cost involved in generating and identifying mutants.

A commonly used method for detecting modified genes relies on the enzymatic cleavage of heteroduplexes formed, after melting and re-annealing, by hybridization of wild-type and mutated DNA strands or two differently mutated DNA strands. A bacteriophage resolvase, such as T7E1, or single-strand-specific plant endonucleases, such as those of the CEL family (commercialized under the brand Surveyor) are used to recognize and digest unpaired heteroduplex DNA independently of the sequence. Therefore, they are suitable for screening of any target sequence [142]. Enzymatic digestion of heteroduplexes has been utilized, for example, by Cai et al. [20] to test target site editing efficiency in soybean hairy roots, by Khan et al. [64] for sgRNA efficiency testing in infiltrated leaves of *N. benthamiana*, and by Brandt et al. [15] to optimize wheat protoplasts transformation with CRISPR-Cas ribonucleoprotein complexes. A side-by-side comparison has shown that T7E1 identifies preferentially insertions and deletions, whereas Surveyor has better sensitivity to recognize substitutions [142]. The T7E1/Surveyor assays are reproducible, inexpensive, and easy to use, but are unable to differentiate between identically mutated (homozygous mutants) and wild-type alleles, or between biallelic mutants and heterozygous monoallelic mutants. Therefore, additional testing is necessary to

detect biallelic mutations, and homozygous mutant clones may be discarded as falsely reported wild type. Furthermore, the T7EI and Surveyor nuclease assays do not provide information about the type of induced mutation or the number of indels, making it impossible to exclude indels of (multiples of) three nucleotides. In addition, in polyploid species, false positive signals can arise from formation of heteroduplex between non-identical paralogs. In some cases, a heteroduplex mobility assay (HMA) using PAGE has been used to analyze heteroduplex formation instead of the enzymatic digestion, as reported by Hoang et al. [45] and Nguyen et al. [108] to test the efficiency of genome editing in hairy roots of papaya and cucumber, respectively.

Another classic method used for mutation detection is polymerase chain reaction (PCR)/restriction enzyme (RE) assay. If a restriction site is present in the target locus, the region around it can be amplified by PCR and run on an agarose gel after digestion with the RE to display the digestion pattern. When a mutation disrupts the restriction site, the amplified fragment remains uncut and appears as a single undigested band. Although it is straightforward and accessible to most laboratories, the PCR/RE method is heavily dependent on the availability of a restriction enzyme site near the target sequence, which is already constrained of a PAM for the nuclease at the cleavage site. Furthermore, each target sequence requires a specific set-up that hinders the general optimization of the protocol. Nevertheless, this approach is widely used for rapid and inexpensive screening of mutagenesis events, especially when optimizing protocols in less explored species such as in wild diploid potato relative [19] and Taraxacum [54] hairy roots, in tobacco and maize protoplasts [85], soybean protoplasts and hairy roots [135] or in agroinfiltrated *N. benthamiana* leaves [141].

Recently, a method based on PCR followed by digestion with purified ribonucleoprotein complexes of SpCas9 or FnCpf1 (known as PCR/RNP method) was reported to detect nuclease induced mutations in both polyploid and diploid plants [84]. This method is more applicable than PCR/RE as the CRISPR nucleases RNP will digest PCR products identical to the guide (wild type) but fail to digest PCR products with mutated sequences (mutants) without the need for the presence of the additional restriction site. According to the authors, the PCR/RNP method is less effective in detecting SNPs than indels, but the latter can still be distinguished from the wild-type sequence. In addition, the PCR/RNP method appears to be superior to the T7EI assay in terms of accuracy, and to Sanger sequencing in terms of sensitivity [84]. The main drawback of this screening strategies is that it requires purified *in vitro* transcribed guides and purified nucleases to preassemble CRISPR ribonucleoprotein (RNP) complexes.

To overcome the limitations of the PCR/RE method, several other PCR-based protocols have been developed, such as annealing at critical temperature PCR (ACT-PCR) [49], double-strand break site-targeted PCR (DST-PCR) [55], and bindel-PCR [124]. In addition, real-time PCR [114] and droplet digital PCR [115] are interesting alternatives to conventional PCR methods because they do not require post-PCR product manipulation. They are fast, high-throughput, and reduce the risk of laboratory contamination. Both protocols require the design of two

differently labelled probes recognizing different parts of the same amplicons. The probe designed outside the expected mutation position will bind to all alleles, allowing the assessment of the total amount of amplicons present in the sample, while the probe designed on the gene-editing target site will only bind to the wild-type sequence, thus revealing the presence of a new mutation. The ratio of the relative amplification values of the two probes in qPCR or the ratio of mutant droplets (positive for one fluorophore) to wild-type droplets (positive for a double fluorophore) in dPCR can then be used to distinguish wild-type, homozygous, and heterozygous mutations and quantify the mutation frequency of gene editing. qPCR and dPCR can detect single nucleotide indels or single nucleotide mutations with high sensitivity, especially qPCR. The cost of the labelled probes, the inability to detect large deletions in homozygous samples, and the need for direct sequencing to determine the exact mutated sequence are the major drawbacks of these two protocols.

Fluorescent PCR-capillary gel electrophoresis/DNA fragment analysis [119] and high-resolution melting curve analysis (HRM) [138] are also PCR based approaches that are successfully employed for genotyping genome editing events. Fluorescent PCR-capillary gel electrophoresis/DNA fragment analysis, of which several variants are available, employs fluorophore-labelled primers to amplify the genomic region containing the expected edited site. The labelled amplicons are then separated by capillary electrophoresis, and sized by comparison to an internal standard mixed with the sample before proceeding with electrophoresis. Data analysis guided by software identifies mutants based on the shifts in fragment size compared to the wild-type fragment. Recently, Carlsen et al. [21] successfully employed a specific fragment analysis strategy called Indel Detection by Amplicon Analysis (IDAA) to evaluate editing efficiency in tetraploid potato protoplasts in a study aimed at improving guide efficiency design [21]. Additionally, High Resolution Fragment Analysis (HRFA) described by Andersson et al. [5] has been utilized to test mutation efficiency during genome editing in tomato protoplasts [89], and the editing and regeneration protocol in rapeseed protoplasts [79]. Fragment analysis efficiently and accurately detects the number of nucleotides inserted or deleted at the target site, but is not accurate in detecting indels larger than 30 base pairs, and does not detect base substitutions or single nucleotide polymorphisms (SNPs). By providing information about the size and the relative abundance of different amplicons, fragment analysis allows detection of different alleles, homo- and heterozygosity, chimerism, sample mixtures, and genome editing efficiency. In addition, it offers a high sensitivity and resolution, being able to discriminate fragments that differ by a single base pair, and is a fast, high-throughput, automatable and multiplexable. On the other hand, it requires specialized equipment and software that are not commonly available and accessible to all laboratories. This also applies for another easy and fast techniques called High Resolution Melting (HRM), that combines PCR and heteroduplex formation. By increasing the temperature after PCR of small amplicons (about 100 bp) containing the target region, a specific and different dissociation profile is observed for homoduplex and heteroduplex of double stranded DNA fluorescently labelled. Subsequently, the Tm and the characteristic different signatures of PCR products are used by dedicated software to identify the presence of

mutant alleles without the need of additional manipulations after PCR. The main advantage of HRM protocol is that it can distinguish individual mutant alleles within a complex background, allowing detection of even rare mutation and chimerism as low as 5%. On the other hand, in addition to the cost of the equipment, a limitation of HRM is that it cannot detect large deletions due to the small size of the amplicons.

Certainly, Sanger sequencing of PCR products is the most direct and definitive approach to obtain detailed information about the type(s) of mutation in a sample, and is used in almost all studies, both to directly analyze amplified target regions and to accurately determine the mutated sequence after screening with one of the aforementioned methods. Although highly informative, sequencing methods are costly for high-throughput genotyping and require bioinformatic skills to analyze the data. In fact, chromatograms of PCR amplicons derived from complex samples (heterozygotes, biallelic mutants, chimera, polyploid species) can contain multiple traces, and several online bioinformatics tools, such as TIDE [17], DSDecode [87], and ICE [29], have been developed to decode the underlying mutation types.

Finally, thanks to its high sensitivity (0.01%), deep sequencing of the amplicons using NGS-based methods represents the gold standard for mutation detection, especially when targeted mutagenesis frequencies are low and rare editing events must be detected among a high background of unmodified alleles. The genomic region around the target site is amplified with a proofreading DNA polymerase, and the PCR products are barcoded (to distinguish the reads from independent amplifications of the target site) and indexed (to enable library multiplexing in the flow cell) to allow high-throughput sequencing. The raw data are then analyzed to calculate mutation efficiency as the percentage of reads containing indels in a defined window around the cleavage site, with the help of specific bioinformatic pipelines such as Cas-Analyzer [113], CRISPResso [116] or CRISPAltRations [72]. Several examples of deep sequencing of amplicons for assessing editing efficiency can be found in the literature, specifically in studies involving protoplast assay [9, 39, 60] or agroinfiltration assay [8, 96].

Unfortunately, due to the high costs and the need for specialized skills and bioinformatic tools, NGS methods are not widely applicable for the initial screening of edited lines.

6 Summary and Conclusions

CRISPR/Cas technology is considered the cutting-edge tool for genome editing for both fundamental and applied research in plants. It relies on a wide repertoire of molecular tools, including nucleases with the ability to target a large spectrum of PAM sequences, as well as precise editing approaches such as Base Editing and Prime Editing. This versatility makes CRISPR/Cas an invaluable technology. However, the challenge lies in obtaining transformed plants that can effectively express the editing machinery, as this step remains challenging for many plant

species. Without verifying the actual efficacy of the selected CRISPR reagents, undertaking a stable plant transformation can be demanding and risky using current transformation and regeneration protocols. Therefore, knowing the actual editing frequencies is crucial in selecting the most efficient guides and planning the editing experiment accordingly.

The development of computational tools enormously facilitates the selection of putative editing sites on target genes by predicting the most specific and efficient guides. The development of these computational models requires the use of large datasets regarding the performance of the experimentally determined guide. To date, computational models are not available for all editing approaches, especially for new nucleases, and it will be difficult to keep up with the continuous emergence of new molecular tools. Furthermore, bioinformatic tools built by using plant-specific datasets are almost completely lacking. For this reason, it is essential to have rapid systems capable of evaluating the performance of CRISPR reagents *in planta*. This allows for screening different guides and choosing the most effective one, as well as testing new editing systems quickly.

The *in vitro* test is extremely simple, but has many limitations, so it can only be considered as a pre-screening system, while the *in vivo* tests, such as agroinfiltration, protoplasts and hairy roots are to be preferred. The choice of the *in vivo* system depends mostly on the species and the desired type of genotypic data, which in turn relies on the analytical method used to determine the editing rate. The disadvantage of agroinfiltration and protoplasts is that they require NGS for accurate genotyping.

Among the *in vivo* platforms, the hairy roots represent a promising tool to test the efficacy of CRISPR reagents in *A. rhizogenes*-susceptible plants. This system combines the advantage of stable transformation with the rapid analysis of transient systems, allowing for accurate and rapid genotyping using PCR-based methods, and potentially Sanger sequencing, without the need for regenerating entire plants.

Acknowledgements The writing of this manuscript was supported by BIOTECH-Cisget project from the Italian Ministry of Agriculture (MiPAAF D.M. 15924) and SMART-BREED project (A0375E0166, POR FESR LAZIO 2014 – 2020), it was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

References

1. Altpeter, F., Springer, N.M., Bartley, L.E., Blechl, A., Brutnell, T.P., Citovsky, V., et al.: Advancing crop transformation in the era of genome editing. Plant Cell. (2016): tpc.00196.2016-tpc..2016
2. Amilia Pratiwi, R., Surya, M.I.: Agrobacterium-mediated transformation. Genet. Transform. Crops IntechOpen. (2020)
3. Anders, C., Jinek, M.: In vitro enzymology of Cas9. Methods Enzymol. **546**, 1–20 (2014)

4. Anderson, M.V., Haldrup, J., Thomsen, E.A., Wolff Jonas, H., Mikkelsen, J.G.: pegIT – a web-based design tool for prime editing. Nucleic Acids Res. **49**(W1), W505–W5W9 (2021)
5. Andersson, M., Turesson, H., Nicolia, A., Fält, A.S., Samuelsson, M., Hofvander, P.: Efficient targeted multiallelic mutagenesis in tetraploid potato (Solanum tuberosum) by transient CRISPR-Cas9 expression in protoplasts. Plant Cell Rep. **36**(1), 117–128 (2017)
6. Anzalone, A.V., Randolph, P.B., Davis, J.R., Sousa, A.A., Koblan, L.W., Levy, J.M., et al.: Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. **576**(7785), 149–157 (2019)
7. Bae, S., Kweon, J., Kim, H.S., Kim, J.-S.: Microhomology-based choice of Cas9 nuclease target sites. Nat. Methods. **11**(7), 705–706 (2014)
8. Baltes, N.J., Hummel, A.W., Konecna, E., Cegan, R., Bruns, A.N., Bisaro, D.M., et al.: Conferring resistance to geminiviruses with the CRISPR-Cas prokaryotic immune system. Nat. Plant. **1**(10) (2015)
9. Banakar, R., Schubert, M., Kurgan, G., Rai, K.M., Beaudoin, S.F., Collingwood, M.A., et al.: Efficiency, specificity and temperature sensitivity of Cas9 and Cas12a RNPs for DNA-free genome editing in plants. Front. Genome Editing. **3** (2022)
10. Bandyopadhyay, A., Kancharla, N., Javalkote, V.S., Dasgupta, S., Brutnell, T.P.: CRISPR-Cas12a (Cpf1): a versatile tool in the plant genome editing tool box for agricultural advancement. Front. Plant Sci. **11** (2020)
11. Bidabadi, S.S., Jain, S.M.: Cellular, molecular, and physiological aspects of in vitro plant regeneration. Plants. **9**(6), 702 (2020)
12. Bortesi, L., Zhu, C., Zischewski, J., Perez, L., Bassié, L., Nadi, R., et al.: Patterns of CRISPR/Cas9 activity in plants, animals and microbes. Plant Biotechnol. J. **14**(12), 2203–2216 (2016)
13. Bouchabké-Coussa, O., Obellianne, M., Linderme, D., Montes, E., Maia-Grondard, A., Vilaine, F., et al.: Wuschel overexpression promotes somatic embryogenesis and induces organogenesis in cotton (Gossypium hirsutum L.) tissues cultured in vitro. Plant Cell Rep. **32**(5), 675–686 (2013)
14. Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L., et al.: Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell. **14**(8), 1737–1749 (2002)
15. Brandt, K.M., Gunn, H., Moretti, N., Zemetra, R.S.: A streamlined protocol for wheat (Triticum aestivum) protoplast isolation and transformation with CRISPR-Cas ribonucleoprotein complexes. Front. Plant Sci. **11** (2020)
16. Bridgeland, A., Biswas, S., Tsakirpaloglou, N., Thomson, M.J., Septiningsih, E.M.: Optimization of gene editing in cowpea through protoplast transformation and agroinfiltration by targeting the phytoene desaturase gene. PLOS One. **18**, e0283837–e0283837 (2023). https://doi.org/10.1371/journal.pone.0283837
17. Brinkman, E.K., Chen, T., Amendola, M., Van Steensel, B.: Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. **42**(22) (2014)
18. Bull, T., Michelmore, R.: Molecular determinants of in vitro plant regeneration: prospects for enhanced manipulation of Lettuce (Lactuca sativa L.). Front. Plant Sci. **13**, 888425 (2022)
19. Butler, N.M., Jansky, S.H., Jiang, J.: First-generation genome editing in potato using hairy root transformation. Plant Biotechnol. J. **18**(11), 2201–2209 (2020)
20. Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., et al.: CRISPR/Cas9-mediated genome editing in soybean hairy roots. PLoS One. **10**(8) (2015)
21. Carlsen, F.M., Johansen, I.E., Yang, Z., Liu, Y., Westberg, I.N., Kieu, N.P., et al.: Strategies for efficient gene editing in protoplasts of Solanum tuberosum theme: determining gRNA efficiency design by utilizing protoplast (research). Front. Genome Editing. **3** (2022)
22. Čermák, T., Baltes, N.J., Čegan, R., Zhang, Y., Voytas, D.F.: High-frequency, precise modification of the tomato genome. Genome Biol. **16**(1) (2015)
23. Chatterjee, P., Jakimo, N., Jacobson, J.M.: Minimal PAM specificity of a highly similar SpCas9 ortholog. Sci. Adv. **4**(10) (2018)

24. Chen, W., McKenna, A., Schreiber, J., Haeussler, M., Yin, Y., Agarwal, V., et al.: Massively parallel profiling and predictive modeling of the outcomes of CRISPR/Cas9-mediated double-strand break repair. Nucleic Acids Res. **47**(15), 7989–8003 (2019)
25. Chimdessa, E.: Composition and preparation of plant tissue culture medium. J. Tissue Cult. Bioeng. (2020). https://doi.org/10.29011/2688-6502.000020
26. Chow, R.D., Chen, J.S., Shen, J., Chen, S.: A web tool for the design of prime-editing guide RNAs. Nat. Biomed. Eng. **5**(2), 190–194 (2020)
27. Ciciani, M., Demozzi, M., Pedrazzoli, E., Visentin, E., Pezzè, L., Signorini, L.F., et al.: Automated identification of sequence-tailored Cas9 proteins using massive metagenomic data. Nat. Commun. **13**(1), 6474 (2022)
28. Collias, D., Beisel, C.L.: CRISPR technologies and the search for the PAM-free nuclease. Nat. Commun. **12**(1) (2021)
29. Conant, D., Hsiau, T., Rossi, N., Oki, J., Maures, T., Waite, K., et al.: Inference of CRISPR edits from sanger trace data. CRISPR J. **5**(1), 123–130 (2022)
30. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., et al.: Multiplex genome engineering using CRISPR/Cas systems. Science. **339**(6121), 819–823 (2013)
31. Conrad, T., Plumbom, I., Alcobendas, M., Vidal, R., Sauer, S.: Maximizing transcription of nucleic acids with efficient T7 promoters. Commun. Biol. **3**(1), 439 (2020)
32. Deguchi, M., Bogush, D., Weeden, H., Spuhler, Z., Potlakayala, S., Kondo, T., et al.: Establishment and optimization of a hemp (Cannabis sativa L.) agroinfiltration system for gene expression and silencing studies. Sci. Rep. **10**(1), 3504 (2020)
33. Dhar, U., Joshi, M.: Efficient plant regeneration protocol through callus for Saussurea obvallata (DC.) Edgew. (Asteraceae): effect of explant type, age and plant growth regulators. Plant Cell Rep. **24**(4), 195–200 (2005)
34. Doench, J.G., Hartenian, E., Graham, D.B., Tothova, Z., Hegde, M., Smith, I., et al.: Rational design of highly active sgRNAs for CRISPR-Cas9–mediated gene inactivation. Nat. Biotechnol. **32**(12), 1262–1267 (2014)
35. Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., et al.: Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat. Biotechnol. **34**(2), 184–191 (2016)
36. Durai, S., Mani, M., Kandavelou, K., Wu, J., Porteus, M.H., Chandrasegaran, S.: Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. Nucleic Acids Res. **33**(18), 5978–5990 (2005)
37. Ebert, P.R., Clarke, A.E.: Transformation and regeneration of the self-incompatible species Nicotiana alata Link & Otto. Plant Mol. Biol. **14**, 815 (1990)
38. El-Mounadi, K., Morales-Floriano, M.L., Garcia-Ruiz, H.: Principles, applications, and biosafety of plant genome editing using CRISPR-Cas9. Front. Plant Sci. **11** (2020)
39. Fierlej, Y., Jacquier, N.M.A., Guille, L., Just, J., Montes, E., Richard, C., et al.: Evaluation of genome and base editing tools in maize protoplasts. Front. Plant Sci. **13** (2022)
40. Gerdakaneh, M., Badakhshan, H., Mohamadi, M., Arji, I.: Effect of different media and growth regulators on micropropagation of GF677. J. Plant Prod. **43**, 241–254 (2020). https://doi.org/10.22055/ppd.2019.27439.1667
41. Hahn, F., Eisenhut, M., Mantegazza, O., Weber, A.P.M.: Homology-directed repair of a defective glabrous gene in arabidopsis with cas9-based gene targeting. Front. Plant Sci. **9** (2018)
42. Hartung, F., Schiemann, J.: Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. Plant J. **78**, 742–752 (2014). https://doi.org/10.1111/tpj.12413
43. Hassan, M.M., Zhang, Y., Yuan, G., De, K., Chen, J.-G., Muchero, W., et al.: Construct design for CRISPR/Cas-based genome editing in plants. Trends Plant Sci. **26**(11), 1133–1152 (2021)
44. Hassan, M.M., Yuan, G., Liu, Y., Alam, M., Eckert, C.A., Tuskan, G.A., et al.: Precision genome editing in plants using gene targeting and prime editing: existing and emerging strategies. Biotechnol. J. **17**(10) (2022)

45. Hoang, T.H.T., Nguyen, N.H., Nguyen, L.T., Bui, T.P., Le, N.T., Dao, N.T., et al.: Developing a robust in vivo hairy root system for assessing transgene expression and genome editing efficiency in papaya. Plant Cell Tissue Organ Culture (PCTOC). **152**(3), 661–667 (2023)
46. Hou, Z., Zhang, Y., Propson, N.E., Howden, S.E., Chu, L.-F., Sontheimer, E.J., et al.: Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. Proc. Natl. Acad. Sci. **110**(39), 15644–15649 (2013)
47. Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., et al.: DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. **31**(9), 827–832 (2013)
48. Hsu, J.Y., Grünewald, J., Szalay, R., Shih, J., Anzalone, A.V., Lam, K.C., et al.: PrimeDesign software for rapid and simplified design of prime editing guide RNAs. Nat. Commun. **12**(1), 1034 (2021)
49. Hua, Y., Wang, C., Huang, J., Wang, K.: A simple and efficient method for CRISPR/Cas9-induced mutant screening. J Genet. Genomics. **44**(4), 207–213 (2017)
50. Hua, K., Han, P., Zhu, J.K.: Improvement of base editors and prime editors advances precision genome engineering in plants. Plant Physiol. **188**(4), 1795–1810 (2022)
51. Huang, T.K., Puchta, H.: CRISPR/Cas-mediated gene targeting in plants: finally a turn for the better for homologous recombination. Plant Cell Rep. **38**(4), 443–453 (2019)
52. Hussein, G.M., Abu El-Heba, G.A., Abdou, S.M., Abdallah, N.A.: Optimization of transient gene expression system in Gerbera jemosonii petals. GM Crops Food. **4**(1), 50–57 (2013)
53. Hwang, G.-H., Jeong, Y.K., Habib, O., Hong, S.-A., Lim, K., Kim, J.-S., et al.: PE-designer and PE-analyzer: web-based design and analysis tools for CRISPR prime editing. Nucleic Acids Res. **49**(W1), W499–W504 (2021)
54. Iaffaldano, B., Zhang, Y., Cornish, K.: CRISPR/Cas9 genome editing of rubber producing dandelion Taraxacum kok-saghyz using Agrobacterium rhizogenes without selection. Ind. Crop. Prod. **89**, 356–362 (2016)
55. Ijaz, F., Nakazato, R., Setou, M., Ikegami, K.: A pair of primers facing at the double-strand break site enables to detect NHEJ-mediated indel mutations at a 1-bp resolution. Sci. Rep. **12**(1) (2022)
56. Jacobs, T.B., Martin, G.B.: High-throughput CRISPR vector construction and characterization of DNA modifications by generation of tomato hairy roots. J. Vis. Exp. **110** (2016)
57. Jedličková, V., Mácová, K., Štefková, M., Butula, J., Staveníková, J., Sedláček, M., et al.: Hairy root transformation system as a tool for CRISPR/Cas9-directed genome editing in oilseed rape (Brassica napus). Front. Plant Sci. **13** (2022)
58. Jha, P., Ochatt, S.J., Kumar, V.: WUSCHEL: a master regulator in plant growth signaling. Plant Cell Rep. **39**(4), 431–444 (2020)
59. Jia, H., Wang, N.: Targeted genome editing of Sweet Orange using Cas9/sgRNA. PLoS One. **9**(4), e93806-e (2014)
60. Jiang, W., Bush, J., Sheen, J.: A versatile and efficient plant protoplast platform for genome editing by Cas9 RNPs. Front. Genome Editing. **3** (2021)
61. Jin, S., Lin, Q., Gao, Q., Gao, C.: Optimized prime editing in monocot plants using PlantPegDesigner and engineered plant prime editors (ePPEs). Nat. Protoc. **18**(3), 831–853 (2023)
62. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E.: A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science. **337**(6096), 816–821 (2012)
63. Kaur, M., Manchanda, P., Kalia, A., Ahmed, F.K., Nepovimova, E., Kuca, K., et al.: Agroinfiltration mediated scalable transient gene expression in genome edited crop plants. Int. J. Mol. Sci. **22**(19), 10882 (2021)
64. Khan, A.A., El-Sayed, A., Akbar, A., Mangravita-Novo, A., Bibi, S., Afzal, Z., et al.: A highly efficient ligation-independent cloning system for CRISPR/Cas9 based genome editing in plants. Plant Methods. **13**(1) (2017)
65. Kim, H., Kim, S.T., Ryu, J., Kang, B.C., Kim, J.S., Kim, S.G.: CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat. Commun. **8** (2017)

66. Kim, H.K., Min, S., Song, M., Jung, S., Choi, J.W., Kim, Y., et al.: Deep learning improves prediction of CRISPR–Cpf1 guide RNA activity. Nat. Biotechnol. **36**(3), 239–241 (2018)
67. Kim, H., Choi, J., Won, K.H.: A stable DNA-free screening system for CRISPR/RNPs-mediated gene editing in hot and sweet cultivars of Capsicum annuum. BMC Plant Biol. **20**(1) (2020)
68. Kim, H.K., Yu, G., Park, J., Min, S., Lee, S., Yoon, S., et al.: Predicting the efficiency of prime editing guide RNAs in human cells. Nat. Biotechnol. **39**(2), 198–206 (2021)
69. Kirchner, T.W., Niehaus, M., Debener, T., Schenk, M.K., Herde, M.: Efficient generation of mutations mediated by CRISPR/Cas9 in the hairy root transformation system of Brassica carinata. PLoS One. **12**(9), e0185429-e (2017)
70. Kleinstiver, B.P., Prew, M.S., Tsai, S.Q., Topkar, V.V., Nguyen, N.T., Zheng, Z., et al.: Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature. **523**(7561), 481–485 (2015)
71. Konstantakos, V., Nentidis, A., Krithara, A., Paliouras, G.: CRISPR–Cas9 gRNA efficiency prediction: an overview of predictive tools and the role of deep learning. Nucleic Acids Res. **50**(7), 3616–3637 (2022)
72. Kurgan, G., Turk, R., Li, H., Roberts, N., Rettig, G.R., Jacobi, A.M., et al.: CRISPAltRations: a validated cloud-based approach for interrogation of double-strand break repair mediated by CRISPR genome editing. Mol. Ther. Method Clin. Dev. **21**, 478–491 (2021)
73. Lardon, R., Geelen, D.: Natural variation in plant pluripotency and regeneration. Plants. **9**(10), 1261 (2020)
74. Lei, Y., Lu, L., Liu, H.Y., Li, S., Xing, F., Chen, L.L.: CRISPR-P: a web tool for synthetic single-guide RNA design of CRISPR-system in plants. Mol. Plant. **7**(9), 1494–1496 (2014)
75. Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., et al.: Multiplex and homologous recombination–mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat. Biotechnol. **31**(8), 688–691 (2013)
76. Li, B., Cui, G., Shen, G., Zhan, Z., Huang, L., Chen, J., et al.: Targeted mutagenesis in the medicinal plant Salvia miltiorrhiza. Sci. Rep. **7**(1), 43320 (2017)
77. Li, T., Yang, X., Yu, Y., Si, X., Zhai, X., Zhang, H., et al.: Domestication of wild tomato is accelerated by genome editing. Nat. Biotechnol. **36**(12), 1160–1163 (2018)
78. Li, Y., Chen, J., Tsai, S.Q., Cheng, Y.: Easy-Prime: a machine learning–based prime editor design tool. Genome Biol. **22**(1), 235 (2021a)
79. Li, X., Sandgrind, S., Moss, O., Guan, R., Ivarson, E., Wang, E.S., et al.: Efficient protoplast regeneration protocol and CRISPR/Cas9-mediated editing of glucosinolate transporter (GTR) genes in Rapeseed (Brassica napus L.). Front. Plant Sci. **12**, 680859 (2021b)
80. Li, C., Chu, W., Gill, R.A., Sang, S., Shi, Y., Hu, X., et al.: Computational tools and resources for CRISPR/Cas genome editing. Genomics Proteomics Bioinformatics. **21**, 108 (2022a)
81. Li, J., Scarano, A., Gonzalez, N.M., D'Orso, F., Yue, Y., Nemeth, K., et al.: Biofortified tomatoes provide a new route to vitamin D sufficiency. Nat. Plants. **8**(6), 611–616 (2022b)
82. Li, J., Zhang, C., He, Y., Li, S., Yan, L., Li, Y., et al.: Plant base editing and prime editing: the current status and future perspectives. J. Integr. Plant Biol. **65**(2), 444–467 (2023)
83. Liang, G., Zhang, H., Lou, D., Yu, D.: Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. Sci. Rep. **6**(1), 21451 (2016)
84. Liang, Z., Chen, K., Yan, Y., Zhang, Y., Gao, C.: Genotyping genome-edited mutations in plants using CRISPR ribonucleoprotein complexes. Plant Biotechnol. J. **16**(12), 2053–2062 (2018)
85. Lin, C.S., Hsu, C.T., Yang, L.H., Lee, L.Y., Fu, J.Y., Cheng, Q.W., et al.: Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. Plant Biotechnol. J. **16**(7), 1295–1310 (2018)
86. Lin, Q., Jin, S., Zong, Y., Yu, H., Zhu, Z., Liu, G., et al.: High-efficiency prime editing with optimized, paired pegRNAs in plants. Nat. Biotechnol. **39**(8), 923–927 (2021)
87. Liu, W., Xie, X., Ma, X., Li, J., Chen, J., Liu, Y.G.: DSDecode: a web-based tool for decoding of sequencing chromatograms for genotyping of targeted mutations. Mol. Plant. **8**(9), 1431–1433 (2015)

88. Liu, G., Lin, Q., Jin, S., Gao, C.: The CRISPR-Cas toolbox and gene editing technologies. Mol. Cell, 333–347 (2022a)
89. Liu, Y., Andersson, M., Granell, A., Cardi, T., Hofvander, P., Nicolia, A.: Establishment of a DNA-free genome editing and protoplast regeneration method in cultivated tomato (Solanum lycopersicum). Plant Cell Rep. **41**(9), 1843–1852 (2022b)
90. Liu, X., Zhu, K., Xiao, J.: Recent advances in understanding of the epigenetic regulation of plant regeneration. aBIOTECH. (2023). https://doi.org/10.1007/s42994-022-00093-2
91. Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., et al.: A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol. Plant. **8**(8), 1274–1284 (2015)
92. Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J., et al.: An updated evolutionary classification of CRISPR-Cas systems. Nat. Rev. Microbiol. **13**(11), 722–736 (2015)
93. Makarova, K.S., et al.: Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. Nat. Rev. Microbiol. **18**, 67–83 (2020). https://doi.org/10.1038/s41579-019-0299-x
94. Malnoy, M., Viola, R., Jung, M.-H., Koo, O.-J., Kim, S., Kim, J.-S., et al.: DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci. **7** (2016)
95. Mangal, M., Sharma, D., Sharma, M., Kumar, S.: In vitro regeneration in olive (Olea europaea L.) cv, 'Frontio' from nodal segments. Indian J. Exp. Biol. **52**, 912 (2014)
96. Martín-Pizarro, C., Triviño, J.C., Posé, D.: Functional analysis of the TM6 MADS-box gene in the octoploid strawberry by CRISPR/Cas9-directed mutagenesis. J. Exp. Bot. **70**(3), 949–961 (2019)
97. Martínez-López, M., García-Pérez, A., Gimeno-Páez, E., Prohens, J., Vilanova, S., García-Fortea, E.: Screening of suitable plant regeneration protocols for several capsicum spp. Through direct organogenesis. Horticulturae. **7**(9) (2021)
98. Mattiello, L., Rütgers, M., Sua-Rojas, M.F., Tavares, R., Soares, J.S., Begcy, K., et al.: Molecular and computational strategies to increase the efficiency of CRISPR-based techniques. Front. Plant Sci. **13** (2022)
99. Michno, J.-M., Wang, X., Liu, J., Curtin, S.J., Kono, T.J.Y., Stupar, R.M.: CRISPR/Cas mutagenesis of soybean and Medicago truncatula using a new web-tool and a modified Cas9 enzyme. GM Crops Food. **6**(4), 243–252 (2015)
100. Mitrofanova, I., Lesnikova-Sedoshenko, N., Mitrofanova, O., Smykov, A., Chelombit, S.: Comparative studies of in vitro regeneration capacity in some breeding forms of Prunus persica (L.) Batsch. BIO Web Conf. **24**, 00055 (2020)
101. Mohanraju, P., Oost, J., Jinek, M., Swarts, D.: Heterologous expression and purification of the CRISPR-Cas12a/Cpf1 protein. Bio-Protocol. **8**(9) (2018)
102. Molla, K.A., Sretenovic, S., Bansal, K.C., Qi, Y.: Precise plant genome editing using base editors and prime editors. Nat. Plant. **7**(9), 1166–1187 (2021)
103. Moreno-Mateos, M.A., Vejnar, C.E., Beaudoin, J.-D., Fernandez, J.P., Mis, E.K., Khokha, M.K., et al.: CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. Nat. Methods. **12**(10), 982–988 (2015)
104. Müller, M., Lee, C.M., Gasiunas, G., Davis, T.H., Cradick, T.J., Siksnys, V., et al.: Streptococcus thermophilus CRISPR-Cas9 systems enable specific editing of the human genome. Mol. Ther. **24**(3), 636–644 (2016)
105. Naim, F., Shand, K., Hayashi, S., O'Brien, M., McGree, J., Johnson, A.A.T., et al.: Are the current gRNA ranking prediction algorithms useful for genome editing in plants? PLoS One. **15**(1), e0227994-e (2020)
106. Najm, F.J., Strand, C., Donovan, K.F., Hegde, M., Sanson, K.R., Vaimberg, E.W., et al.: Orthologous CRISPR–Cas9 enzymes for combinatorial genetic screens. Nat. Biotechnol. **36**(2), 179–189 (2018)

107. Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J.D.G., Kamoun, S.: Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat. Biotechnol. **31**(8), 691–693 (2013)
108. Nguyen, D.V., Hoang, T.T.H., Le, N.T., Tran, H.T., Nguyen, C.X., Moon, Y.H., et al.: An efficient hairy root system for validation of plant transformation vector and CRISPR/Cas construct activities in cucumber (Cucumis sativus L.). Front. Plant Sci. **12** (2022)
109. Nicolia, A., Andersson, M., Hofvander, P., Festa, G., Cardi, T.: Tomato protoplasts as cell target for ribonucleoprotein (RNP)-mediated multiplexed genome editing. Plant Cell Tissue Organ Cult. **144**(2) (2021)
110. Orzaez, D., Mirabel, S., Wieland, W.H., Granell, A.: Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. Plant Physiol. **140**(1), 3–11 (2006)
111. Osakabe, K., Wada, N., Miyaji, T., Murakami, E., Marui, K., Ueta, R., et al.: Genome editing in plants using CRISPR type I-D nuclease. Commun. Biol. **3**(1) (2020)
112. Ozyigit, I.I., Yucebilgili Kurtoglu, K.: Particle bombardment technology and its applications in plants. Mol. Biol. Rep. **47**(12), 9831–9847 (2020)
113. Park, J., Lim, K., Kim, J.S., Bae, S.: Cas-analyzer: an online tool for assessing genome editing results using NGS data. Bioinformatics. **33**(2), 286–288 (2017)
114. Peng, C., Wang, H., Xu, X., Wang, X., Chen, X., Wei, W., et al.: High-throughput detection and screening of plants modified by gene editing using quantitative real-time polymerase chain reaction. Plant J. **95**(3), 557–567 (2018)
115. Peng, C., Zheng, M., Ding, L., Chen, X., Wang, X., Feng, X., et al.: Accurate detection and evaluation of the gene-editing frequency in plants using droplet digital PCR. Front. Plant Sci. **11** (2020)
116. Pinello, L., Canver, M.C., Hoban, M.D., Orkin, S.H., Kohn, D.B., Bauer, D.E., et al.: Analyzing CRISPR genome-editing experiments with CRISPResso. Nat. Biotechnol. **34**(7), 695–697 (2016)
117. Puchta, H.: Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. Curr. Opin. Plant Biol. **36**, 1–8 (2017)
118. Ricroch, A., Clairand, P., Harwood, W.: Use of CRISPR systems in plant genome editing: toward new opportunities in agriculture. Emerg. Top. Life Sci. **1**, 169–182 (2017). https://doi.org/10.1042/ETLS20170085
119. Ramlee, M.K., Yan, T., Cheung, A.M.S., Chuah, C.T.H., Li, S.: High-throughput genotyping of CRISPR/Cas9-mediated mutants using fluorescent PCR-capillary gel electrophoresis. Sci. Rep. **5** (2015)
120. Ran, F.A., Cong, L., Yan, W.X., Scott, D.A., Gootenberg, J.S., Kriz, A.J., et al.: In vivo genome editing using Staphylococcus aureus Cas9. Nature. **520**(7546), 186–191 (2015)
121. Ron, M., Kajala, K., Pauluzzi, G., Wang, D., Reynoso, M.A., Zumstein, K., et al.: Hairy root transformation using Agrobacterium rhizogenes as a tool for exploring cell type-specific gene expression and function using tomato as a model. Plant Physiol. **166**(2) (2014)
122. Rozov, S.M., Permyakova, N.V., Deineko, E.V.: The problem of the low rates of CRISPR/Cas9-mediated knock-ins in plants: approaches and solutions. Int. J. Mol. Sci. **20**(13) (2019)
123. Sagarbarria, M.G.S., Caraan, J.A.M.: Usefulness of current sgRNA design guidelines and *in vitro* cleavage assays for plant CRISPR/Cas genome editing: a case in eggplant (*Solanum melongena* L.). bioRxiv. (2023).:2023.03.19.532877-2023.03.19
124. Sakurai, T., Kamiyoshi, A., Takei, N., Watanabe, S., Sato, M., Shindo, T.: Bindel-PCR: a novel and convenient method for identifying CRISPR/Cas9-induced biallelic mutants through modified PCR using Thermus aquaticus DNA polymerase. Sci. Rep. **9**(1) (2019)
125. Shi, J., Wang, E., Milazzo, J.P., Wang, Z., Kinney, J.B., Vakoc, C.R.: Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. Nat. Biotechnol. **33**(6), 661–667 (2015)
126. Shu, H., Luo, Z., Peng, Z., Wang, J.: The application of CRISPR/Cas9 in hairy roots to explore the functions of AhNFR1 and AhNFR5 genes during peanut nodulation. BMC Plant Biol. **20**(1), 417 (2020)

127. Siegner, S.M., Karasu, M.E., Schröder, M.S., Kontarakis, Z., Corn, J.E.: PnB Designer: a web application to design prime and base editor guide RNAs for animals and plants. BMC Bioinformatics. **22**(1), 101 (2021)
128. Skoog, F., Miller, C.O.: Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. **11**, 118–130 (1957)
129. Smits, A.H., Ziebell, F., Joberty, G., Zinn, N., Mueller, W.F., Clauder-Münster, S., et al.: Biological plasticity rescues target activity in CRISPR knock outs. Nat. Methods. **16**(11), 1087–1093 (2019)
130. Sparkes, I.A., Runions, J., Kearns, A., Hawes, C.: Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat. Protoc. **1**(4), 2019–2025 (2006)
131. Srinivasan, C., Heidmann, I., Supena, E.D.J., Fukuoka, H., Joosen, R., Lambbalk, J., et al.: Heterolougus expression of the BABY BOOM AP2/ERF transcription factor enhances the regeneration capacity of tobacco (Nicotiana tabacum L.). Planta. **225**, 341–351 (2007)
132. Standage-Beier, K., Tekel, S.J., Brafman, D.A., Wang, X.: Prime editing guide RNA design automation using PINE-CONE. ACS Synth. Biol. **10**(2), 422–427 (2021)
133. Steinert, J., Schiml, S., Fauser, F., Puchta, H.: Highly efficient heritable plant genome engineering using Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus*. Plant J. **84**(6), 1295–1305 (2015)
134. Sternberg, S.H., Redding, S., Jinek, M., Greene, E.C., Doudna, J.A.: DNA interrogation by the CRISPR RNAguided endonuclease Cas9. Nature. **507**, 62–67 (2014). https://doi.org/10.1038/nature13011
135. Sun, X., Hu, Z., Chen, R., Jiang, Q., Song, G., Zhang, H., et al.: Targeted mutagenesis in soybean using the CRISPR-Cas9 system. Sci. Rep. **5** (2015)
136. Syombua, E.D., Zhang, Z., Tripathi, J.N., Ntui, V.O., Kang, M., George, O.O., et al.: A CRISPR/Cas9-based genome-editing system for yam (*Dioscorea* spp.). Plant Biotechnol. J. **19**(4), 645–647 (2021)
137. Teixeira da Silva, J.A., Gulyás, A., Magyar-Tábori, K., Wang, M.R., Wang, Q.C., Dobránszki, J.: In vitro tissue culture of apple and other Malus species: recent advances and applications. Planta. **249**(4), 975–1006 (2019)
138. Thomas, H.R., Percival, S.M., Yoder, B.K., Parant, J.M.: High-throughput genome editing and phenotyping facilitated by high resolution melting curve analysis. PLoS One. **9**, e114632–e114632 (2014). https://doi.org/10.1371/journal.pone.0114632
139. Triozzi, P.M., Schmidt, H.W., Dervinis, C., Kirst, M., Conde, D.: Simple, efficient and open-source CRISPR/Cas9 strategy for multi-site genome editing in *Populus tremula* × *alba*. Tree Physiol. **41**(11), 2216–2227 (2021)
140. Van Gent, D.C., Van Der Burg, M.: Non-homologous end-joining, a sticky affair. Oncogene. **26**(56), 7731–7740 (2007)
141. Vazquez-Vilar, M., Bernabé-Orts, J.M., Fernandez-del-Carmen, A., Ziarsolo, P., Blanca, J., Granell, A., et al.: A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard. Plant Methods. **12**(1) (2016)
142. Vouillot, L., Thélie, A., Pollet, N.: Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3: genes. Genomes Genet. **5**(3), 407–415 (2015)
143. Wada, N., Osakabe, K., Osakabe, Y.: Expanding the plant genome editing toolbox with recently developed CRISPR–Cas systems. Plant Physiol. **188**(4), 1825–1837 (2022)
144. Walton, R.T., Christie, K.A., Whittaker, M.N., Kleinstiver, B.P.: Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. Science. **368**(6488), 290–296 (2020)
145. Wang, T., Wei, J.J., Sabatini, D.M., Lander, E.S.: Genetic screens in human cells using the CRISPR-Cas9 system. Science. **343**(6166), 80–84 (2014)
146. Wang, J., Zhang, X., Cheng, L., Luo, Y.: An overview and metanalysis of machine and deep learning-based CRISPR gRNA design tools. RNA Biol. **17**(1), 13–22 (2020)

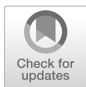
147. Wroblewski, T., Tomczak, A., Michelmore, R.: Optimization of agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. Plant Biotechnol. J. **3**(2), 259–273 (2005)
148. Xu, H., Xiao, T., Chen, C.-H., Li, W., Meyer, C.A., Wu, Q., et al.: Sequence determinants of improved CRISPR sgRNA design. Genome Res. **25**(8), 1147–1157 (2015)
149. Yan, J., Chuai, G., Zhou, C., Zhu, C., Yang, J., Zhang, C., et al.: Benchmarking CRISPR on-target sgRNA design. Brief. Bioinform. **19**(4), 721–724 (2018)
150. Yan, J., Xue, D., Chuai, G., Gao, Y., Zhang, G., Liu, Q.: Benchmarking and integrating genome-wide CRISPR off-target detection and prediction. Nucleic Acids Res. **48**(20), 11370–11379 (2020)
151. Yaseen, M., Ahmad, T., Sablok, G., Standardi, A., Hafiz, I.A.: Review: role of carbon sources for in vitro plant growth and development. Mol. Biol. Rep. **40**(4), 2837–2849 (2013)
152. Yue, J.-J., Yuan, J.-L., Wu, F.-H., Yuan, Y.-H., Cheng, Q.-W., Hsu, C.-T., et al.: Protoplasts: from isolation to CRISPR/Cas genome editing application. Front. Genome Editing. **3** (2021)
153. Zaidi, S.S.-E.-A., Mahfouz, M.M., Mansoor, S.: CRISPR-Cpf1: a new tool for plant genome editing. Trends Plant Sci. **22**(7), 550–553 (2017)
154. Zhang, G., Dai, Z., Dai, X.: C-RNNCrispr: prediction of CRISPR/Cas9 sgRNA activity using convolutional and recurrent neural networks. Comput. Struct. Biotechnol. J. **18**, 344–354 (2020a)
155. Zhang, N., Roberts, H.M., Van Eck, J., Martin, G.B.: Generation and molecular characterization of CRISPR/Cas9-induced mutations in 63 immunity-associated genes in tomato reveals specificity and a range of gene modifications. Front. Plant Sci. **11** (2020b)
156. Zhao, Q.-H., Fisher, R., Auer, C.: Developmental phases and STM expression during Arabidopsis shoot organogenesis. Plant Growth Regul. **37**(3), 223–231 (2002)
157. Zsögön, A., Čermák, T., Naves, E.R., Notini, M.M., Edel, K.H., Weinl, S., et al.: De novo domestication of wild tomato using genome editing. Nat. Biotechnol. **36**(12), 1211–1216 (2018)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Part II
Applications of Genome Editing

Chapter 7
Genome Editing of a Macroalgae with Possible Global Impacts



Hilde-Gunn Opsahl-Sorteberg and Espen Evju

Abstract Kelp forests are major marine ecosystems and key sources of biodiversity comparable to tropical forests, as pointed out by Darwin on the Beagle in 1834: *"Yet if in any country a forest was destroyed, I do not believe nearly so many species of animals would perish as would here, from the destruction of the kelp"*. Despite the key roles supporting marine life, our understanding of their biology lags far behind that of land plants. Kelp mitigates the effects of climate change, sequesters CO_2, reduces eutrophication while providing biomass for food, feed, and materials. Genome editing together with functional genomics can map genetic diversity potentials for temperature tolerance, important since they already face the upper tolerance limits in some regions. This chapter considers the major genome editing prerequisites; the transformation methods for introducing DNA/RNA and annotated genomes for predicting results. Risk assessments are discussed. These uses of genome editing show how widely applicable the techniques can be used from basic science to securing the global environment for our existence.

1 Genome Editing Prerequisites

Disappearance of species due to climate change is causing catastrophic changes to **biodiversity** within our ecosystems, affecting environmental and human health including threatening our survival. Policy makers recognize the need for actions to halt these losses and prevent extreme weather catastrophes, hopefully acting, leading to increased **science**-based activities **to secure our globe's future**. Species loss threatens human survival and quality of life directly by losing space, food/feed, and water resources and indirectly by reducing our health. Actions to halt or reverse planetary warming must involve policy leaders from across the globe and across the globe's environments. In the marine environment, **kelp forests** are highly relevant environments because they represent essential ecosystems supporting high levels of

H.-G. Opsahl-Sorteberg (✉) · E. Evju
Faculty of Biosciences, Norwegian University of Life Sciences (NMBU), Ås, Norway
e-mail: hilde-gunn.sorteberg@nmbu.no

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_7

biodiversity that cover extensive regions of our coastlines. Kelp species have many uses from **harvested wild** habitats and **aquaculture** production globally, where sugar kelp is **facing extinction** from the warmer ranges of its habitat and arctic populations are challenged by melting ice caps and reduced sea salt content [1, 2]. Losses of kelp forests affects **whole ecosystems** because these environments are habitats for numerous species ranging from microorganisms through photosynthetic plants to a range of marine animals including mammals. Kelp forest disappearance would also be disastrous for the global ecosystem because kelp is highly efficient at sequestering CO_2. **Newly available technologies** provide tools to evaluate genetic diversity and resilience. Uses of kelp have a long history, however fundamental and applied research are underfinanced, and progress lags behind other fields of plant and animal sciences. This situation is improving with the advent of increasing numbers of annotated genome sequences for kelp and the development of associated genomics-enabled tools including **genome editing**. The **aquaculture potential** of kelps in the blue economy should ideally be kept in balance with and isolated from wild populations [3, 4]. In addition, cultivation of local strains as material for rewilding needs to be considered. However, Europe is still missing clear guidelines for macroalgae management ranging from harvesting wild populations, cultivation systems, and breeding strategies and methods including genome editing. The Phycomorph COST action produced a report [5] providing a background for the development of guidelines for good practice in macroalgae exploitation. This chapter presents an updated overview of the **potential of applying genome editing** to secure future benefits from **macroalgae for global health** in the face of climate change.

The CRISPR/Cas9 system, discovered from the adaptive immune system of bacteria, has revolutionized the field of genome editing by providing a simple, efficient, and precise method for targeted gene modification [6].

Genome editing for research and applied purposes still depends on (1) adapted **gene transfer methods** and (2) **annotated genomes** for efficient and safe predictable uses. To secure reliable results and predictable outcomes, independent of positional effects and additional genome variation caused by somaclonal variation due to time in vitro casing mutations, robust gene transfer methods with minimal time in vitro are paramount [7]. This gene transfer is still species dependent in plants. The gene transfer methods are mostly limited to two; particle bombardment and agrobacterial T-DNA transfer. The number, accuracy and annotation quality of plant genome sequences are rapidly increasing since the Arabidopsis genome was released in 2000 [8]. Since the techniques for sequencing whole genomes have improved massively this prerequisite is mostly met both regarding directing the location of gene transfer when using CRISPR/Cas9 and the means to do predictable high quality risk assessment of well characterized modified land-plants. However, some species are lagging behind such as macroalgae where the molecular characterization is just emerging with promising insights to basic biological understanding and correlated applied opportunities [9].

1.1 Genetic Transformation Methods

Gene transfer was first achieved by a range of techniques in the 1970s and 1980s [10]. The first GM bacteria were produced in 1973 [11], human insulin production from GM bacteria was achieved in 1978 and FDA approved 1982 and in 1994 the first GM crop plant was approved (see e.g. ISAAA statistics: https://www.isaaa.org/resources/publications/briefs/05/download/isaaa-brief-05-1997.pdf). Since then, transgenics and resulting plants have been established for most species including many economically important crops, generating over 30 GM crop plants which are approved in over 40 countries including the USA, Canada, Argentina, EU, Norway, UK and Switzerland covering over 190 million hectares.

The two preferred gene transfer routes are still agrobacterium transfection if it is adapted to the plant species and particle bombardment mostly when agrobacterium cannot be applied. Agrobacterium is preferred since it is a “cleaner” method transferring less transgene copies, and into more appropriate genome locations causing less unintended effects. This could be because of natural selection pressure favoring successful integration for Agrobacterium [12]. *Agrobacterium tumefaciens* is a plant pathogenic bacterium commonly found in soil, which has a long history of transferring a portion of its Ti-plasmid, the T-DNA segment into host genomes for infection and survival. In nature the T-DNA allow the introduction of exogenous genes and subsequent expression of the corresponding proteins [13]. This has been adapted for research and gene transfer purposes, by replacing the agrobacterium tumor causing T-DNA and introducing any DNA of interest. The cloned DNA in the T-DNA region is placed into a binary vector before being introduced to the *Agrobacterium* cells. As the plant tissue becomes infected, the T-DNA integrates itself into the host’s genome, facilitating the stable expression of new genes [14].

Tobacco and tomato easily incorporate added DNA, while crop plants like cereals took decades of research before predictable transformation frequencies and reproducible results were achieved. Frequencies of regenerated transgenic plants have recently improved considerably for such recalcitrant species. This was partly due to an improved understanding of the cell cycles achieved by the introduction of related meristem developmental regulatory genes. Adding a growth regulating factor and a cofactor, greatly increased regeneration frequencies in wheat, triticale, rice and citrus [15]. CRISPR/Cas9 modifications of plant stem cells and the floral meristems have shown how the cell cycle and stem cells are related to growth and biomass production as well as yield from flowers, fruits and/or seeds [16–19]. As also seen in medical science, stem cell research is central to both basic and applied science, and plant breeding to meet sustainability goals for food production [20]. Further improvement by directing integration or integrating transgene constructs by homologous recombination by CRISPR/Cas9 has greatly improved predictability of targeted genome knock-out or transgene integration, while simultaneously reducing off-target effects by avoiding multiple inserted copies that are common when using particle bombardment for transgenesis.

Risk assessments and approval for commercialization of transgenic crops require characterization of the transgene integration, to secure single copy integration and detect possible unintended positional effects. This minimizes the risk of silencing the transgene as well as other parts of the genome. Somaclonal variation due to mutant effects of the transformation or time in tissue culture, has led to technological improvements that minimize time in culture, reducing unintended effects. Stable transfer depends on integration of transgenes into chromosomes. Some methods such as those to produce maximal amounts of transgene products, might have transgenes transiently active without nuclear integration or integration in organelles such as plastids.

1.2 Annotated Genomes

Genome sequences and analyses keep revolutionizing our understanding of basic biology such as stem cell functions and cell cycle implications, phylogeny, and evolution, providing a robust basis for deeper understanding of basic biological relationships and applications [21, 22]. This is because sequencing techniques have become increasingly accurate, efficient, and accessible. Also, shot-gun sequencing combined with long sequence reads provides reading through long repetitive sequences, such as centromeres, making it possible to identify unique regions for CRISPR/Cas9 specific alterations within any targeted genome part. This allows mapping the unknown parts of genomes, changing them from what some still refer as “junk-DNA” to informed molecular understanding [23]. Updated knowledge shows most of the genome is being transcribed to variable sized RNA molecules involved in gene regulation by RNAi like mechanisms [24, 25].

Specifically, such genomic methods are promising to fill the gaps in our understanding of microtubule directed cell cycle control, division planes, meristem functions and growth. The information generated is of great importance to medicine, food production and global health through allowing informed decisions for optimal sustainable uses of resources. Additionally, genome editing depends on well annotated genomes for risk assessments and applications. Thus, genome sequencing currently developed for new species allows adapting the CRISPR/Cas9 system in macroalgae. The other main limiting factor to applying genome editing to macroalgae are the gene transfer methods. Currently gene transfer is still only established for the green algae *Ulva* and the model brown algae *Ectocarpus*. This is expected to be extended to more species shortly like *S. latissima* from current efforts in Europe and Japan [9, 26].

1.3 Macroalgae Cultivation and Breeding

Macroalgae vegetate coastal and inshore areas and have been exploited by humans since prehistoric times. They are also important for providing a range of ecosystem services. Research is lagging behind land plants possibly partly because they are

positioned between land-based and fishery interests that are mainly further out at sea. They additionally compete with fish aquaculture e.g., the salmon industry, which often has higher financial significance in sea water areas. To secure native macroalgae populations from non-sustainable exploitation, some technology-based aquaculture production is developed for the dominant species such as the brown algae *S japonica* in the Eastern coastal regions of China, Japan, and Australia while *S latissima* occurs in the Atlantic cooler sea regions. Challenges for the exploitation of GM and GE algae are that sea regions interconnect and so cannot be easily contained as land plants. This means that regulations need to be established and agreed internationally.

Genome sequences are important to map genetic potential to survive strong selection pressures. Genome editing would be the best method to do both functional studies linking genes to temperature tolerance, and to enable breeding to meet requirements for sustainable algal strains that meet human needs.

The European Commission is responsible for EU's joint marine resources, but no regulatory frameworks and laws for macroalgae exploitation have been established at neither national nor European level [27]. The Phycomorph EU COST action FA1406 (2015–2019) drafted an extensive 200 page guideline "Pegasus" on sustainable aquaculture of seaweeds [5]. The impact of this on the EU Commission is unknown. An issue raised in several European countries is whether breeding can be applied to macroalgae being cultivated, where current cultivation of seaweed is largely carried out by local populations. Breeding would result in pre-cultivated gametophytes intercrossed in laboratory facilities to generate sporophyte "seedlings" for sea-based aquaculture cultivation of novel strains. So far only minimal breeding has been exploited, by crossing selected parental strains in *S. japonica*, and hardly any for *S. latissima* yet. Breeding might be needed to adapt sugar kelp for survival in warming sea locations; since temperature rise appears to be the main reason that sugar kelp populations have decreased.

2 Genome Editing – Important to Global Health for Mapping and Increasing Biodiversity to Survive Increasing Temperatures

Brown algae (*Phaeophyceae*) are ubiquitous, covering app. 25% of the world's coastlines and about half of them are in the Laminariales and Fucales. Kelps are efficient CO_2 sequesters and very efficient energy producers since they do not require terrestrial space, fresh water or added fertilizer. They therefore represent an important resource in the effort to mitigate the effects of global warming. In addition, kelps are effective purifying systems, removing organic pollutants from marine waters and they can also serve as monitors of the impact of climate change, particularly increased temperatures. Kelp growth is controlled by carbon allocation, primarily influenced by light, temperature, nutrient availability, and their genomic competence. The growth of the endemic Brazilian deep kelp *L. abyssalis*, for

example, is limited to the Austral summers while they tend to decrease in the Austral winter [1]. Consequently, little is known regarding the temporal variations in density and biomass of standing stock (individuals per m^2) for harvesting, commercial applications, or conservation of these species and, consequently, protecting the environment they inhabit. This is addressed in an ongoing Biodiversa+ project with 11 European and 2 Brazilian partners with some additional French and Norwegian associated partners in RESTORESEAS (https://www.restoreseas.net).

Generally, organisms can respond to changes in the environment by acclimation (phenotypic plasticity), or evolution (local adaptation). Local adaptation requires heritable genetic variation for traits that increase tolerance to the new environment, therefore, mapping the genetic variation for temperature tolerance is important to predict effects of rising seawater temperature and to possibly take actions to meet expected future sea water temperatures. Temperature tolerance in kelps has been shown to vary and is linked to genetic variation in the northern hemisphere for *Saccharina latissima* [28, 29] and *Laminaria digitata* [30]. For the cold-water kelps and the Laminaria pockets by the Brazilian and Moroccan coasts, the populations are declining and expected to disappear if they cannot adapt to warming sea regions. Comparative crosses within and between an arctic (Spitsbergen) and a temperate North Sea (Helgoland) population have shown heterosis effects positively affecting increased temperature tolerance in both populations, probably caused by increased heterozygosity. This even occurs if the introduced new alleles are from the northern population with less high temperature tolerance [30]. Previously genome sequencing in *Saccharina japonica* showed that the genetic variation within cultivated types were low compared to the genetic variation in the wild, since all cultivated populations descended from a few collected individuals from the wild [31]. This demonstrates that it is important to generate whole genome mapping/understanding to get accurate understanding of the genetic potential, also to avoid possible inbreeding depression in cultivated macroalgae due to accumulated reduced levels of genetic variation.

Pan genomes help anchoring newly sequenced individuals or closely related species as deeply sequenced and well annotated new accessions are generated, in addition to new genotypes from which to utilize the application of new tools including genome editing. Genome editing can facilitate fundamental functional studies to unravel seaweed biology from single genes to genomes and systems biology, including the kelps' coexisting biota. Annotated genomes will give us better mapping of genetic variation for important traits for survival of wild populations, as well as the productivity of cultivated populations. Mapping genetic variation for elevated temperature tolerance and variation in iodine content would build a solid foundation for selective breeding. The importance of understanding biodiversity was demonstrated when whole genomes of wild and cultivated *S. japonica* were re-sequenced in China, since that revealed existing genetic variation and the potentials from the populations [31].

A high quality annotated brown algae genome sequence is available for *Ectocarpus* (https://bioinformatics.psb.ugent.be/orcae/overview/EctsiV2). A chromosome-scale assembly of the *S. latissima* genome generated by the French

Genomique large-scale sequencing project Phaeoexplorer (https://phaeoexplorer.sb-roscoff.fr/home/) is expected to be released in early 2024 while the *L. ochroleuca* genome is currently being sequenced by the Restoresea project. This will add to the two available genomes: *S. japonica* and *Undaria pinnatifida* [32, 33], for interspecific comparisons. Genes that potentially play key roles in determining resilience to climate change, particularly climate warming, can be identified by combining results of the transcriptomic analyses with geographically correlated polymorphism information from the genomic analysis of population structure. Genes differentially regulated under temperature stress compared with control conditions and that show geographically correlated patterns of polymorphism, can be functionally assessed by CRISPR/Cas9 to select genes that are correlated with temperature tolerance. This approach may allow functionally annotate genes linked to resilience to temperature stress, with applied applications for breeding and survival in increasingly warmer seas [30].

3 Future Perspectives on Macroalgae Socioeconomics: From Ecology to Ecosystem Services

Genome editing is a powerful tool to meet important challenges in plant improvement that we face. GE allows **modelling** to predict the effects of temperature tolerance in wide crosses of kelp populations, to assess the effects of macroalgal growth on ecosystem dynamics [34, 35] and determine the potential for carbon sequestration under different climate scenarios [36]. The impact of climate change can be assessed based on no intervention on current kelp populations, a 2 °C temperature rise and the effects of above 2 °C increase in sea temperature [34, 37].

Growth as an integrative parameter of all physiological processes is controlled by carbon allocation that is primarily influenced by light, temperature, and nutrient availability, and their seasonal variations and interaction. Generally, most Northern Atlantic kelp species exhibit rapid growth from mid-winter to spring or early summer [38]. The pressure from anthropogenic activities on marine ecosystems have been driving severe changes in kelp distribution and abundance globally [39]. The decline in kelps forests can additionally affect a wide range of ecosystem services which are vital to human well-being (e.g., recreational and commercial fisheries activities). Examples of indirect influence on human well-being are: habitat provision for marine species, primary production, climate control, carbon storage, nutrient filtering and coastline protection [40].

Marine macroalgae (seaweed) net primary productivity (NPP) is of major ecological importance in the global carbon balance. Seaweeds form the largest and most productive underwater vegetated habitat on Earth, comparable to the terrestrial Amazon rain forest [36]. Global NPP datasets for 246 seaweed taxa from 429 individual sites distributed on all continents, from the intertidal to 55 m depth, underpin our increasing understanding of the importance of our ocean forests ecosystem

services. Their ecological contribution to annual aerial carbon production as well as to carbon production volumes are estimated to global averages of 656 and 1711 gC m^{-2} $year^{-1}$ in the subtidal and intertidal regions [41]. More than half of the macro algae species are brown algae; mainly Laminariales and Fucales. Brown algae (Phaeophyceae) are ubiquitous, dominating app. 25% of the world's coastlines and representing the major foundation of temperate coastal ecosystems, conservatively estimated to amount to an ecosystem value of $500,000–$1,000,000 per year per km of coastline [42]. The brown algae provide ecosystem services indirectly by increasing coastal production and habitat provision, and directly as fuel, feed, food and specialized products [43]. Well annotated genomes, functional genomics linking gene sequences with functions and application of this in breeding might be crucial for future survival of many macroalgae species and populations. GE is our best tool to answer fundamental questions about their biology, predict possibilities and generate solutions for their many services to the globe's ecology, climate, and more direct human interests.

References

1. Anderson, A.B., et al.: Global warming assessment suggests the endemic Brazilian kelp beds to be an endangered ecosystem. Mar. Environ. Res. **168**, 105307 (2021)
2. Diehl, N., Roleda, M.Y., Bartsch, I., Karsten, U., Bischof, K.: (PANGAEA, 2021)
3. Araújo, G.S., et al.: Sustainable Global Resources of Seaweeds Volume 1: Bioresources, Cultivation, Trade and Multifarious Applications. (Ranga Rao, A., Ravishankar, G.A. eds.) pp. 63–73. Springer, Cham (2022)
4. Corrigan, S., Brown, A.R., Ashton, I.G.C., Smale, D.A., Tyler, C.R.: Quantifying habitat provisioning at macroalgal cultivation sites. Rev. Aquac. **14**, 1671–1694 (2022)
5. Barbier, M., et al.: Development and objectives of the PHYCOMORPH European Guidelines for the Sustainable Aquaculture of Seaweeds (PEGASUS). Bot. Mar. **63**, 5–16 (2020)
6. Doudna, J.A., Charpentier, E.: Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science. **346**, 1258096 (2014)
7. Keshavareddy, G., Kumar, A.R.V., Ramu, S.V.: Methods of plant transformation- a review. Int. J. Curr. Microbiol. App. Sci. **7**, 2656–2668 (2018)
8. The Arabidopsis Genome Initiative: Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature. **408**, 796–815 (2000)
9. Badis, Y., et al.: Targeted CRISPR-Cas9-based gene knockouts in the model brown alga Ectocarpus. New Phytol. **231**, 2077–2091 (2021)
10. Horn, M.E., Woodard, S.L., Howard, J.A.: Plant molecular farming: systems and products. Plant Cell Rep. **22**, 711–720 (2004)
11. Cohen, S.N., Chang, A.C., Boyer, H.W., Helling, R.B.: Construction of biologically functional bacterial plasmids in vitro. Proc. Natl. Acad. Sci. U. S. A. **70**, 3240–3244 (1973)
12. Gelvin, S.B.: Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. Rev. **67**, 16–37, table of contents (2003)
13. Tiwari, M., Mishra, A.K., Chakrabarty, D.: Agrobacterium-mediated gene transfer: recent advancements and layered immunity in plants. Planta. **256**, 37 (2022)
14. Hwang, H.H., Yu, M., Lai, E.M.: Agrobacterium-mediated plant transformation: biology and applications. Arabidopsis Book. **15**, e0186 (2017)
15. Debernardi, J.M., et al.: A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat. Biotechnol. **38**, 1274–1279 (2020)

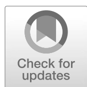
16. Liu, L., et al.: Enhancing grain-yield-related traits by CRISPR–Cas9 promoter editing of maize CLE genes. Nat. Plant. **7**, 287–294 (2021)
17. Yuste-Lisbona, F.J., et al.: *ENO* regulates tomato fruit size through the floral meristem development network. Proc. Natl. Acad. Sci. **117**, 8187–8195 (2020)
18. Roxrud, I., Lid, S.E., Fletcher, J.C., Schmidt, E.D.L., Opsahl-Sorteberg, H.-G.: GASA4, one of the 14-member Arabidopsis GASA family of small polypeptides, regulates flowering and seed development. Plant Cell Physiol. **48**, 471–483 (2007)
19. Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., Simon, R.: Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science. **289**, 617–619 (2000)
20. Fletcher, J.C.: The CLV-WUS stem cell signaling pathway: a roadmap to crop yield optimization. Plants (Basel). **7** (2018)
21. Bringloe, T.T., et al.: Phylogeny and evolution of the Brown Algae. Crit. Rev. Plant Sci. **39**, 281–321 (2020)
22. Spang, A., Mahendrarajah, T.A., Offre, P., Stairs, C.W.: Evolving perspective on the origin and diversification of cellular life and the Virosphere. Genome Biol. Evol. **14** (2022)
23. Naish, M., et al.: The genetic and epigenetic landscape of the Arabidopsis centromeres. Science. **374**, eabi7489 (2021)
24. Agrawal, N., et al.: RNA interference: biology, mechanism, and applications. Microbiol. Mol. Biol. Rev. **67**, 657–685 (2003)
25. Taning, C.N., et al.: RNA-based biocontrol compounds: current status and perspectives to reach the market. Pest Manag. Sci. **76**, 841–845 (2020)
26. Blomme, J., Liu, X., Jacobs, T.B., De Clerck, O.: A molecular toolkit for the green seaweed Ulva mutabilis. Plant Physiol. **186**, 1442–1454 (2021)
27. Lähteenmäki-Uutela, A., et al.: European Union legislation on macroalgae products. Aquac. Int. **29**, 487–509 (2021)
28. Li, H., et al.: Responses of the kelp Saccharina latissima (Phaeophyceae) to the warming Arctic: from physiology to transcriptomics. Physiol. Plant. **168**, 5–26 (2020)
29. Guzinski, J., et al.: Seascape genomics of the sugar kelp Saccharina latissima along the North Eastern Atlantic Latitudinal Gradient. Gene. **11**, 1503 (2020)
30. Liesner, D., et al.: Increased heat resilience of intraspecific outbred compared to inbred lineages in the kelp Laminaria digitata: physiology and transcriptomics. Front. Mar. Sci. **9** (2022)
31. Ye, N., et al.: Saccharina genomes provide novel insight into kelp biology. Nat. Commun. **6**, 6986 (2015)
32. Liu, T., et al.: Evolution of complex thallus alga: genome sequencing of Saccharina japonica. Front. Genet. **10**, 378 (2019)
33. Shan, T., et al.: First genome of the Brown Alga Undaria pinnatifida: chromosome-level assembly using PacBio and Hi-C technologies. Front. Genet. **11**, 140 (2020)
34. Jiang, L., et al.: Carrying capacity of Saccharina latissima cultivation in a Dutch coastal bay: a modelling assessment. ICES J. Mar. Sci. **79**, 709–721 (2022)
35. Campbell, I., et al.: The environmental risks associated with the development of seaweed farming in Europe – prioritizing key knowledge gaps. Front. Mar. Sci. **6** (2019)
36. Duarte, C.M., Bruhn, A., Krause-Jensen, D.: A seaweed aquaculture imperative to meet global sustainability targets. Nat. Sustain. **5**, 185–193 (2022)
37. Broch, O.J., Slagstad, D.: Modelling seasonal growth and composition of the kelp Saccharina latissima. J. Appl. Phycol. **24**, 759–776 (2012)
38. Kain, J.M., Jones, N.S.: The biology of Laminaria Hyperborea. VIII. Growth on cleared areas. J. Mar. Biol. Assoc. UK. **56**, 267–290 (1976)
39. Manuela Bernardes, B.: Kelps' long-distance dispersal: role of ecological/oceanographic processes and implications to marine forest conservation. Diversity. **10**, 11 (2018)
40. Wernberg, T., Krumhansl, K., Filbee-Dexter, K., Pedersen, M.F.: Status and trends for the World's Kelp forests. In: World Seas: An Environmental Evaluation. Elsevier (2019)
41. Pessarrodona, A., et al.: A global dataset of seaweed net primary productivity. Sci. Data. **9**, 484 (2022)

42. Filbee-Dexter, K., Wernberg, T.: Rise of turfs: a new battlefront for globally declining kelp forests. Bioscience. **68**, 64–76 (2018)
43. Ortega, A., et al.: Important contribution of macroalgae to oceanic carbon sequestration. Nat. Geosci. **12**, 748–754 (2019)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 8
A Short Review of Advances in Plant-Based Antigen Production Strategies and the Production of Viral Vaccine Antigens Derived from CRISPR/Cas9 Genome Edited *N. benthamiana* Plants for Enhanced Vaccine Efficacy



Espen Evju and Hilde-Gunn Opsahl-Sorteberg

Abstract Plant-based antigen manufacturing procedures have transformed vaccine research and industry by offering a cost-effective, scalable, and safe alternative to traditional protein production systems. This chapter discusses genome editing applications for plant-based protein production systems, antigen, and antibody manufacturing, as well as their future and current developments. The chapter briefly summarizes the several advantages of plant-based protein manufacturing platforms, including lower production costs, faster response to developing risks, and the absence of animal-derived components, which contributes to a lower risk of contamination and allergic responses. The chapter provides a basic overview of recent advances in plant-based antigen production, with a focus on vaccine antigens generated from CRISPR/Cas9 genome edited *Nicotiana benthamiana* to improve immunogenicity by altering plant glycosylation patterns to be more compatible with human glycosylation. Solving this could revolutionize existing vaccine production from plants to meet sustainable production objectives while also benefiting human health. These applications of genome editing demonstrate how versatile the approaches may be, from basic science to improving human health.

E. Evju (✉) · H.-G. Opsahl-Sorteberg
Faculty of Biosciences, Norwegian University of Life Sciences (NMBU), Ås, Norway
e-mail: espen.evju@nmbu.no

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_8

1 Production of Plant-Based Therapeutic Antigens and Antibodies

The growing demand for recombinant proteins, particularly for therapeutic uses, drives the development of alternative efficient and cost-effective production systems. Current widely used platforms for recombinant protein production include bacterial, yeast, insect, and mammalian cells. These traditional systems are limited by high production costs, human pathogen contamination, improper protein folding, and glycosylation patterns [1].

1.1 Background to Plant-Based Protein Production Systems

Plant-based expression systems exhibit increasing interest due to their innate ability to generate complex proteins with precise folding patterns and post-translational modifications, and their potential for scalability and reduced risk of pathogenic contamination [2]. In 2012, the United States Food and Drug Administration (FDA) approved taliglucerase alfa, a therapeutic protein produced in plants, a groundbreaking milestone for plant-based protein production [3]. Numerous other biopharmaceuticals have been generated since and been through rigorous clinical evaluations, exhibiting the practicality and viability of plant-based protein manufacturing platforms [4]. Plant-based production can be based on transient, chloroplast, or stable nuclear expression [5]. **Transient** expression systems involve the temporary activity from transferred genes of interest into plant cells' cytoplasm, typically via Agrobacterium-mediated infiltration or viral vectors [6]. This approach enables rapid protein production and has been used to produce a wide range of proteins, including monoclonal antibodies, vaccines, enzymes, and therapeutic proteins, such as human serum albumin and human somatotropin, in crops like rice and tobacco [7]. **Stable** transformation systems involve the integration of the target gene into the plant's nuclear or chloroplast genomes, generating transgenic plants that express the protein of interest through their life span and following generations if transferred to offspring by classical cross-breeding [8].

1.2 The Use of **Nicotiana benthamiana** *in Transient Expression Systems*

Nicotiana benthamiana, a close relative of tobacco (*Nicotiana tabacum*), is a diploid herbaceous plant native to Australia, which can grow through 6–8 weeks from seed to harvest of vegetative parts. The small genome size, approximately 3 Gb, facilitates genetic modification, while its high rate of inbreeding promotes genetic uniformity [9]. *Nicotiana benthamiana* is there for one preferred model plant for

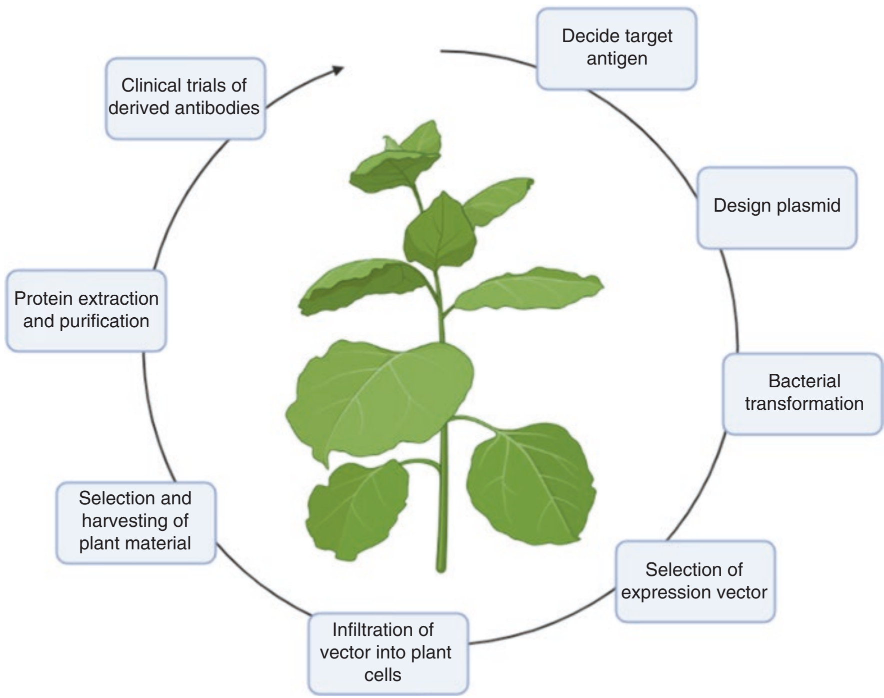


Fig. 8.1 An overview of the steps to produce proteins in a transient plant-based expression system

plant-based protein production due to its high transformation frequencies, rapid growth, high biomass yield, and "accepting" immune system, making it susceptible to many common biotechnology tools [10].

N. benthamiana has been widely employed for the expression of a diverse range of recombinant proteins, including antibodies, enzymes, and vaccine antigens. The transient expression system further allows for rapid production and evaluation of protein candidates, which is particularly valuable to respond to emerging infectious diseases, such as the production of ZMapp, a monoclonal antibody mix against Ebola virus [11], and generation of vaccine antigens in response to influenza viruses [12] (Fig. 8.1).

2 Antigen/Antibody Production for Sustainable Health Solutions

The *N. benthamiana* was decoded and compiled in 2012, offering an essential tool for exploring the functional genomics of *N. benthamiana* [13]. The primary assembly, Nb-1.0, spanned 3.1 Gb and comprised 46,220 anticipated protein-encoding genes. In 2023, a de novo whole-genome assembly was carried out in *N.*

benthamiana using Hifi reads, resulting in 1668 contigs with a combined length of 3.1 Gb [14]. The 21 lengthiest scaffolds, considered pseudomolecules, held a 2.8-Gb sequence, covering 95.6% of the assembled genome. A sum of 57,583 gene sequences with high confidence was anticipated.

The functional annotation of the *N. benthamiana* genome is a process that predicts gene functions by comparing them to known genes from other species and assessing experimental data. This method is vital for determining gene functionality and control, as well as aiding in pinpointing potential genome editing targets. Various tools have been established for the functional annotation of the *N. benthamiana* genome, such as the Sol Genomics Network (SGN) [15]. These repositories grant researchers access to genomic data, gene annotations, and functional projections, thus laying the groundwork for experimental inquiries guided by hypotheses and applications in biotechnology.

2.1 The Role of Glycosylation in Protein Function and Stability

Glycosylation is a post-translational modification of macromolecules, such as proteins and lipids, that involves the addition of carbohydrates to the final molecule. This modification affects the stability, activity, and immunogenicity of the molecule, and is particularly important in the production of recombinant proteins [16].

Despite the many benefits of plant-based protein manufacturing systems, the glycosylation patterns of plant and human proteins differ, which is a considerable challenge for plant-based production systems [17]. Protein folding, stability, and function depend on glycosylation, while such glycosylation patterns can affect plant-produced protein efficacy and immunogenicity in humans [18]. As such, the comprehension and modification of plant glycosylation patterns to emulate those of humans is essential and must be solved for efficient development and application of plant-derived biopharmaceuticals.

2.2 Key Differences Between Human and Plant Glycosylation Patterns

N-linked glycosylation, the covalent attachment of oligosaccharides to asparagine residues within the consensus sequence Asn-X-Ser/Thr, is a conserved modification among eukaryotes, including humans and plants [18]. However, despite conserving the core glycan structure, significant differences exist in the processing and maturation of N-glycans between humans and plants [19].

In humans, the intricate N-glycans exhibit prominent adornment with sialic acid residues and β1,4-galactose, which are nonexistent in plants. Additionally, the

Fig. 8.2 The key differences in post-translational glycosylation patterns between humans and plants

human N-glycans display core α1,6-fucosylation, while devoid of plant-specific β1,2-xylose and core α1,3-fucose residues (Fig. 8.2). These variances in N-glycan configurations can significantly impact the effectiveness and immunogenicity of recombinant proteins manufactured by plant-based expression systems, thus creating a significant obstacle that must be solved to develop plant-based biopharmaceuticals [20].

2.3 CRISPR/Cas9-Mediated Engineering of Glycosylation Patterns in **N. benthamiana**

In *Nicotiana benthamiana*, CRISPR/Cas9-mediated knockouts of the XylT and FucT genes have effectively eliminated plant-specific glycan structures, resulting in recombinant proteins with glycosylation profiles that more closely resemble human glycoproteins [21].

The study utilized CRISPR-Cas9 to create knockout lines of *N. benthamiana* plants to produce biopharmaceutical glycoproteins. The target genes XylT1, XylT2, FucT1, FucT2, FucT3, FucT4, and FucT5 were identified, and gRNAs were designed to target all genes in each group [21]. The gRNAs were tested by transient expression and then inserted into the binary pPAM vector carrying a plant-codon

optimized cas9 cassette with intron and a gRNA expression cassette under the control of the *A. thaliana* U6 promoter. Three variants of the knockout construct were prepared, one targeting XylT1 and 2, one targeting FucT1-4, and one with all seven gRNAs combined. The CRISPR constructs were then transformed into *N. benthamiana* plants using agroinfiltration. High-resolution melt analysis and Western blotting were used to confirm successful knockout of the targeted genes [21].

The CRISPR-Cas9 system works by introducing a double-strand break at a specific location in the genome, which is then repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). In the case of this study, the goal was to disrupt the function of specific genes involved in glycosylation, which can affect the efficacy of biopharmaceuticals. By creating knockout lines of *N. benthamiana* plants using CRISPR-Cas9, the researchers were able to produce glycoproteins with reduced or eliminated plant-specific glycans, which have comparable affinity to gold standard biopharmaceuticals produced in by using cells from Chinese hamster ovaries. (CHO).

A very recent paper utilizing this newly double knockout line of *N. benthamiana* investigated the impact of plant N-glycosylation on the immunogenic properties of a chimeric Hepatitis B Virus (HBV) S/L vaccine candidate produced in wild-type and XylT and FucT knockout lines of *N. benthamiana* [22]. The study found that prevention of b-1,2-xylose, and a-1,3-fucose attachment to the HBV antigen significantly increased the immune response in mice compared to the wild-type plant-produced counterpart. Notably, the antibodies triggered by the knockout-made antigen neutralized both wild-type HBV and a clinically relevant vaccine escape mutant more efficiently. The study validates the glycoengineered *N. benthamiana* as a substantially improved host for plant production of glycoprotein vaccines.

This work provides evidence that glyco-engineering of plants can significantly enhance the immunogenicity of plant-produced vaccines. Further research is ongoing to explore the potential of the CRISPR/Cas9 system in enhancing the immunogenicity of plant-produced antigens for human use. Using CRISPR/Cas9 in plant-based vaccine production provides an innovative avenue for developing novel cost-effective vaccines.

3 Socioeconomics of Plant-Based Protein Production Including Regulatory Issues

CRISPR/Cas9-mediated engineering of plant glycosylation patterns can produce human-like therapeutic proteins at lower cost, higher scalability, and with less contamination risk than traditional protein production platforms [23]. However, to ensure the safe and responsible development and use of plant-produced recombinant proteins, this technology's introduction into the biopharmaceutical industry raises several ethical, regulatory, and commercial concerns.

CRISPR/Cas9 enables quick and easy alteration of glycosylation patterns in plants. Consequently, apprehensions regarding health and environmental hazards associated with genetically modified organisms (GMOs) emerge [24]. While not exclusive to plant-based protein production, considering long-term repercussions of introducing genetically altered flora into ecosystems and devising strategies to mitigate potential risks is vital. CRISPR/Cas9-mediated plant engineering also falls under GMO regulations, which exhibit considerable variation across nations. Such regulations impact the development, manufacture, and distribution of plant-made recombinant proteins. Businesses must adapt their operations to ensure compliance within this intricate framework. Streamlining international regulations and enhancing the transparency of the approval process could facilitate wider utilization of this technology.

Regulatory approval and patient safety are contingent upon the safety and quality of plant-produced recombinant proteins. Regulatory bodies, including the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), mandate comprehensive safety and efficacy data for the authorization of therapeutic proteins—even those generated in plants. Due to the lower costs associated with plant-based protein production, life-saving medications could become accessible in low-income regions where cutting-edge medical treatments often remain elusive. From an ethical perspective, it is crucial to ensure that technological advancements' benefits are distributed equitably, preventing exacerbation of existing global health disparities.

The recent research into the intricacies of plant-specific glycosyltransferases, such as β1,2-xylosyltransferase (XylT) and core α1,3-fucosyltransferase (FucT), has laid the foundation for gene-editing methods to further engineer plant glycosylation patterns. By employing CRISPR/Cas9 technology, the modification of plant glycosylation patterns becomes more streamlined, enabling alterations to specific genes with exceptional accuracy and effectiveness, ultimately obtaining engineered plants that produce glycoproteins resembling their human equivalents. The ongoing fine-tuning of CRISPR/Cas9, which include the creation of new Cas9 variations, guide RNA designs, and the adoption of high-throughput screening methods, has the capacity to further advance gene-editing procedures within plants, making them even more precise and efficient.

The modification of glycosylation patterns in plants holds the promise to transform the biopharmaceutical industry. Plant-based expression systems can contribute to the discovery of innovative therapeutic proteins that boost enhanced pharmacological properties by offering an affordable, scalable, and secure alternatives to mammalian cell culture. Continued efforts in this area could potentially give rise to new treatments for a broad spectrum of illnesses.

References

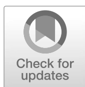
1. Tripathi, N.K., Shrivastava, A.: Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. Front. Bioeng. Biotechnol. **7**, 420 (2019)
2. Kulshreshtha, A., Sharma, S., Padilla, C.S., Mandadi, K.K.: Plant-based expression platforms to produce high-value metabolites and proteins. Front. Plant Sci. **13**, 1043478 (2022)
3. Zimran, A., Wajnrajch, M., Hernandez, B., Pastores, G.M.: Taliglucerase alfa: safety and efficacy across 6 clinical studies in adults and children with Gaucher disease. Orphanet J. Rare Dis. **13**, 36 (2018)
4. Shanmugaraj, B., Phoolcharoen, W.: Plant molecular farming: a viable platform for recombinant biopharmaceutical production. Plants (Basel). **9**, 842 (2020)
5. Santoni, M., Gecchele, E., Zampieri, R., Avesani, L.: Plant-based systems for vaccine production. Methods Mol. Biol. **2412**, 95–115 (2022)
6. Canto, T.: Transient expression systems in plants: potentialities and constraints. Adv. Exp. Med. Biol. **896**, 287–301 (2016)
7. Fahad, S., et al.: Recent developments in therapeutic protein expression technologies in plants. Biotechnol. Lett. **37**, 265–279 (2015)
8. Lorence, A., Verpoorte, R.: Gene transfer and expression in plants. Methods Mol. Biol. **267**, 329–350 (2004)
9. Derevnina, L., Kamoun, S., Wu, C.-H.: Dude, where is my mutant? Nicotiana benthamiana meets forward genetics. New Phytol. **221**, 607–610 (2019)
10. Eidenberger, L., Kogelmann, B., Steinkellner, H.: Plant-based biopharmaceutical engineering. Nat. Rev. Bioeng. **1**, 426 (2023)
11. Budzianowski, J.: Tobacco against Ebola virus disease. Przegl. Lek. **72**, 567–571 (2015)
12. Shanmugaraj, B., Rattanapisit, K., Manopwisedjaroen, S., Thitithanyanont, A., Phoolcharoen, W.: Monoclonal antibodies B38 and H4 produced in Nicotiana benthamiana neutralize SARS-CoV-2 in vitro. Front. Plant Sci. **11**, 589995 (2020)
13. Bombarely, A., et al.: A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. Mol. Plant-Microbe Interact. **25**, 1523–1530 (2012)
14. Kurotani, K.I., et al.: Genome sequence and analysis of Nicotiana benthamiana, the model Plant for Interactions between organisms. Plant Cell Physiol. **64**, 248–257 (2023)
15. Fernandez-Pozo, N., et al.: The Sol Genomics Network (SGN)–from genotype to phenotype to breeding. Nucleic Acids Res. **43**, D1036–D1041 (2015)
16. Berger, M., Kaup, M., Blanchard, V.: Protein glycosylation and its impact on biotechnology. Adv. Biochem. Eng. Biotechnol. **127**, 165–185 (2012)
17. Schillberg, S., Spiegel, H.: Recombinant protein production in plants: a brief overview of strengths and challenges. Methods Mol. Biol. **2480**, 1–13 (2022)
18. Liu, L.: Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and Fc-fusion proteins. J. Pharm. Sci. **104**, 1866–1884 (2015)
19. Fogarty, C.A., Harbison, A.M., Dugdale, A.R., Fadda, E.: How and why plants and human N-glycans are different: insight from molecular dynamics into the “glycoblocks” architecture of complex carbohydrates. Beilstein J. Org. Chem. **16**, 2046–2056 (2020)
20. Viinikangas, T., Khosrowabadi, E., Kellokumpu, S.: N-glycan biosynthesis: basic principles and factors affecting its outcome. Exp. Suppl. **112**, 237–257 (2021)
21. Jansing, J., Sack, M., Augustine, S.M., Fischer, R., Bortesi, L.: CRISPR/Cas9-mediated knockout of six glycosyltransferase genes in Nicotiana benthamiana for the production of recombinant proteins lacking beta-1,2-xylose and core alpha-1,3-fucose. Plant Biotechnol. J. **17**, 350–361 (2019)
22. Pantazica, A.M., et al.: The “humanized” N-glycosylation pathway in CRISPR/Cas9-edited Nicotiana benthamiana significantly enhances the immunogenicity of a S/preS1 Hepatitis B Virus antigen and the virus-neutralizing antibody response in vaccinated mice. Plant Biotechnol. J. **21**, 1176 (2023)

23. Ahmad, A., Pereira, E.O., Conley, A.J., Richman, A.S., Menassa, R.: Green biofactories: recombinant protein production in plants. Recent Pat. Biotechnol. **4**, 242–259 (2010)
24. Tsatsakis, A.M., et al.: Environmental impacts of genetically modified plants: a review. Environ. Res. **156**, 818–833 (2017)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 9
Precise Gene Editing of Cereals Using CRISPR/Cas Technology



Pouneh Pouramini and Goetz Hensel

Abstract Targeted mutagenesis using CRISPR/Cas technology has become routine in elucidating biological processes or their application in breeding and agriculture. This means that the change to be achieved can be accurately predicted. However, knockout of a gene function is not always desirable, as reducing activity or affecting a protein domain can influence its properties and, thus, the phenotype. This chapter will therefore focus on precise genome modification in temperate cereals. The methods used, including some representative examples, are summarised here.

Time is an essential factor to consider in developing new agricultural varieties. Since domestication, plant breeders have steadily expanded their toolbox, but establishing a new cereal variety takes an average of 8–10 years [1]. Developments in genome sequencing (barley [2], wheat [3], rye [4]), oat [5], and molecular biology methods for genome-assisted breeding (marker-assisted breeding [6]) have provided tools and techniques for the breeding process that positively influence the process and workload.

In this chapter, temperate cereals of the botanical tribe Triticeae (barley, wheat, rye, and oats) grown in Europe will be considered. Due to limitations in the availability of genomic sequences (rye and oat only in the last two years) and the lack of efficient transformation protocols, there are currently only reports from barley and wheat. These are also among the more essential cereals in Europe in terms of cultivated area (FAO Stat). While diploid barley is mainly used for animal feed, beer, and whisky, tetra- and hexaploid wheat are essential for pasta and bakery products. Oat and rye are the main components of breakfast cereals.

Targeted mutagenesis induced by endonucleases such as TALEN [7] and CRISPR/Cas [8] has enabled an incredible number of applications in a wide range of species since their first biotechnological application in 2012 [9]. Thus, the results have helped many new insights into basic research and show promise for

P. Pouramini · G. Hensel (✉)
Heinrich-Heine-University, Düsseldorf, Germany
e-mail: goetz.hensel@hhu.de

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_9

applications. Targeting an organism's genomic sequence has never been so easy. CRISPR/Cas technology is a two-component system in which a target-specific guide RNA guides a double-strand-inducing Cas enzyme to the desired location in the target genome [8]. The cell's repair mechanisms then repair the induced double-strand breaks. A more detailed description of the technology and repair mechanisms is described in Chap. 1.

Applications of precise genome editing in plants have been summarised several times (for review, see [10–12]). These reach into all areas of modern plant research. Starting with pure basic research, i.e., the identification and characterization of genes and their phenotypic expressions, the methods are also used to master the current challenges of agriculture. Here, agronomic parameters play a similarly important role in improving product characteristics. Plants with enhanced resistance to fungal or viral pathogens are being developed and tested. Plants should be better adapted to changing climatic conditions and thus have better water and nutrient utilization. Plants should become heat or drought-tolerant but also be able to grow with an increased salt concentration in the soil. There are examples of all these experiments in barley and/or wheat (Fig. 9.1). The only publication on applying CRISPR/Cas technology in triticale, a cross between wheat and rye, shows its functionality exclusively in protoplasts [13].

Even if the targeted induction of a double-strand break can be carried out precisely, the result is random. According to previous reports, the most common

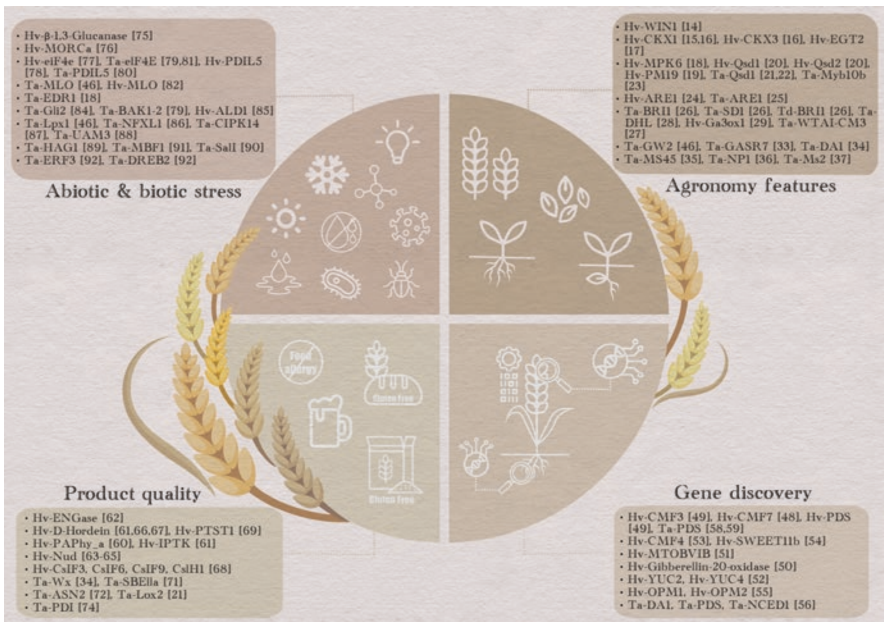


Fig. 9.1 Summary of barley and wheat genes with functions in agronomic features [14–47], gene discovery [48–59], product quality [21, 34, 60–74], and abiotic and biotic stress [46, 75–92]

outcome for CRISPR/Cas9 is a deletion of a few (≤10) nucleotides or the insertion of one nucleotide (InDel) [93]. However, it is impossible to predict precisely whether loss or insertion will occur, and it is also somewhat random which base is inserted. Although there are reports (for review, see [94]) that a certain percentage can be predicted using microhomology-dependent repair mechanisms, the outcome remains undetermined in most applications.

To achieve a prediction of the mutation result and thus precise genome editing, one can consider several possibilities. When using two gRNAs, one gets an exact deletion between the two induced double-strand breaks in a part of the mutated cells. In this case, the choice of gRNA binding sites can, for example, influence the function of a protein domain [49]. By not inducing a knockout, a reduced or altered functionality remains, similar to the RNAi effect but is genetically fixed in contrast.

Another possibility is to transfer a repair template with the desired sequence simultaneously with the double-strand-inducing reagent. However, the challenge here is to bring a sufficient number of repair templates to this site at the time of the double-strand break repair. One possibility is the biolistic transfer of the repaired DNA [95]. However, this method has all the previously described disadvantages that have led to the preferential use of *Agrobacteria*-mediated transformation [96].

Initial results at the cellular level in barley showed targeted allelic exchange of the fluorescence protein GFP. GFP and YFP differ in only one amino acid; thus, exchanging two nucleotides causes a change in the emission spectrum [97]. It was shown that 3% of the mutant epidermal cells had integrated the non-functional YFP fragment in the genome, thus exhibiting a shift in the lambda scan. These results were even surpassed when pre-assembled RNP complexes were biolistically transferred with Cas9 instead of plasmid DNA [95]. Here, it was shown that up to 8% of GFP-mutated epidermal cells exhibited such an allelic exchange. A typical application for allelic exchange is the creation of herbicide resistance [98]. Since this allows the selection of the correctly modified cells, such a method is easier to apply. However, the efficiency is expected to be lower if the modification has no selection advantage during creation.

An improvement here is the prime editing method [99]. In contrast to the Cas9 technology, the Moloney Murine Leukemia Virus reverse transcriptase (MLV-RT) domain was added to the Cas endonuclease. At the same time, the gRNA was extended by the part of the repair template. However, there have been few reports of plant applications so far, suggesting that the technology still needs improvement.

To precisely incorporate large DNA fragments in plants, a PrimeRoot-named method was recently described [100]. Third-generation PrimeRoot editors use optimized prime editing guide RNA designs, an improved plant prime editor, and superior recombinases to enable precise large DNA insertions of up to 11.1 kilobases into plant genomes. The authors describe using PrimeRoot to introduce gene regulatory elements into the rice. Applications in temperate cereals have not yet been described.

Base editing (BE) is another technology for the precise modification of genomes (DNA) or transcriptomes (RNA) of living cells at single-base resolution (for review, see [101]). BEs comprise a catalytically impaired cas nuclease fused with a

nucleotide deaminase and sometimes DNA repair proteins. BEs can introduce single nucleotide variants at desired sites into the DNA (nuclear or organellar) or RNA of both dividing and non-dividing cells. There are two types of BEs – DNA BEs, which directly induce targeted point mutations in DNA, and RNA BEs, which convert one ribonucleotide to another in RNA. The currently available DNA BEs can be further divided into cytosine BEs (CBEs), adenine BEs (ABEs), C-to-G BEs (CGBEs), dual-base editors, and organellar BEs. These categories are discussed below [101]. After protoplast testing, C to T substitutions was successfully detected in two heterozygous wheat plants [102]. To increase the efficiency, further improvements such as the NLS, crRNA, LbCas12a nuclease, adenine deaminase, and linker were undertaken, achieving up to 55% efficiency in stable mutants (*TaLOX* and *TaMLO*) [103]. In other plants, further improvements, such as placing a N-terminal reverse transcriptase–Cas9 nickase fusion performed better in rice than the commonly applied C-terminal fusion [104]. In addition, introducing multiple-nucleotide substitutions in the reverse transcriptase template stimulated prime editing with enhanced efficiency. Additionally, it was shown that using two pegRNAs that encode the same edits but target complementary DNA strands highly promotes the desired outcome [105].

However, all the previously mentioned methods are still subject to particular challenges. These concern all parts of the process, such as selecting, using, and transferring appropriate gRNAs and Cas and proteins, general tissue culture, genotype dependence, detection of induced mutations, and identification of transgene-free, etc. homozygous progeny [106]. Further sequencing of genomes and enzyme evolution will undoubtedly lead to other plant improvements. It is crucial that the plants produced in this way also find use in European agriculture and that outdated regulations do not prevent their use.

References

1. Morrison, R.A., Evans, D.A.: Haploid plants from tissue culture: new plant varieties in a shortened time frame. Nat. Biotechnol. **6**, 684–690 (1988). https://doi.org/10.1038/nbt0688-684
2. Mascher, M., Gundlach, H., Himmelbach, A., et al.: A chromosome conformation capture ordered sequence of the barley genome. Nature. **544**, 427–433 (2017). https://doi.org/10.1038/nature22043
3. Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science. **361** (2018) https://doi.org/10.1126/science.aar7191
4. Rabanus-Wallace, M.T., Hackauf, B., Mascher, M., et al.: Chromosome-scale genome assembly provides insights into rye biology, evolution and agronomic potential. Nat. Genet. **53**, 564–573 (2021). https://doi.org/10.1038/s41588-021-00807-0
5. Kamal, N., Tsardakas Renhuldt, N., Bentzer, J., et al.: The mosaic oat genome gives insights into a uniquely healthy cereal crop. Nature. **606**, 113–119 (2022). https://doi.org/10.1038/s41586-022-04732-y
6. Collard, B.C.Y., Mackill, D.J.: Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. **363**, 557–572 (2008). https://doi.org/10.1098/rstb.2007.2170

7. Boch, J., Scholze, H., Schornack, S., et al.: Breaking the code of DNA binding specificity of TAL-type III effectors. Science. **326**, 1509–1512 (2009). https://doi.org/10.1126/science.1178811
8. Jinek, M., Chylinski, K., Fonfara, I., et al.: A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. **337**, 816–821 (2012). https://doi.org/10.1126/science.1225829
9. Moon, S.B., Kim, D.Y., Ko, J.-H., et al.: Recent advances in the CRISPR genome editing tool set. Exp. Mol. Med. **51**, 1–11 (2019). https://doi.org/10.1038/s12276-019-0339-7
10. Kumlehn, J., Pietralla, J., Hensel, G., et al.: The CRISPR/Cas revolution continues: from efficient gene editing for crop breeding to plant synthetic biology. J. Integr. Plant Biol. **60**, 1127–1153 (2018). https://doi.org/10.1111/jipb.12734
11. Zhu, H., Li, C., Gao, C.: Applications of CRISPR-Cas in agriculture and plant biotechnology. Nat. Rev. Mol. Cell Biol. **21**, 661–677 (2020). https://doi.org/10.1038/s41580-020-00288-9
12. Capdeville, N., Schindele, P., Puchta, H.: Getting better all the time – recent progress in the development of CRISPR/Cas-based tools for plant genome engineering. Curr. Opin. Biotechnol. **79**, 102854 (2023). https://doi.org/10.1016/j.copbio.2022.102854
13. Michalski, K., Hertig, C., Mańkowski, D.R., et al.: Functional validation of cas9/guideRNA constructs for site-directed mutagenesis of Triticale ABA8′OH1 loci. Int. J. Mol. Sci. **22** (2021). https://doi.org/10.3390/ijms22137038
14. Gerasimova, S.V., Kolosovskaya, E.V., Vikhorev, A.V., et al.: WAX INDUCER 1 regulates β-Diketone biosynthesis by mediating expression of the Cer-cqu gene cluster in Barley. Int. J. Mol. Sci. **24** (2023). https://doi.org/10.3390/ijms24076762
15. Holubová, K., Hensel, G., Vojta, P., et al.: Modification of barley plant productivity through regulation of Cytokinin content by reverse-genetics approaches. Front. Plant Sci. **9**, 1676 (2018). https://doi.org/10.3389/fpls.2018.01676
16. Gasparis, S., Przyborowski, M., Kała, M., et al.: Knockout of the HvCKX1 or HvCKX3 gene in Barley (Hordeum vulgare L.) by RNA-guided Cas9 nuclease affects the regulation of Cytokinin metabolism and root morphology. Cells. **8** (2019). https://doi.org/10.3390/cells8080782
17. Kirschner, G.K., Rosignoli, S., Guo, L., et al.: ENHANCED GRAVITROPISM 2 encodes a STERILE ALPHA MOTIF-containing protein that controls root growth angle in barley and wheat. Proc. Natl. Acad. Sci. U. S. A. **118** (2021). https://doi.org/10.1073/pnas.2101526118
18. Křenek, P., Chubar, E., Vadovič, P., et al.: CRISPR/Cas9-induced loss-of-function mutation in the barley mitogen-activated protein kinase 6 gene causes abnormal embryo development leading to severely reduced grain germination and seedling Shootless phenotype. Front. Plant Sci. **12**, 670302 (2021). https://doi.org/10.3389/fpls.2021.670302
19. Lawrenson, T., Shorinola, O., Stacey, N., et al.: Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. Genome Biol. **16**, 258 (2015). https://doi.org/10.1186/s13059-015-0826-7
20. Hisano, H., Hoffie, R.E., Abe, F., et al.: Regulation of germination by targeted mutagenesis of grain dormancy genes in barley. Plant Biotechnol. J. **20**, 37–46 (2022). https://doi.org/10.1111/pbi.13692
21. Kamiya, Y., Abe, F., Mikami, M., et al.: A rapid method for detection of mutations induced by CRISPR/Cas9-based genome editing in common wheat. Plant Biotechnol. (Tokyo). **37**, 247–251 (2020). https://doi.org/10.5511/plantbiotechnology.20.0404b
22. Abe, F., Haque, E., Hisano, H., et al.: Genome-edited triple-recessive mutation alters seed dormancy in wheat. Cell Rep. **28**, 1362–1369.e4 (2019). https://doi.org/10.1016/j.celrep.2019.06.090
23. Zhu, Y., Lin, Y., Fan, Y., et al.: CRISPR/Cas9-mediated restoration of Tamyb10 to create pre-harvest sprouting-resistant red wheat. Plant Biotechnol. J. **21**, 665–667 (2023). https://doi.org/10.1111/pbi.13981
24. Karunarathne, S.D., Han, Y., Zhang, X.-Q., et al.: CRISPR/Cas9 gene editing and natural variation analysis demonstrate the potential for HvARE1 in improvement of nitrogen use efficiency in barley. J. Integr. Plant Biol. **64**, 756–770 (2022). https://doi.org/10.1111/jipb.13214

25. Zhang, J., Zhang, H., Li, S., et al.: Increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9. J. Integr. Plant Biol. **63**, 1649–1663 (2021). https://doi.org/10.1111/jipb.13151
26. Budhagatapalli, N., Halbach, T., Hiekel, S., et al.: Site-directed mutagenesis in bread and durum wheat via pollination by cas9/guide RNA-transgenic maize used as haploidy inducer. Plant Biotechnol. J. **18**, 2376–2378 (2020). https://doi.org/10.1111/pbi.13415
27. Camerlengo, F., Frittelli, A., Sparks, C., et al.: CRISPR-Cas9 multiplex editing of the α-amylase/trypsin inhibitor genes to reduce allergen proteins in durum wheat. Front. Sustain. Food Syst. **4** (2020). https://doi.org/10.3389/fsufs.2020.00104
28. Guo, B., Jin, X., Chen, J., et al.: ATP-dependent DNA helicase (TaDHL), a novel reduced-height (Rht) gene in wheat. Genes (Basel). **13** (2022). https://doi.org/10.3390/genes13060979
29. Cheng, J., Hill, C., Han, Y., et al.: New semi-dwarfing alleles with increased coleoptile length by gene editing of gibberellin 3-oxidase 1 using CRISPR-Cas9 in barley (Hordeum vulgare L.). Plant Biotechnol. J. **21**, 806–818 (2023). https://doi.org/10.1111/pbi.13998
30. Jiang, Y., Li, J., Liu, B., et al.: Novel Hina alleles created by genome editing increase grain hardness and reduce grain width in barley. Transgenic Res. **31**, 637–645 (2022). https://doi.org/10.1007/s11248-022-00324-8
31. Zeng, Z., Han, N., Liu, C., et al.: Functional dissection of HGGT and HPT in barley vitamin E biosynthesis via CRISPR/Cas9-enabled genome editing. Ann. Bot. **126**, 929–942 (2020). https://doi.org/10.1093/aob/mcaa115
32. Wang, W., Pan, Q., Tian, B., et al.: Gene editing of the wheat homologs of TONNEAU1-recruiting motif encoding gene affects grain shape and weight in wheat. Plant J. **100**, 251–264 (2019). https://doi.org/10.1111/tpj.14440
33. Zhang, Y., Liang, Z., Zong, Y., et al.: Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat. Commun. **7**, 12617 (2016). https://doi.org/10.1038/ncomms12617
34. Zhang, S., Zhang, R., Song, G., et al.: Targeted mutagenesis using the agrobacterium tumefaciens-mediated CRISPR-Cas9 system in common wheat. BMC Plant Biol. **18**, 302 (2018). https://doi.org/10.1186/s12870-018-1496-x
35. Singh, M., Kumar, M., Albertsen, M.C., et al.: Concurrent modifications in the three homeologs of Ms45 gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (Triticum aestivum L.). Plant Mol. Biol. **97**, 371–383 (2018). https://doi.org/10.1007/s11103-018-0749-2
36. Li, J., Wang, Z., He, G., et al.: CRISPR/Cas9-mediated disruption of TaNP1 genes results in complete male sterility in bread wheat. J. Genet. Genomics. **47**, 263–272 (2020). https://doi.org/10.1016/j.jgg.2020.05.004
37. Tang, H., Liu, H., Zhou, Y., et al.: Fertility recovery of wheat male sterility controlled by Ms2 using CRISPR/Cas9. Plant Biotechnol. J. **19**, 224–226 (2021). https://doi.org/10.1111/pbi.13482
38. Zhang, R., Zhang, S., Li, J., et al.: CRISPR/Cas9-targeted mutagenesis of TaDCL4, TaDCL5 and TaRDR6 induces male sterility in common wheat. Plant Biotechnol. J. **21**, 839–853 (2023). https://doi.org/10.1111/pbi.14000
39. de Souza Moraes, T., van Es, S.W., Hernández-Pinzón, I., et al.: The TCP transcription factor HvTB2 heterodimerizes with VRS5 and controls spike architecture in barley. Plant Reprod. **35**, 205–220 (2022). https://doi.org/10.1007/s00497-022-00441-8
40. Wang, C., Yang, X., Zhang, Y., et al.: Barley FASCIATED EAR genes determine inflorescence meristem size and yield traits. Crop J. (2022). https://doi.org/10.1016/j.cj.2022.10.001
41. Chen, Z., Ke, W., He, F., et al.: A single nucleotide deletion in the third exon of FT-D1 increases the spikelet number and delays heading date in wheat (Triticum aestivum L.). Plant Biotechnol. J. **20**, 920–933 (2022). https://doi.org/10.1111/pbi.13773
42. Errum, A., Rehman, N., Uzair, M., et al.: CRISPR/Cas9 editing of wheat Ppd-1 gene homoeologs alters spike architecture and grain morphometric traits. Funct. Integr. Genomics. **23**, 66 (2023). https://doi.org/10.1007/s10142-023-00989-2

43. Wang, W., Yu, Z., He, F., et al.: Multiplexed promoter and gene editing in wheat using a virus-based guide RNA delivery system. Plant Biotechnol. J. **20**, 2332–2341 (2022). https://doi.org/10.1111/pbi.13910
44. Miroshnichenko, D., Timerbaev, V., Klementyeva, A., et al.: CRISPR/Cas9-induced modification of the conservative promoter region of VRN-A1 alters the heading time of hexaploid bread wheat. Front. Plant Sci. **13**, 1048695 (2022). https://doi.org/10.3389/fpls.2022.1048695
45. Gupta, A., Hua, L., Zhang, Z., et al.: CRISPR-induced miRNA156-recognition element mutations in TaSPL13 improve multiple agronomic traits in wheat. Plant Biotechnol. J. **21**, 536–548 (2023). https://doi.org/10.1111/pbi.13969
46. Wang, W., Pan, Q., He, F., et al.: Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. CRISPR J. **1**, 65–74 (2018). https://doi.org/10.1089/crispr.2017.0010
47. Luo, J., Li, S., Xu, J., et al.: Pyramiding favorable alleles in an elite wheat variety in one generation by CRISPR-Cas9-mediated multiplex gene editing. Mol. Plant. **14**, 847–850 (2021). https://doi.org/10.1016/j.molp.2021.03.024
48. Li, M., Hensel, G., Mascher, M., et al.: Leaf variegation and impaired chloroplast development caused by a truncated CCT domain gene in albostrians barley. Plant Cell. **31**, 1430–1445 (2019). https://doi.org/10.1105/tpc.19.00132
49. Li, M., Hensel, G., Melzer, M., et al.: Mutation of the ALBOSTRIANS Ohnologous gene HvCMF3 impairs chloroplast development and thylakoid architecture in barley. Front. Plant Sci. **12**, 732608 (2021). https://doi.org/10.3389/fpls.2021.732608
50. Han, Y., Broughton, S., Liu, L., et al.: Highly efficient and genotype-independent barley gene editing based on anther culture. Plant Commun. **2**, 100082 (2021). https://doi.org/10.1016/j.xplc.2020.100082
51. Steckenborn, S., Cuacos, M., Ayoub, M.A., et al.: The meiotic topoisomerase VI B subunit (MTOPVIB) is essential for meiotic DNA double-strand break formation in barley (Hordeum vulgare L.). Plant Reprod. **36**, 1–15 (2023). https://doi.org/10.1007/s00497-022-00444-5
52. Amanda, D., Frey, F.P., Neumann, U., et al.: Auxin boosts energy generation pathways to fuel pollen maturation in barley. Curr. Biol. **32**, 1798–1811.e8 (2022). https://doi.org/10.1016/j.cub.2022.02.073
53. Huang, Y., Kamal, R., Shanmugaraj, N., et al.: A molecular framework for grain number determination in barley. Sci. Adv. **9**, eadd0324 (2023). https://doi.org/10.1126/sciadv.add0324
54. Radchuk, V., Belew, Z.M., Gündel, A., et al.: SWEET11b transports both sugar and cytokinin in developing barley grains. Plant Cell. (2023). https://doi.org/10.1093/plcell/koad055
55. Yang, X., Wilkinson, L.G., Aubert, M.K., et al.: Ovule cell wall composition is a maternal determinant of grain size in barley. New Phytol. **237**, 2136–2147 (2023). https://doi.org/10.1111/nph.18714
56. Li, J., Zhang, S., Zhang, R., et al.: Efficient multiplex genome editing by CRISPR/Cas9 in common wheat. Plant Biotechnol. J. **19**, 427–429 (2021). https://doi.org/10.1111/pbi.13508
57. Hyde, L., Osman, K., Winfield, M., et al.: Identification, characterization, and rescue of CRISPR/Cas9 generated wheat SPO11-1 mutants. Plant Biotechnol. J. **21**, 405–418 (2023). https://doi.org/10.1111/pbi.13961
58. Zhou, H., Xu, L., Li, F., et al.: Transcriptional regulation by CRISPR/dCas9 in common wheat. Gene. **807**, 145919 (2022). https://doi.org/10.1016/j.gene.2021.145919
59. Howells, R.M., Craze, M., Bowden, S., et al.: Efficient generation of stable, heritable gene edits in wheat using CRISPR/Cas9. BMC Plant Biol. **18**, 215 (2018). https://doi.org/10.1186/s12870-018-1433-z
60. Holme, I.B., Wendt, T., Gil-Humanes, J., et al.: Evaluation of the mature grain phytase candidate HvPAPhy_a gene in barley (Hordeum vulgare L.) using CRISPR/Cas9 and TALENs. Plant Mol. Biol. **95**, 111–121 (2017). https://doi.org/10.1007/s11103-017-0640-6
61. Vlčko, T., Ohnoutková, L.: Allelic variants of CRISPR/Cas9 induced mutation in an inositol trisphosphate 5/6 kinase gene manifest different phenotypes in barley. Plants (Basel). **9** (2020). https://doi.org/10.3390/plants9020195

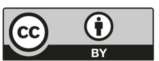
62. Kapusi, E., Corcuera-Gómez, M., Melnik, S., et al.: Heritable genomic fragment deletions and small Indels in the putative ENGase gene induced by CRISPR/Cas9 in Barley. Front. Plant Sci. **8**, 540 (2017). https://doi.org/10.3389/fpls.2017.00540
63. Gasparis, S., Kała, M., Przyborowski, M., et al.: A simple and efficient CRISPR/Cas9 platform for induction of single and multiple, heritable mutations in barley (Hordeum vulgare L.). Plant Methods. **14**, 111 (2018). https://doi.org/10.1186/s13007-018-0382-8
64. Gerasimova, S.V., Hertig, C., Korotkova, A.M., et al.: Conversion of hulled into naked barley by Cas endonuclease-mediated knockout of the NUD gene. BMC Plant Biol. **20**, 255 (2020). https://doi.org/10.1186/s12870-020-02454-9
65. Zang, Y., Gong, Q., Xu, Y., et al.: Production of conjoined transgenic and edited barley and wheat plants for Nud genes using the CRISPR/SpCas9 system. Front. Genet. **13**, 873850 (2022). https://doi.org/10.3389/fgene.2022.873850
66. Li, Y., Liu, D., Zong, Y., et al.: New D hordein alleles were created in barley using CRISPR/Cas9 genome editing. Cereal Res. Commun. **48**, 131–138 (2020). https://doi.org/10.1007/s42976-020-00023-2
67. Yang, Q., Zhong, X., Li, Q., et al.: Mutation of the d-hordein gene by RNA-guided Cas9 targeted editing reducing the grain size and changing grain compositions in barley. Food Chem. **311**, 125892 (2020). https://doi.org/10.1016/j.foodchem.2019.125892
68. Garcia-Gimenez, G., Barakate, A., Smith, P., et al.: Targeted mutation of barley (1,3;1,4)-β-glucan synthases reveals complex relationships between the storage and cell wall polysaccharide content. Plant J. **104**, 1009–1022 (2020). https://doi.org/10.1111/tpj.14977
69. Zhong, Y., Blennow, A., Kofoed-Enevoldsen, O., et al.: Protein targeting to starch 1 is essential for starchy endosperm development in barley. J. Exp. Bot. **70**, 485–496 (2019). https://doi.org/10.1093/jxb/ery398
70. Yang, Q., Ding, J., Feng, X., et al.: Editing of the starch synthase IIa gene led to transcriptomic and metabolomic changes and high amylose starch in barley. Carbohydr. Polym. **285**, 119238 (2022). https://doi.org/10.1016/j.carbpol.2022.119238
71. Li, J., Jiao, G., Sun, Y., et al.: Modification of starch composition, structure and properties through editing of TaSBEIIa in both winter and spring wheat varieties by CRISPR/Cas9. Plant Biotechnol. J. **19**, 937–951 (2021). https://doi.org/10.1111/pbi.13519
72. Raffan, S., Sparks, C., Huttly, A., et al.: Wheat with greatly reduced accumulation of free asparagine in the grain, produced by CRISPR/Cas9 editing of asparagine synthetase gene TaASN2. Plant Biotechnol. J. **19**, 1602–1613 (2021). https://doi.org/10.1111/pbi.13573
73. Ibrahim, S., Saleem, B., Rehman, N., et al.: CRISPR/Cas9 mediated disruption of Inositol Pentakisphosphate 2-Kinase 1 (TaIPK1) reduces phytic acid and improves iron and zinc accumulation in wheat grains. J. Adv. Res. **37**, 33–41 (2022). https://doi.org/10.1016/j.jare.2021.07.006
74. Hu, J., Yu, M., Chang, Y., et al.: Functional analysis of TaPDI genes on storage protein accumulation by CRISPR/Cas9 edited wheat mutants. Int. J. Biol. Macromol. **196**, 131–143 (2022). https://doi.org/10.1016/j.ijbiomac.2021.12.048
75. Kim, S.-Y., Bengtsson, T., Olsson, N., et al.: Mutations in two aphid-regulated β-1,3-Glucanase genes by CRISPR/Cas9 do not increase barley resistance to Rhopalosiphum padi L. Front. Plant Sci. **11**, 1043 (2020). https://doi.org/10.3389/fpls.2020.01043
76. Galli, M., Martiny, E., Imani, J., et al.: CRISPR/SpCas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth. Plant Biotechnol. J. **20**, 89–102 (2022). https://doi.org/10.1111/pbi.13697
77. Hoffie, R.E., Otto, I., Perovic, D., et al.: Targeted knockout of eukaryotic translation initiation factor 4E confers Bymovirus resistance in winter barley. Front. Genome Editing. **3**, 784233 (2021). https://doi.org/10.3389/fgeed.2021.784233
78. Hoffie, R.E., Perovic, D., Habekuß, A., et al.: Novel resistance to the Bymovirus BaMMV established by targeted mutagenesis of the PDIL5-1 susceptibility gene in barley. Plant Biotechnol. J. **21**, 331–341 (2023). https://doi.org/10.1111/pbi.13948

79. Hahn, F., Sanjurjo Loures, L., Sparks, C.A., et al.: Efficient CRISPR/Cas-mediated targeted mutagenesis in spring and winter wheat varieties. Plants (Basel). **10** (2021). https://doi.org/10.3390/plants10071481
80. Kan, J., Cai, Y., Cheng, C., et al.: Simultaneous editing of host factor gene TaPDIL5-1 homoeoalleles confers wheat yellow mosaic virus resistance in hexaploid wheat. New Phytol. **234**, 340–344 (2022). https://doi.org/10.1111/nph.18002
81. Kan, J., Cai, Y., Cheng, C., et al.: CRISPR/Cas9-guided knockout of eIF4E improves wheat yellow mosaic virus resistance without yield penalty. Plant Biotechnol. J. **21**, 893–895 (2023). https://doi.org/10.1111/pbi.14002
82. Koide, H., Hisano, H., Yaeno, T.: CRISPR/Cas9-based generation of mlo mutants for allelic complementation experiments to elucidate MLO function in barley. J. Gen. Plant Pathol. **89**, 153–158 (2023). https://doi.org/10.1007/s10327-023-01120-w
83. Zhang, Y., Bai, Y., Wu, G., et al.: Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. Plant J. **91**, 714–724 (2017). https://doi.org/10.1111/tpj.13599
84. Sánchez-León, S., Gil-Humanes, J., Ozuna, C.V., et al.: Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol. J. **16**, 902–910 (2018). https://doi.org/10.1111/pbi.12837
85. Brambilla, A., Lenk, M., Ghirardo, A., et al.: Pipecolic acid synthesis is required for systemic acquired resistance and plant-to-plant-induced immunity in barley. J. Exp. Bot. (2023). https://doi.org/10.1093/jxb/erad095
86. Brauer, E.K., Balcerzak, M., Rocheleau, H., et al.: Genome editing of a Deoxynivalenol-induced transcription factor confers resistance to fusarium graminearum in wheat. Mol. Plant-Microbe Interact. **33**, 553–560 (2020). https://doi.org/10.1094/mpmi-11-19-0332-r
87. He, F., Wang, C., Sun, H., et al.: Simultaneous editing of three homoeologues of TaCIPK14 confers broad-spectrum resistance to stripe rust in wheat. Plant Biotechnol. J. **21**, 354–368 (2023). https://doi.org/10.1111/pbi.13956
88. Huang, X., Bai, X., Qian, C., et al.: TaUAM3, a UDP-Ara mutases protein, positively regulates wheat resistance to the stripe rust fungus. Food Energy Secur. (2023). https://doi.org/10.1002/fes3.456
89. Zheng, M., Lin, J., Liu, X., et al.: Histone acetyltransferase TaHAG1 acts as a crucial regulator to strengthen salt tolerance of hexaploid wheat. Plant Physiol. **186**, 1951–1969 (2021). https://doi.org/10.1093/plphys/kiab187
90. Mohr, T., Horstman, J., Gu, Y.Q., et al.: CRISPR-Cas9 gene editing of the Sal1 gene family in wheat. Plants (Basel). **11** (2022). https://doi.org/10.3390/plants11172259
91. Tian, X., Qin, Z., Zhao, Y., et al.: Stress granule-associated TaMBF1c confers thermotolerance through regulating specific mRNA translation in wheat (Triticum aestivum). New Phytol. **233**, 1719–1731 (2022). https://doi.org/10.1111/nph.17865
92. Kim, D., Alptekin, B., Budak, H.: CRISPR/Cas9 genome editing in wheat. Funct. Integr. Genomics. **18**, 31–41 (2018). https://doi.org/10.1007/s10142-017-0572-x
93. Bortesi, L., Zhu, C., Zischewski, J., et al.: Patterns of CRISPR/Cas9 activity in plants, animals and microbes. Plant Biotechnol. J. **14**, 2203–2216 (2016). https://doi.org/10.1111/pbi.12634
94. van Vu, T., Thi Hai Doan, D., Kim, J., et al.: CRISPR/Cas-based precision genome editing via microhomology-mediated end joining. Plant Biotechnol. J. **19**, 230–239 (2021). https://doi.org/10.1111/pbi.13490
95. Becker, M., Hensel, G.: Ribonucleoprotein (RNP)-mediated allele replacement in barley (Hordeum vulgare L.) leaves. Methods Mol. Biol. **2653**, 199–205 (2023). https://doi.org/10.1007/978-1-0716-3131-7_13
96. Harwood, W.A.: Advances and remaining challenges in the transformation of barley and wheat. J. Exp. Bot. **63**, 1791–1798 (2012). https://doi.org/10.1093/jxb/err380
97. Budhagatapalli, N., Rutten, T., Gurushidze, M., et al.: Targeted modification of gene function exploiting homology-directed repair of TALEN-mediated double-strand breaks in barley. G3 (Bethesda). **5**, 1857–1863 (2015). https://doi.org/10.1534/g3.115.018762

98. Svitashev, S., Schwartz, C., Lenderts, B., et al.: Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat. Commun. **7**, 13274 (2016). https://doi.org/10.1038/ncomms13274
99. Anzalone, A.V., Randolph, P.B., Davis, J.R., et al.: Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. **576**, 149–157 (2019). https://doi.org/10.1038/s41586-019-1711-4
100. Sun, C., Lei, Y., Li, B., et al.: Precise integration of large DNA sequences in plant genomes using PrimeRoot editors. Nat. Biotechnol. (2023). https://doi.org/10.1038/s41587-023-01769-w
101. Molla, K.A., Sretenovic, S., Bansal, K.C., et al.: Precise plant genome editing using base editors and prime editors. Nat. Plant. **7**, 1166–1187 (2021). https://doi.org/10.1038/s41477-021-00991-1
102. Zong, Y., Wang, Y., Li, C., et al.: Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. Nat. Biotechnol. **35**, 438–440 (2017). https://doi.org/10.1038/nbt.3811
103. Gaillochet, C., Peña Fernández, A., Goossens, V., et al.: Systematic optimization of Cas12a base editors in wheat and maize using the ITER platform. Genome Biol. **24**, 6 (2023). https://doi.org/10.1186/s13059-022-02836-2
104. Xu, W., Yang, Y., Yang, B., et al.: A design optimized prime editor with expanded scope and capability in plants. Nat. Plant. **8**, 45–52 (2022). https://doi.org/10.1038/s41477-021-01043-4
105. Lin, Q., Jin, S., Zong, Y., et al.: High-efficiency prime editing with optimized, paired pegRNAs in plants. Nat. Biotechnol. **39**, 923–927 (2021). https://doi.org/10.1038/s41587-021-00868-w
106. Cardi, T., Murovec, J., Bakhsh, A., et al.: CRISPR/Cas-mediated plant genome editing: outstanding challenges a decade after implementation. Trends Plant Sci. **28**, 1144–1165 (2023). https://doi.org/10.1016/j.tplants.2023.05.012

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.



Chapter 10
Implementing Genome Editing in Barley Breeding

Liina Jakobson, Signem Oney Birol, and Ljudmilla Timofejeva

Abstract This chapter summarizes the status of the genome editing efforts in *Hordeum vulgare* L. and provides an overview of the technical advances and obstacles of applying genome editing in barley. It also highlights the potential of genome editing in barley breeding with the focus on breeding for high yielding, disease resistant and stable varieties. The CRISPR/Cas technology is a breakthrough in genome editing due to its robustness and easy to use programming, especially for generating targeted mutations to switch off genes that have a negative impact on food quality, increase susceptibility to pathogens, or divert metabolic flux away from useful end products. Genome editing studies are expected to advance barley breeding by accelerating the breeding process and enabling easier multiplexing of traits. The chapter offers an outlook on the future of barley genome editing techniques based on CRISPR/Cas system.

1 Barley Breeding

Barley (*Hordeum vulgare* L.) is one of the first crops to be domesticated and on the other hand one of the most genetically diverse cereal species [1]. The cultivation history of the barley was started with the first seeding about 10,000 years ago by the farmers in Near East [1, 2]. In Europe, barley is nowadays the second most important cereal crop. It is mainly used for animal feed and beverage production. Although human diet is not the primary use, barley offers several health benefits and is still an important source of calories in Northern and Eastern Europe as well as in other parts of the world such as North Africa, Middle East and Asia.

L. Jakobson (✉) · L. Timofejeva
Plant Biotechnology Department, Centre of Estonian Rural Research and Knowledge, Jõgeva, Estonia
e-mail: liina.jakobson@metk.agri.ee

S. Oney Birol
Department of Molecular Biology and Genetics, Faculty of Science, Burdur Mehmet Akif Ersoy University, Burdur, Turkiye

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_10

Over decades, breeders have utilized many strategies to introduce novel heritable mutations into plant genomes in order to develop new improved varieties. The use of numerous physical, chemical and biological mutagens such as gamma rays [3], X-rays [4], ethyl methanesulfonate [5], sodium azide [6], *Agrobacterium tumefaciens* [7] and transposon-based molecular markers [8] has facilitated the rapid extension of genetic diversity throughout the last century. However, these approaches have significant limitations, including the non-specific character of the produced mutations, the huge number of unnecessary mutations and the occasional undesired deletions, duplications or rearrangements of large genomic fragments [9]. Methods of haploid production are also an important tool in barley breeding, being time-saving and providing genetically fixed breeding lines.

Current breeding goals for barley depend on local conditions and vary a lot. In order to meet the increasing demand for livestock feed, starch, and a range of alcoholic (such as beer, whisky, and ethanol) and non-alcoholic (such as barley tea, barley coffee, and malt drink) beverages, barley breeding must focus on developing high-yielding and stress-tolerant varieties that can thrive even in challenging climatic conditions. Global changes such as the predicted increase in human population and diet, set some common goals both for breeding and agricultural crop production [10]. Thus, breeding for disease resistance, high yielding and stability are crucial components worldwide to ensure food security and satisfy the increasing demand. Together with the accelerating global changes, barley breeding has been challenged to speed up the process and multiplex a variety of traits in new varieties.

2 Genome Editing Advancements in Barley

Among gene editing technologies there are three major classes of synthetic endonucleases applied in plants: (a) **zinc finger nucleases (ZFNs)** are endonucleases linked to a multi-zinc-finger DNA-binding domain [11]; (b) **transcription activator-like effector nucleases (TALENs)** are composed of multiple transcription-factor-like DNA-binding domains linked to endonuclease domain [12]; (c) the widely used **CRISPR/Cas** (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system is composed of single guide RNAs (sgRNAs) enabling targeting multiple genes simultaneously and the Cas nuclease [13, 14]. First, TALEN-mediated genetic modifications in barley were induced in embryogenic pollen and leaf epidermis of winter barley variety ‘Igri’ [15, 16]. At the same time, Lawrenson and colleagues demonstrated for the first time the use of CRISPR/Cas9 technology in immature embryos of barley variety ‘Golden Promise’ to generate stable and inheritable mutations [17]. Biolistic transformation method for inducing CRISPR/Cas9-mediated InDels in ‘Golden Promise’ was presented only couple of years later [18].

Barley genome assembly was completed on the North American barley variety 'Morex' in 2017 [19]. Soon thereafter several protocols optimizing CRISPR/Cas protocols for barley were published [20–23]. The first Cas nuclease utilised in site-directed mutagenesis of barley was *Sp*Cas9 originated from *Streptococcus pyogenes*. Recently, the use of two versions of high efficiency endonuclease *Lb*Cas12a from *Lachnospiraceae bacterium* coupled with CRISPR was reported in barley [24].

Most CRISPR/Cas-mediated approaches focus on "negative effects" and generation of null alleles or loss-of-function alleles by targeting coding regions, while many agronomically important traits are associated with gain-of-function alleles. New strategies employed in other plants **target non-coding promoter regions**. Li and co-workers [25] engineered allelic variation by editing tomato KLUH promoter around a single-nucleotide polymorphism (SNP) located in a conserved putative cis-regulatory element [25]. Among twenty-one mutant alleles with various insertions and deletions, five mutant alleles showed a consistent increase in fruit weight. Moreover, promoter editing has proven useful in altering plant architecture in tomato [26], in developing resistance against *Xanthomonas* in rice [27, 28] and citrus [29], and in engineering drought tolerance in maize [30, 31]. Editing of non-coding cis-regulatory elements (CRE) offers considerable potential for crop improvement via fine-tuning of gene expression that cannot be achieved by simple knockout mutations. However, its widespread application is still hampered by the lack of precise knowledge about functional motifs in CRE [32]. Recent advancement CRISPR-Combo enables genome editing (targeted mutagenesis or base editing) and gene activation in plants simultaneously [33].

The above-mentioned findings have opened the great potential of rapid characterisation of gene function in barley, followed by advancement in precision and increased speed in breeding. **New breeding techniques (NBTs)** now enable switching on/off target genes in barley or convert allelic variants into more advantageous alleles without genetic linkage drag. These approaches could support traditional breeding by overcoming the limits of random mutagenesis and at the same time without developing transgenic plants. However, there are several bottlenecks in the successful utilisation of CRISPR/Cas system in barley such as transformation efficiency and the risk for off-targets. Transformation efficiency is one of the obstacles hindering the use of CRISPR/Cas in the production of new barley varieties. The risk of off-target mutations can be minimized with the help of large number of bioinformatic and computational tools developed to date, which facilitate gRNA site selection and evaluation of the probability for off-target events [34].

Thus, genome editing with the CRISPR/Cas system has been presented to be applicable in barley and a suitable technology for precision breeding. So far, the examples of using genome editing in barley have mainly focused on the validation of mutagenesis protocols and gene function, however, many genes and traits applicable in breeding are yet to be tested and verified.

3 Using Genome Editing to Target Disease Resistance, Yield and Stability

The discovery of the CRISPR/Cas system about ten years ago has brought an extensive precision to the portfolio of site-specific mutagens and a wide range of inducible modifications, which have many putative applications in breeding. Efficient, easy to use and highly target-specific single-guide RNAs (sgRNA) enable crop breeders to boost specifically either yield, biomass, abiotic/biotic stress tolerance, disease, pest resistance or any other trait [35–37]. Recent experiments combining customisable endonucleases and doubled haploid technology facilitate and accelerate the induction of multiple homozygous and inheritable mutations even further [38]. There are also several recent reviews available about the potential of using CRISPR/Cas genome editing in barley [39–45]. Here we shall highlight the potential of genome editing in barley breeding with the focus on breeding for high yielding, disease resistant and stable varieties.

Overall plant immunity and genome stabilization could be one of the targets to induce disease resistant barley varieties. There are a few examples in barley, where disease resistance has been tackled with the aid of CRISPR/Cas technology. The cosmopolitan fungal pathogen *Fusarium graminearum* causes fusarium head blight (FHB), which not only reduces crop yield but also accumulates mycotoxins in barley grains. 2-oxoglutarate Fe(II)-dependent oxygenase (2OGO) has been identified as a susceptibility factor and plant immunity suppressor in *Arabidopsis*. Barley orthologue *Hv*2OGO was shown to complement the CRISPR/Cas9-induced knock out mutation in *Arabidopsis* and may have a similar role in controlling resistance to FHB in barley [46]. In addition, seven MORC proteins in barley, paralogs of Microrchidia (MORC) protein family, were shown to be involved in plant immunity. CRISPR/Cas-induced double knockout mutants of *HvMORC1* and *HvMORC6a* showed increased disease resistance to fungal pathogens *Blumeria graminis* and *Fusarium graminearum* [47, 48]. A large number of *mildew locus o* (*mlo*) mutants have been found or generated in various barley varieties, which exhibit strong resistance to powdery mildew fungus *Blumeria graminis f. sp. hordei*. Recently, CRISPR/Cas9-mediated reverse genetics approach was employed to elucidate the molecular function of *MLO* [49].

Wheat dwarf virus (WDV) is an economically important, insect-transmitted DNA virus, which infects also barley, causing severe yield losses. Direct antiviral utilization of the CRISPR/Ca9 system was presented in barley by establishing WDV resistance via targeting sgRNA sequences against viral genome [50]. Eukaryotic virus translation initiation factor E (eIF4E) is a plant cellular translation initiation factor and an essential target in potyvirus infection. Barley plants with modified *HveIF4E* were generated, but viral resistance is yet to be tested [51].

Soil-borne bymoviruses *barley yellow mosaic virus* (BaYMV) and *barley mild mosaic virus* (BaMMV) infect young winter barley seedlings in autumn and can

cause yield loss up to 50%. PROTEIN DISULFIDE ISOMERASE LIKE 5–1 (PDIL5-1) from ancient landraces and wild relatives of barley confers resistance to all known strains of these viruses. Novel genome-edited *PDIL5-1* alleles were shown also to be resistant to BaMMV, without any adverse effects on growth or yield [52].

Yield stability is the genotype's ability to produce consistently high yield in diverse environments. Breeding for high yield and stability is a complex process that requires the consideration of various factors such as genetics, environment and management practices. Breeding for yield requires the selection of high-yielding genotypes with desirable agronomic traits such as plant height [53, 54], physiological maturity [55], disease and pest resistance [56], and lodging resistance [57]. Previous studies on CRISPR/Cas editing technology in barley targeted *HvPM19* multi-copy genes (*PM19-1 and PM19-3*), associated with grain dormancy [17]. Lawrenson and colleagues transformed the two *PM19* genes independently into variety 'Golden Promise'. Genome-editing of the cytokinin oxidase/dehydrogenases (*HvCKX1* and *HvCKX3*) in barley, which are regulating endogenous cytokinin metabolism, has shown their importance in regulating root length, tillering and yield [20, 58]. Galli and colleagues described transformation of *HvMORC1* and *HvMORC6a* CRISPR/SpCas9 constructs to regulate transposable elements to increase biotic stress resistance and agronomic traits in barley [48]. Cellulose synthase-like gene superfamily (*HvCslF3, HvCslF6, HvCslF9* and *HvCslH1*) genes are related to low grain (1,3; 1,4)-β-glucan content in barley, which is a preferred trait in brewing and distillation processes [59, 60]. D-hordein gene *(HvHor3)* has also been targeted to change the D-hordein composition and other grain phenotypic features [61, 62]. Gene-editing of the caffeic acid O-methyltransferase 1 (*COMT1*) for use in lignocellulosic biomass and lignin biosynthesis has also been utilized [63–65].

The terms "phenotypic stability and yield stability" are often used to refer to phenotypic variations of the genotypes. Moreover, according to Becker and Leon a stable genotype is one that performs consistently despite significant statistical differences in environmental variables [66]. Stability of quality traits to produce superior varieties in cereals is very important for breeders because genotype ranks affect selection efficiency for genotypes that perform well under different environmental stress factors such as drought [67], salinity [68], diseases [69] to produce superior varieties in cereals. Thus, several studies have reported that the yield stability is correlated with the biomass [70], photosynthetic capacity [71], flowering time [72]. Newly developed targeting and genome editing technologies provide an opportunity to manipulate specific genomic sequences for improved yield stability. Recently, most of the genome editing studies in barley focus on grain quality, by targeting phytase activity [73], flavoenzyme activity [20], high amylose content [74], D-hordein content [62], grain size and composition [75], lignocellulosic content in secondary cell walls [63] and vitamin E biosynthesis in grains [76].

4 Challenges in Barley Genome Editing

Although, the CRISPR/Cas is currently actively studied in many research centres and breeding companies, there are still a number of limitations in the application of the protocol. The limiting factors are: small number of suitable barley genotypes, low transformation efficiency, possible off-targets in other parts of the genome exhibiting high sequence similarity, availability of mutable cut sites in the target sequence, and biallelic mutations due to inefficient cutting of the genomic DNA [20, 77, 78].

Callus regeneration is the final step in the *Agrobacterium*-mediated transformation and CRISPR/Cas genome editing protocols used mainly for barley. However, the varietal dependency in the efficiency of barley callus regeneration was observed already in 1980-ies both for winter and spring barley [79]. Barley variety 'Golden Promise' has since then been used as the standard for callus regeneration and transformation. Thus, genotypic restrictions on plant regeneration have hindered the implementation of transformation and genome editing tools on most barley varieties for over four decades.

Barley transformation protocols have been optimized, updated and improved over time. Several enhanced protocols have been published, mainly for *Agrobacterium*-mediated transformation of immature embryos, which provide average transformation efficiencies of 25% in the background of 'Golden Promise' [80–83]. For instance dicamba in the callus induction and maintenance media was generally superior to 2,4-D in promoting transformation and addition of $CuSO_4$ resulted in formation of more green plants [84]. The addition of L-cysteine as an antioxidant was reported to hinder the browning of embryos and boost the efficiency of transformation [85]. Albinism, which can appear among regenerated barley, is caused by the inability of proplastids to transform into chloroplasts. Pre-treatment with mannitol may help to reduce albino barley plants [86]. Recently, anther culture-based system was shown to enable effective creation of transgenic plants not only from 'Golden Promise' but also from four other Australian commercial barley varieties [87].

In addition to changing hormone and nutrient levels, techniques that modify the innate gene expression of plants could enhance the effectiveness of transformation. There are examples from maize, rice, sorghum and sugarcane (*Saccharum officinarum*) that overexpressing *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes produced high transformation frequencies in previously nontransformable lines [88]. BBM is a transcription factor among the superfamily of the APETALA 2/ETHYLENE RESPONSE FACTOR (AP2/ERF) DNA-binding domain, subfamily AP2 [89, 90]. Two *TaBBM* genes in wheat have been identified as orthologues for maize *Bbm* [91], however, barley counterparts are still to be uncovered. WUS is a bifunctional homeodomain transcription factor, which mainly acts as a repressor but can become also an activator [92, 93].

Another promising approach for increasing transformation efficiency, could be the expression of fusion protein combining wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) [94]. The concept was proven to have the desired effect in wheat, rice, triticale as well as in the dicot crop citrus hybrid Carrizo citrange [94]. Studying the effect of GRF-GIF fusion protein in reducing varietal gap in barley transformation would be a compelling avenue for exploration.

Lately, there have been several reports addressing the transformation efficiency by aiming to dissect its genetic determinants in transformable genotypes. Three significant and seven suggestive Transformation Amenability (TFA) regions were identified in 'Golden Promise', which likely include necessary factors for *Agrobacterium*-mediated transformation in barley [7, 95]. Additionally, the transformation efficiency (*TRA1*) locus was identified in the barley mutant M1460 on chromosome 2H incorporating 225 gene sequences [96]. Thus, there are suggestions in the literature that certain genetic components could affect the amenability to *Agrobacterium*-mediated transformation. However, further research is needed before this data could be used to overcome recalcitrance.

5 Social and Legislative Aspects of Using Genome Editing in Barley Breeding

Transgenic and genome editing technologies have a number of challenges, including regulatory barriers, public acceptance and the time and cost of risk assessments needed prior to commercialization. Genome edited products do not align with the prevailing definitions of genetically modified organisms (GMOs) in the majority of legal frameworks [75, 97]. The United States Department of Agriculture (USDA) [98] and the Australian Government Office of the Gene Technology Regulator [99] have determined that CRISPR-edited crops without foreign DNA are exempt from regulation as genetically modified organisms with regard to the regulation and commercialization of such products that enables to support and usage of the genome edited crop production. Afterwards, the European Parliamentary Research Service has ruled the legislation process based on the societal acceptance to promote the safety of humans, other animals, the non-living environment, and safe agriculture based on current developments in international genome editing laws in any genome editing application organized with the CRISPR/Cas system [100]. Recent developments in England reveal a change in the law permitting the commercial use of the gene edited products [101]. Cambridge-based researchers support utilizing this technology to develop crop varieties that are resilient to climate change and anticipate that it will create new employment opportunities. By enabling the production of better-adapted crops in less time and facilitating prompt market access, this approach holds significant promise.

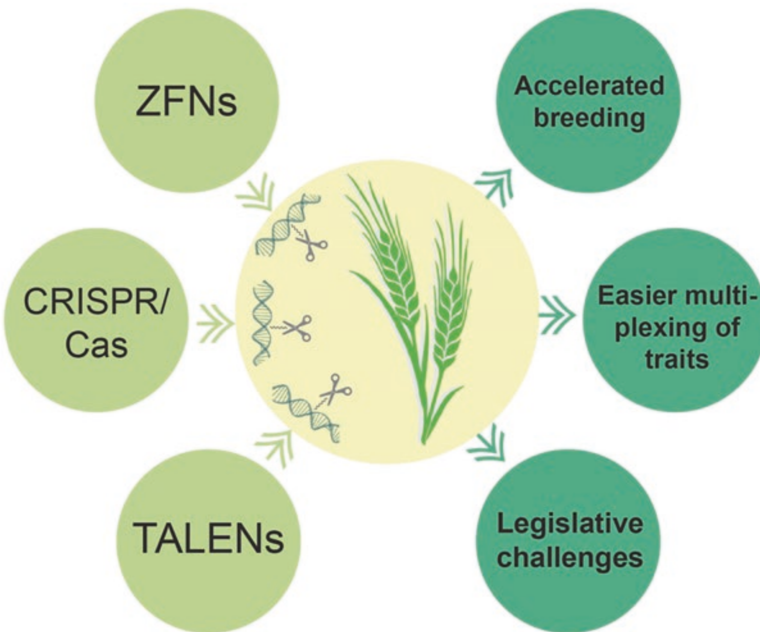


Fig. 10.1 Implementing genome editing in barley breeding. NBTs such as ZFNs, TALENs and CRISPR/Cas system enable to accelerate breeding and multiplex different traits. Application of NBTs in breeding is yet accompanied by the challenges in legislative rules

Consequently, genome editing enables to breed in less time and with greater precision, maximize crop genetic potential, generate germplasms that are more resistant to pests, biotic and abiotic challenges, and extend shelf life of plant products to reduce food waste. However, barley genome editing protocols still have a number of limitations. Acquiring knowledge about genome editing techniques and their applications is crucial for shaping regulatory frameworks that will impact the feasibility of utilizing novel barley varieties and support food security (Fig. 10.1).

References

1. Baik, B.-K., Ullrich, S.E.: Barley for food: characteristics, improvement, and renewed interest. J. Cereal Sci. **48**, 233–242 (2008). https://doi.org/10.1016/j.jcs.2008.02.002
2. Newman, C.W., Newman, R.K.: A brief history of barley foods. Cereal Foods World. **51**, 4–7 (2006)
3. Bado, S., Forster, B.P., Nielen, S., Ali, A.M., Lagoda, P.J.L., Till, B.J., Laimer, M.: Plant mutation breeding: current Progress and future assessment. In: Plant Breeding Reviews, vol. 39, pp. 23–88. Wiley (2015)
4. Ohnoutkova, L.: Mutation breeding in barley: historical overview. In: Harwood, W.A. (ed.) Barley: Methods and Protocols, pp. 7–19. Springer, New York (2019)

5. Lee, L.S., Till, B.J., Hill, H., Huynh, O.A., Jankowicz-Cieslak, J.: Mutation and mutation screening. In: Henry, R.J., Furtado, A. (eds.) Cereal Genomics: Methods and Protocols, pp. 77–95. Humana Press, Totowa (2014)
6. Olsen, O., Wang, X., von Wettstein, D.: Sodium azide mutagenesis: preferential generation of A.T-->G.C transitions in the barley Ant18 gene. Proc. Natl. Acad. Sci. U.S.A. **90**, 8043–8047 (1993)
7. Hisano, H., Sato, K.: Genomic regions responsible for amenability to agrobacterium-mediated transformation in barley. Sci. Rep. **6**, 37505 (2016). https://doi.org/10.1038/srep37505
8. Singh, S., Nandha, P.S., Singh, J.: Transposon-based genetic diversity assessment in wild and cultivated barley. Crop J. **5**, 296–304 (2017)
9. Goldberg, M.L., Hartwell, L.H., Fischer, J.A., Hood, L.E.: Genetics: From Genes to Genomes. McGraw-Hill Education (2017)
10. Ray, D.K., Mueller, N.D., West, P.C., Foley, J.A.: Yield trends are insufficient to double global crop production by 2050. PLoS One. **8**, e66428 (2013). https://doi.org/10.1371/journal.pone.0066428
11. Kim, Y.G., Cha, J., Chandrasegaran, S.: Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc. Natl. Acad. Sci. U.S.A. **93**, 1156–1160 (1996). https://doi.org/10.1073/pnas.93.3.1156
12. Christian, M., Cermak, T., Doyle, E.L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A.J., Voytas, D.F.: Targeting DNA double-strand breaks with TAL effector nucleases. Genetics. **186**, 757–761 (2010). https://doi.org/10.1534/genetics.110.120717
13. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E.: A programmable dual RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. **337**, 816–821 (2012). https://doi.org/10.1126/science.1225829
14. Doudna, J.A., Charpentier, E.: Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science. **346**, 1258096 (2014). https://doi.org/10.1126/science.1258096
15. Budhagatapalli, N., Rutten, T., Gurushidze, M., Kumlehn, J., Hensel, G.: Targeted modification of gene function exploiting homology-directed repair of TALEN-mediated double-strand breaks in Barley. G3 GenesGenomesGenetics. **5**, 1857–1863 (2015). https://doi.org/10.1534/g3.115.018762
16. Gurushidze, M., Hensel, G., Hiekel, S., Schedel, S., Valkov, V., Kumlehn, J.: True-breeding targeted Gene Knock-Out in barley using designer TALE-nuclease in haploid cells. PLoS One. **9**, e92046 (2014). https://doi.org/10.1371/journal.pone.0092046
17. Lawrenson, T., Shorinola, O., Stacey, N., Li, C., Østergaard, L., Patron, N., Uauy, C., Harwood, W.: Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. Genome Biol. **16**, 258 (2015). https://doi.org/10.1186/s13059-015-0826-7
18. Kapusi, E., Corcuera-Gómez, M., Melnik, S., Stoger, E.: Heritable genomic fragment deletions and small Indels in the putative ENGase gene induced by CRISPR/Cas9 in Barley. Front. Plant Sci. **8**, 540 (2017). https://doi.org/10.3389/fpls.2017.00540
19. Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S.O., Wicker, T., Radchuk, V., Dockter, C., Hedley, P.E., Russell, J., Bayer, M., Ramsay, L., Liu, H., Haberer, G., Zhang, X.-Q., Zhang, Q., Barrero, R.A., Li, L., Taudien, S., Groth, M., Felder, M., Hastie, A., Šimková, H., Staňková, H., Vrána, J., Chan, S., Muñoz-Amatriaín, M., Ounit, R., Wanamaker, S., Bolser, D., Colmsee, C., Schmutzer, T., Aliyeva-Schnorr, L., Grasso, S., Tanskanen, J., Chailyan, A., Sampath, D., Heavens, D., Clissold, L., Cao, S., Chapman, B., Dai, F., Han, Y., Li, H., Li, X., Lin, C., McCooke, J.K., Tan, C., Wang, P., Wang, S., Yin, S., Zhou, G., Poland, J.A., Bellgard, M.I., Borisjuk, L., Houben, A., Doležel, J., Ayling, S., Lonardi, S., Kersey, P., Langridge, P., Muehlbauer, G.J., Clark, M.D., Caccamo, M., Schulman, A.H., Mayer, K.F.X., Platzer, M., Close, T.J., Scholz, U., Hansson, M., Zhang, G., Braumann, I., Spannagl, M., Li, C., Waugh, R., Stein, N.: A chromosome conformation capture ordered sequence of the barley genome. Nature. **544**, 427–433 (2017). https://doi.org/10.1038/nature22043
20. Gasparis, S., Kała, M., Przyborowski, M., Łyżnik, L.A., Orczyk, W., Nadolska-Orczyk, A.: A simple and efficient CRISPR/Cas9 platform for induction of single and multiple, heri-

table mutations in barley (Hordeum vulgare L.). Plant Method. **14**, 111 (2018). https://doi.org/10.1186/s13007-018-0382-8

21. Kapusi, E., Stöger, E.: Detection of CRISPR/Cas9-induced genomic fragment deletions in barley and generation of homozygous edited lines via embryogenic pollen culture. In: Pereira, C. (ed.) Plant Vacuolar Trafficking: Methods and Protocols, pp. 9–20. Springer, New York (2018)
22. Lawrenson, T., Harwood, W.A.: Creating targeted gene knockouts in barley using CRISPR/Cas9. In: Harwood, W.A. (ed.) Barley: Methods and Protocols, pp. 217–232. Springer, New York (2019)
23. Jiang, Q., Yang, Q., Harwood, W., Tang, H., Wei, Y., Zheng, Y.: A CRISPR/Cas9 protocol for target gene editing in barley. In: Yang, B., Harwood, W., Que, Q. (eds.) Plant Genome Engineering: Methods and Protocols, pp. 287–296. Springer, New York (2023)
24. Lawrenson, T., Hinchliffe, A., Forner, M., Harwood, W.: Highly efficient genome editing in barley using novel LbCas12a variants and impact of sgRNA architecture. https://www.biorxiv.org/content/10.1101/2022.04.28.489853v1 (2022)
25. Li, Q., Feng, Q., Snouffer, A., Zhang, B., Rodríguez, G.R., van der Knaap, E.: Increasing fruit weight by editing a cis-regulatory element in tomato KLUH promoter using CRISPR/Cas9. Front. Plant Sci. **13** (2022)
26. Rodríguez-Leal, D., Lemmon, Z.H., Man, J., Bartlett, M.E., Lippman, Z.B.: Engineering quantitative trait variation for crop improvement by genome editing. Cell. **171**, 470–480.e8 (2017). https://doi.org/10.1016/j.cell.2017.08.030
27. Hummel, A.W., Doyle, E.L., Bogdanove, A.J.: Addition of transcription activator-like effector binding sites to a pathogen strain-specific rice bacterial blight resistance gene makes it effective against additional strains and against bacterial leaf streak. New Phytol. **195**, 883–893 (2012). https://doi.org/10.1111/j.1469-8137.2012.04216.x
28. Zafar, K., Khan, M.Z., Amin, I., Mukhtar, Z., Yasmin, S., Arif, M., Ejaz, K., Mansoor, S.: Precise CRISPR-Cas9 mediated genome editing in super basmati Rice for resistance against bacterial blight by targeting the major susceptibility gene. Front. Plant Sci. **11**, 575 (2020). https://doi.org/10.3389/fpls.2020.00575
29. Peng, A., Chen, S., Lei, T., Xu, L., He, Y., Wu, L., Yao, L., Zou, X.: Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene CsLOB1 promoter in citrus. Plant Biotechnol. J. **15**, 1509–1519 (2017). https://doi.org/10.1111/pbi.12733
30. Shi, J., Gao, H., Wang, H., Lafitte, H.R., Archibald, R.L., Yang, M., Hakimi, S.M., Mo, H., Habben, J.E.: ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol. J. **15**, 207–216 (2017). https://doi.org/10.1111/pbi.12603
31. Pandiarajan, R., Grover, A.: In vivo promoter engineering in plants: are we ready? Plant Sci. Int. J. Exp. Plant Biol. **277**, 132–138 (2018). https://doi.org/10.1016/j.plantsci.2018.10.011
32. Wolter, F., Puchta, H.: Application of CRISPR/Cas to understand cis- and trans-regulatory elements in plants. Method Mol. Biol. Clifton NJ. **1830**, 23–40 (2018). https://doi.org/10.1007/978-1-4939-8657-6_2
33. Pan, C., Li, G., Malzahn, A.A., Cheng, Y., Leyson, B., Sretenovic, S., Gurel, F., Coleman, G.D., Qi, Y.: Boosting plant genome editing with a versatile CRISPR-combo system. Nat. Plant. **8**, 513–525 (2022). https://doi.org/10.1038/s41477-022-01151-9
34. Uniyal, A.P., Mansotra, K., Yadav, S.K., Kumar, V.: An overview of designing and selection of sgRNAs for precise genome editing by the CRISPR-Cas9 system in plants. 3 Biotech. **9**, 223 (2019). https://doi.org/10.1007/s13205-019-1760-2
35. Chen, K., Wang, Y., Zhang, R., Zhang, H., Gao, C.: CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu. Rev. Plant Biol. **70**, 667–697 (2019). https://doi.org/10.1146/annurev-arplant-050718-100049
36. Biswas, S., Zhang, D., Shi, J.: CRISPR/Cas systems: opportunities and challenges for crop breeding. Plant Cell Rep. **40**, 979–998 (2021). https://doi.org/10.1007/s00299-021-02708-2

37. Miladinovic, D., Antunes, D., Yildirim, K., Bakhsh, A., Cvejić, S., Kondić-Špika, A., Marjanovic Jeromela, A., Opsahl-Sorteberg, H.-G., Zambounis, A., Hilioti, Z.: Targeted plant improvement through genome editing: from laboratory to field. Plant Cell Rep. **40**, 935–951 (2021). https://doi.org/10.1007/s00299-020-02655-4
38. Hoffie, R.E., Otto, I., Hisano, H., Kumlehn, J.: Site-directed mutagenesis in barley using RNA-guided Cas endonucleases during microspore-derived generation of doubled haploids. Methods Mol. Biol. Clifton NJ. **2287**, 199–214 (2021). https://doi.org/10.1007/978-1-0716-1315-3_9
39. Karunarathne, S.D., Han, Y., Zhang, X.-Q., Li, C.: Advances in understanding the molecular mechanisms and potential genetic improvement for nitrogen use efficiency in barley. Agronomy. **10**, 662 (2020). https://doi.org/10.3390/agronomy10050662
40. Sakellariou, M., Mylona, P.V.: New uses for traditional crops: the case of barley biofortification. Agronomy. **10**, 1964 (2020). https://doi.org/10.3390/agronomy10121964
41. Riaz, A., Kanwal, F., Börner, A., Pillen, K., Dai, F., Alqudah, A.M.: Advances in genomics-based breeding of barley: molecular tools and genomic databases. Agronomy. **11**, 894 (2021). https://doi.org/10.3390/agronomy11050894
42. Garcia-Gimenez, G., Jobling, S.A.: Gene editing for barley grain quality improvement. J. Cereal Sci. **103**, 103394 (2022). https://doi.org/10.1016/j.jcs.2021.103394
43. Patial, M., Chauhan, R., Chaudhary, H.K., Pramanick, K.K., Shukla, A.K., Kumar, V., Verma, R.P.S.: Au-courant and novel technologies for efficient doubled haploid development in barley (Hordeum vulgare L.). Crit. Rev. Biotechnol. **0**, 1–19 (2022). https://doi.org/10.1080/07388551.2022.2050181
44. Chen, Z., Li, L., Halford, N.G., Xu, H., Huang, L., Gao, R., Lu, R., Liu, C.: Advances in barley breeding for improving nitrogen use efficiency. Agronomy. **12**, 1682 (2022). https://doi.org/10.3390/agronomy12071682
45. Hameed, A., Poznanski, P., Noman, M., Ahmed, T., Iqbal, A., Nadolska-Orczyk, A., Orczyk, W.: Barley resistance to Fusarium graminearum infections: from transcriptomics to field with food safety concerns. J. Agric. Food Chem. **70**, 14571–14587 (2022). https://doi.org/10.1021/acs.jafc.2c05488
46. Low, Y.C., Lawton, M.A., Di, R.: Validation of barley 2OGO gene as a functional orthologue of Arabidopsis DMR6 gene in Fusarium head blight susceptibility. Sci. Rep. **10**, 9935 (2020). https://doi.org/10.1038/s41598-020-67006-5
47. Kumar, N., Galli, M., Ordon, J., Stuttmann, J., Kogel, K.-H., Imani, J.: Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system. Plant Biotechnol. J. **16**, 1892–1903 (2018). https://doi.org/10.1111/pbi.12924
48. Galli, M., Martiny, E., Imani, J., Kumar, N., Koch, A., Steinbrenner, J., Kogel, K.-H.: CRISPR/SpCas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth. Plant Biotechnol. J. **20**, 89–102 (2021). https://doi.org/10.1111/pbi.13697
49. Koide, H., Hisano, H., Yaeno, T.: CRISPR/Cas9-based generation of mlo mutants for allelic complementation experiments to elucidate MLO function in barley. J. Gen. Plant Pathol. (2023). https://doi.org/10.1007/s10327-023-01120-w
50. Kis, A., Hamar, É., Tholt, G., Bán, R., Havelda, Z.: Creating highly efficient resistance against wheat dwarf virus in barley by employing CRISPR/Cas9 system. Plant Biotechnol. J. **17**, 1004–1006 (2019). https://doi.org/10.1111/pbi.13077
51. Kershanskaya, O.I., Yessenbaeva, G.L., Nelidova, D.S., Karabekova, A.N., Sadullaeva, Z.N.: CRISPR/Cas genome editing perspectives for barley breeding. Physiol. Plant. **174**, e13686 (2022). https://doi.org/10.1111/ppl.13686
52. Hoffie, R.E., Perovic, D., Habekuß, A., Ordon, F., Kumlehn, J.: Novel resistance to the Bymovirus BaMMV established by targeted mutagenesis of the PDIL5-1 susceptibility gene in barley. Plant Biotechnol. J. **21**, 331–341 (2023). https://doi.org/10.1111/pbi.13948
53. Weltzien, E., Christinck, A.: Methodologies for priority setting in plant breeding programs. Presented at the January 1 (2009)
54. Ceccarelli, S.: Efficiency of plant breeding. Crop Sci. **55**, 87–97 (2015). https://doi.org/10.2135/cropsci2014.02.0158

55. Voltas, J., Eeuwijk, F., Igartua, E., Moral, del, Molina-Cano, J., Romagosa, I.: Genotype by environment interaction and adaptation in barley breeding: basic concepts and methods of analysis. Barley Sci. Recent Adv. Mol. Biol. Agron. Yield Qual. (2002)
56. Xu, Y., Lu, Y., Xie, C., Gao, S., Wan, J., Prasanna, B.M.: Whole-genome strategies for marker-assisted plant breeding. Mol. Breed. **29**, 833–854 (2012). https://doi.org/10.1007/s11032-012-9699-6
57. Niu, Y., Chen, T., Zhao, C., Zhou, M.: Improving crop lodging resistance by adjusting plant height and stem strength. Agronomy. **11**, 2421 (2021). https://doi.org/10.3390/agronomy11122421
58. Holubová, K., Hensel, G., Vojta, P., Tarkowski, P., Bergougnoux, V., Galuszka, P.: Modification of barley plant productivity through regulation of Cytokinin content by reverse-genetics approaches. Front. Plant Sci. **9** (2018)
59. Chen, L., Zhao, J., Song, J., Jameson, P.E.: Cytokinin dehydrogenase: a genetic target for yield improvement in wheat. Plant Biotechnol. J. **18**, 614–630 (2020). https://doi.org/10.1111/pbi.13305
60. Garcia-Gimenez, G., Barakate, A., Smith, P., Stephens, J., Khor, S.F., Doblin, M.S., Hao, P., Bacic, A., Fincher, G.B., Burton, R.A., Waugh, R., Tucker, M.R., Houston, K.: Targeted mutation of barley (1,3;1,4)-β-glucan synthases reveals complex relationships between the storage and cell wall polysaccharide content. Plant J. **104**, 1009–1022 (2020). https://doi.org/10.1111/tpj.14977
61. Yang, Q., Zhong, X., Li, Q., Lan, J., Tang, H., Qi, P., Ma, J., Wang, J., Chen, G., Pu, Z., Li, W., Lan, X., Deng, M., Harwood, W., Li, Z., Wei, Y., Zheng, Y., Jiang, Q.: Mutation of the d-hordein gene by RNA-guided Cas9 targeted editing reducing the grain size and changing grain compositions in barley. Food Chem. **311**, 125892 (2020). https://doi.org/10.1016/j.foodchem.2019.125892
62. Li, Y., Liu, D., Zong, Y., Jiang, L., Xi, X., Cao, D., Shen, Y., Zhang, H., Liu, B.: New D hordein alleles were created in barley using CRISPR/Cas9 genome editing. Cereal Res. Commun. **48**, 131–138 (2020). https://doi.org/10.1007/s42976-020-00023-2
63. Lee, J.H., Won, H.J., Hoang Nguyen Tran, P., Lee, S., Kim, H.-Y., Jung, J.H.: Improving lignocellulosic biofuel production by CRISPR/Cas9-mediated lignin modification in Barley. GCB Bioenergy. **13**, 742–752 (2021). https://doi.org/10.1111/gcbb.12808
64. Kumar, D., Yadav, A., Ahmad, R., Dwivedi, U.N., Yadav, K.: CRISPR-based genome editing for nutrient enrichment in crops: a promising approach toward global food security. Front. Genet. **13** (2022)
65. Liu, C.-J., Eudes, A.: Lignin synthesis and bioengineering approaches toward lignin modification. In: Sibout, R. (ed.) Advances in Botanical Research, pp. 41–96. Academic Press (2022)
66. Becker, H.C., Léon, J.: Stability analysis in plant breeding. Plant Breed. **101**, 1–23 (1988). https://doi.org/10.1111/j.1439-0523.1988.tb00261.x
67. Araus, J.L., Cairns, J.E.: Field high-throughput phenotyping: the new crop breeding frontier. Trends Plant Sci. **19**, 52–61 (2014). https://doi.org/10.1016/j.tplants.2013.09.008
68. Witcombe, J.R., Hollington, P.A., Howarth, C.J., Reader, S., Steele, K.A.: Breeding for abiotic stresses for sustainable agriculture. Philos. Trans. R. Soc. B Biol. Sci. **363**, 703–716 (2007). https://doi.org/10.1098/rstb.2007.2179
69. Miedaner, T.: Breeding strategies for improving plant resistance to diseases. In: Al-Khayri, J.M., Jain, S.M., Johnson, D.V. (eds.) Advances in Plant Breeding Strategies: Agronomic, Abiotic and Biotic Stress Traits, pp. 561–599. Springer, Cham (2016)
70. Křen, J., Klem, K., Svobodová, I., Míša, P., Neudert, L.: Yield and grain quality of spring barley as affected by biomass formation at early growth stages. Plant Soil Environ. **60**, 221–227 (2014)
71. Araus, J.L., Slafer, G.A., Reynolds, M.P., Royo, C.: Plant breeding and drought in C3 cereals: what should we breed for? Ann. Bot. **89**. Spec No, 925–940 (2002)
72. Stracke, S., Haseneyer, G., Veyrieras, J.-B., Geiger, H.H., Sauer, S., Graner, A., Piepho, H.-P.: Association mapping reveals gene action and interactions in the determination of

flowering time in barley. Theor. Appl. Genet. **118**, 259–273 (2009). https://doi.org/10.1007/s00122-008-0896-y

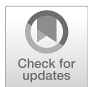
73. Holme, I.B., Dionisio, G., Madsen, C.K., Brinch-Pedersen, H.: Barley HvPAPhy_a as transgene provides high and stable phytase activities in mature barley straw and in grains. Plant Biotechnol. J. **15**, 415–422 (2017). https://doi.org/10.1111/pbi.12636
74. Zhong, Y., Liu, C., Qi, X., Jiao, Y., Wang, D., Wang, Y., Liu, Z., Chen, C., Chen, B., Tian, X., Li, J., Chen, M., Dong, X., Xu, X., Li, L., Li, W., Liu, W., Jin, W., Lai, J., Chen, S.: Mutation of ZmDMP enhances haploid induction in maize. Nat. Plant. **5**, 575–580 (2019). https://doi.org/10.1038/s41477-019-0443-7
75. Yang, B.: Grand challenges in genome editing in plants. Front. Genome Editing. **2** (2020)
76. Zeng, Z., Han, N., Liu, C., Buerte, B., Zhou, C., Chen, J., Wang, M., Zhang, Y., Tang, Y., Zhu, M., Wang, J., Yang, Y., Bian, H.: Functional dissection of HGGT and HPT in barley vitamin E biosynthesis via CRISPR/Cas9-enabled genome editing. Ann. Bot. **126**, 929–942 (2020). https://doi.org/10.1093/aob/mcaa115
77. Rukavtsova, E.B., Zakharchenko, N.S., Lebedev, V.G., Shestibratov, K.A.: CRISPR-Cas genome editing for horticultural crops improvement: advantages and prospects. Horticulturae. **9**, 38 (2023). https://doi.org/10.3390/horticulturae9010038
78. Ma, X., Chen, L., Zhu, Q., Chen, Y., Liu, Y.-G.: Rapid decoding of sequence-specific nuclease-induced heterozygous and Biallelic mutations by direct sequencing of PCR products. Mol. Plant. **8**, 1285–1287 (2015). https://doi.org/10.1016/j.molp.2015.02.012
79. Lührs, R., Lörz, H.: Plant regeneration in vitro from embryogenic cultures of spring- and winter-type barley (Hordeum vulgare L.) varieties. Theor. Appl. Genet. **75**, 16–25 (1987). https://doi.org/10.1007/BF00249136
80. Hensel, G., Kumlehn, J.: Genetic transformation of Barley (Hordeum Vulgare L.) by co-culture of immature embryos with agrobacterium. In: Curtis, I.S. (ed.) Transgenic Crops of the World: Essential Protocols, pp. 35–44. Springer, Dordrecht (2004)
81. Harwood, W.A.: A protocol for high-throughput agrobacterium-mediated barley transformation. Methods Mol. Biol. Clifton NJ. **1099**, 251–260 (2014). https://doi.org/10.1007/978-1-62703-715-0_20
82. Hinchliffe, A., Harwood, W.A.: Agrobacterium-mediated transformation of barley immature embryos. Methods Mol. Biol. Clifton NJ. **1900**, 115–126 (2019). https://doi.org/10.1007/978-1-4939-8944-7_8
83. Marthe, C., Kumlehn, J., Hensel, G.: Barley (Hordeum vulgare L.) transformation using immature embryos. Methods Mol. Biol. Clifton NJ. **1223**, 71–83 (2015). https://doi.org/10.1007/978-1-4939-1695-5_6
84. Trifonova, A., Madsen, S., Olesen, A.: Agrobacterium-mediated transgene delivery and integration into barley under a range of in vitro culture conditions. Plant Sci. **161**, 871–880 (2001). https://doi.org/10.1016/S0168-9452(01)00479-4
85. Hensel, G., Valkov, V., Marthe, C., Kumlehn, J.: Efficient agrobacterium-mediated transformation of various Barley (Hordeum vulgare L.) genotypes. In: Xu, Z., Li, J., Xue, Y., Yang, W. (eds.) Biotechnology and Sustainable Agriculture 2006 and Beyond, pp. 143–145. Springer, Dordrecht (2007)
86. Broughton, S., Sidhu, P.K., Davies, P.A.: In vitro culture for doubled haploids: tools for molecular breeding. Method Mol. Biol. Clifton NJ. **1145**, 167–189 (2014). https://doi.org/10.1007/978-1-4939-0446-4_14
87. Han, Y., Broughton, S., Liu, L., Zhang, X.-Q., Zeng, J., He, X., Li, C.: Highly efficient and genotype-independent barley gene editing based on anther culture. Plant Commun. **2**, 100082 (2020). https://doi.org/10.1016/j.xplc.2020.100082
88. Lowe, K., Wu, E., Wang, N., Hoerster, G., Hastings, C., Cho, M.-J., Scelonge, C., Lenderts, B., Chamberlin, M., Cushatt, J., Wang, L., Ryan, L., Khan, T., Chow-Yiu, J., Hua, W., Yu, M., Banh, J., Bao, Z., Brink, K., Igo, E., Rudrappa, B., Shamseer, P.M., Bruce, W., Newman, L., Shen, B., Zheng, P., Bidney, D., Falco, C., Register, J., Zhao, Z.-Y., Xu, D., Jones, T., Gordon-Kamm, W.: Morphogenic regulators baby boom and wuschel improve monocot transformation. Plant Cell. **28**, 1998–2015 (2016). https://doi.org/10.1105/tpc.16.00124

89. Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.-M., van Lammeren, A.A.M., Miki, B.L.A., Custers, J.B.M., van Lookeren Campagne, M.M.: Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell. **14**, 1737–1749 (2002). https://doi.org/10.1105/tpc.001941
90. Kim, S., Soltis, P.S., Wall, K., Soltis, D.E.: Phylogeny and domain evolution in the APETALA2-like gene family. Mol. Biol. Evol. **23**, 107–120 (2006). https://doi.org/10.1093/molbev/msj014
91. Bilichak, A., Luu, J., Jiang, F., Eudes, F.: Identification of BABY BOOM homolog in bread wheat. Agri. Gene. **7**, 43–51 (2018). https://doi.org/10.1016/j.aggene.2017.11.002
92. Zuo, J., Niu, Q.-W., Frugis, G., Chua, N.-H.: The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J. Cell Mol. Biol. **30**, 349–359 (2002). https://doi.org/10.1046/j.1365-313x.2002.01289.x
93. Ikeda, M., Mitsuda, N., Ohme-Takagi, M.: Arabidopsis WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning. Plant Cell. **21**, 3493–3505 (2009). https://doi.org/10.1105/tpc.109.069997
94. Debernardi, J.M., Tricoli, D.M., Ercoli, M.F., Hayta, S., Ronald, P., Palatnik, J.F., Dubcovsky, J.: A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat. Biotechnol. **38**, 1274–1279 (2020). https://doi.org/10.1038/s41587-020-0703-0
95. Hisano, H., Meints, B., Moscou, M.J., Cistue, L., Echávarri, B., Sato, K., Hayes, P.M.: Selection of transformation-efficient barley genotypes based on TFA (transformation amenability) haplotype and higher resolution mapping of the TFA loci. Plant Cell Rep. **36**, 611–620 (2017). https://doi.org/10.1007/s00299-017-2107-2
96. Orman-Ligeza, B., Harwood, W., Hedley, P.E., Hinchcliffe, A., Macaulay, M., Uauy, C., Trafford, K.: TRA1: a locus responsible for controlling agrobacterium-mediated transformability in barley. Front. Plant Sci. **11** (2020)
97. Wolt, J.D., Yang, B., Wang, K., Spalding, M.H.: Regulatory aspects of genome-edited crops. Vitro Cell. Dev. Biol. Plant. **52**, 349–353 (2016). https://doi.org/10.1007/s11627-016-9784-3
98. Waltz, E.: With a free pass, CRISPR-edited plants reach market in record time. Nat. Biotechnol. **36**, 6–7 (2018). https://doi.org/10.1038/nbt0118-6b
99. Mallapaty, S.: Australian gene-editing rules adopt 'middle ground'. Nature. (2019). https://doi.org/10.1038/d41586-019-01282-8
100. Laaninen, T.: New genomic techniques: European Commission study and first reactions (2021)
101. Ghosh, P.: Commercial development of gene-edited food now legal in England. https://www.bbc.com/news/science-environment-64596453 (2023)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 11
Current Status and Future Prospective of Genome Editing Application in Maize



Serena Varotto

Abstract Maize (*Zea mays* ssp. *mays*) is the most world-widely cultivated agricultural crop and over the past century, its yield per unit land area has increased consistently due to both breeding efforts and improvement in management. Cross breeding, mutation breeding, and transgenic breeding are the main methodologies adopted for maize improvement. The first maize transgenic hybrids were commercialized more than 20 years ago, and till now more than 150 different events of transgenic maize have been approved for commercial cultivation. The sequencing of the maize genome and the development of advanced genomic tools provided the biologists with the theoretical information necessary to attempt the genome modification at the pre-intended genomic loci. The tremendous advances brought about by CRISPR/Cas systems from first applications to nowadays has made genome editing a powerful tool for precise maize improvement. Although many CRISPR-Cas-edited genes have been documented to improve maize traits of agronomic interest, only a few lines have been tested in field trials; additional work for determining potential breeding values of edited maize lines must be done in terms of field tests. The integration of CRISPR-Cas technology in the breeding of new maize varieties also depends on existing and future regulatory policies that will be adopted worldwide.

1 Introduction

Maize (*Zea mays* ssp. *mays*) is the most world-widely cultivated agricultural crop and a renowned experimental model plant for molecular and genetic studies. Maize domestication started about 9000 years ago from the wild grass Z. mays subsp. Parviglumis in the Balsas region of southwest Mexico [1, 2]. Morphological observations, genetic and genomic studies have elucidated how from *Z. mays* subsp. *Parviglumis* (also called "teosinte") *Z. mays* subsp. *mays* was

S. Varotto (✉)
Department of Agronomy Animal, Food Natural Resources and Environment (DAFNAE), University of Padova – Agripolis Viale dell'Università, Legnaro (PD), Italy
e-mail: serena.varotto@unipd.it

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_11

domesticated. Although a few major morphological differences distinguish these two subspecies, and genes responsible for these traits have been identified, it has emerged that several genomic regions have contributed to maize domestication [3]. Among the grasses, maize has a medium-sized genome of approximately 2.4 Gb [4], which is characterized by an exceptional genomic structural diversity. Large insertions and deletions that includes tandem repeats cluster and transposable elements are common between maize inbred lines. This structural diversity, characterized by copy number variants (CNVs) and presence/absence variants (PAVs) is important for maize adaptation and has offered a rich pool of genetic diversity to breeders for creating improved germplasm [5]. At the gene sequence level, single nucleotide polymorphisms (SNPs) are frequent in introns and untranslated regions of maize genome. SNPs surveys and subsequent quantitative traits genome wide association studies (QTL/GWAS) were adopted for linking genetic and phenotypic variations. In parallel, the sequencing of the B73 reference genome, which quality has been greatly improved during the last few years by the development of long-read sequencing (mainly by Pacific Biosciences' PacBio) single molecule real-time (SMRT) sequencing [6] have shed light on both the complexity of the maize genome and the contributions of different variations to phenotypic differences. In addition to the characterization of genetic variations, recent research in maize was aimed at understanding the relationships among gene expression, epigenetic modifications, chromatin interactions, and metabolic, proteome, and phenotype variations. The development of high-quality genome assembly tools together with the precise characterization of genomic diversity and the association of genetic variants with yield-related traits has greatly improved maize genomic research. Till now, thousands of diverse and representative maize lines have been genotyped [7] and the integration of multiple annotated reference genomes has been facilitated by comprehensive databases that store, maintain, analyze, and visualize the multi-omics data, such as MaizeGDB [8] and ZEAMAP [9]. The development of genomic technologies has allowed a deeply exploration of diversity at all levels in different environments and the identification of genes that determine maize yield: the mechanistic understanding of gene function and the precise modification of genes in different genomic background can accelerate breeding for yield in the coming years.

2 A Glance Over Conventional Maize Breeding

Over the past century, maize yield per unit land area increased consistently (over sevenfold) due to both breeding efforts and improvement in management [7]. Breeding of hybrid crops was pioneered in maize since the observation, in early 1900s, that hybrid cultivars provided higher yield compared to pure lines and open pollinated varieties [10]. The constitution of modern maize hybrids relays on

development of elite parental inbred lines and their subsequent evaluation in single cross combination. The original methods for hybrid production introduced by Shull in 1908 (referred as “pure line method of corn breeding”) [11] underwent several modifications over years. This allowed both a more efficient production of inbred lines and identification of superior hybrid combinations between them [12]. In modern maize breeding, the activity of evaluation of inbred lines for hybrid performance is the most critical and expensive phase. Inbred lines are collected in heterotic groups with the aim of facilitating the identification of superior hybrid combinations [10]. Additionally, to increase the number of lines having good potential for hybrid performance, population improvement methods are adopted, and double haploid (DH) technology is used to generate homozygous lines [13]. To introduce desirable alleles into a desired elite inbred line background by genetic crosses, 8–10 selfing generations are required. Moreover, extensive background screening and evaluation of large-sized progenies are necessary to increase the chance of genetic recombination and reduce possible linkage drag effects, when the desirable trait is closely linked to an undesirable trait [7]. The advantage of DH technology relays on the much quicker development of homozygous lines compared to 8–10 generations of inbreeding by selfing or sib-crossing necessary to develop inbreds [14]. Nowadays in maize breeding programs, DH are routinely obtained by pollinating the plants with haploid inducer (HI) lines. Subsequently, chromosomes can be doubled spontaneously or artificially, by treatment with mitotic inhibitors such as colchicine, for generating DH lines.

Cross breeding, mutation breeding, and transgenic breeding are the main methodologies adopted for maize improvement. As illustrated above, in maize to introduce desirable alleles by cross breeding, and use genetic recombination to produce genetic variability are long and costly procedure. Additionally, for some agronomic traits genetic variability has been greatly reduced by domestication and directed selection [15]. To overcome these limits, mutation breeding has created genetic variation by introducing random mutations in the maize genome [16]. However, the stochastic nature of the mutations produced and the need to screen large numbers of mutant genotypes makes mutation breeding a time-consuming and laborious procedure that cannot enhance selection efficiency, even if marker-assisted breeding approach are adopted. Transgenic breeding through the transfer of exogenous genes into commercial elite varieties can accelerate the improvement of important agronomic traits. However, along with some limitations of the methodology, such as the random insertion of the transgene and the low number of sequences that can be introduce in the genome by genetic transformation, the long and costly deregulation processes, and public concerns about transgenic crops, limit the commercialization of genetically modified maize plants [17]. In the following chapter we will highlight how the introduction of targeted mutagenesis and the combination of these novel technologies with conventional breeding procedures can solve some of the main limiting factors for a more sustainable maize breeding.

3 Maize Genetic Transformation

Technological innovation and scientific discoveries have always had a big impact on maize research and breeding, and genetic transformation has been an indispensable biotechnology in both applied and basic maize research. In basic research transformation technologies were widely adopted to study gene regulation and function, mainly through the obtention of mutant plants in which the target sequences are over-expressed, expressed ectopically, downregulated or silenced. Additionally, transgenesis allows the study of gene promoters and other regulatory sequences regulating gene expression in the coding portion of the genome. As already mentioned above, in applied maize research, the introgression of beneficial target genes from one line (donor) to another (recipient) by conventional breeding requires many years of backcrossing after hybridization and may lead to linkage drug effect on the recipient line genome [7]. Conversely, genetic transformation introduces well characterized DNA regulatory and coding sequences into the plant genome. The goal to transform maize with a high efficiency technology, providing high-quality transgenic events has been essential to improve specific maize traits [18]. Initially, the production of genetically modified maize varieties has encountered enormous difficulties, mainly for the genotype-associated recalcitrance to transformation. In late 80', progresses in genetic engineering and biotechnology resulted in stable transformation of maize [19]. Fromm and colleagues stably transformed maize cells for resistance to kanamycin by electroporation-mediated DNA transfer of a chimeric gene encoding neomycin phosphotransferase. In 1987 Grimsley and colleagues reported that maize plants developed symptoms of viral infection when inoculated with strains of Agrobacterium carrying copies of maize streak virus (MSV) genomes in their T-DNA, thus demonstrating that Agrobacterium could transfer DNA to maize [20]. The first genetically transformed infertile maize plants were obtained from embryogenic cell derived protoplasts treated with plasmid DNA containing a gene coding for neomycin phosphotransferase (NPT II) driven by the 35S promoter region of cauliflower mosaic virus [21]. Finally, fertile transgenic maize plants were produced from embryogenic cell suspension transformed with the bacterial gene bar, encoding for phosphinothricin acetyltransferase (PAT), using microprojectile bombardment [22]. Although several protocols for Agrobacterium- and polyethylene glycol (PEG) protoplast-mediated transformation were developed before 2000s [23, 24] transformation efficiency and successful *in vitro* plantlet regeneration through tissue culture was highly dependent on genotypes. Therefore, hybrid lines showing the ability to produce highly transformable calluses were selected: High type II callus (Hi II), containing both A188 and B73 inbred genetic background, became one of the most widely used hybrids for maize transformation in both academic and plant industrial labs [25, 26]. Commonly for maize transformation, embryogenic callus started from immature embryos and cell suspension cultures of embryogenic callus were used [27–29]. Nowadays, although maize transformation is routinely performed, a few genotypes (A188, B104) have been reported to have acceptable transformation efficiency comparable to Hi-II, which have remained the

most popular lines for commercial transformation. For instance, the maize inbred line B73 that is the first inbred to be sequenced and an important genetic resource is strongly recalcitrant to transformation, as well as most of the commercial elite maize inbred lines. The discovery that transgenic maize genotypes overexpressing BABY Boom (ZmBbm), WUSHEL (ZmWus2) and OVULE DEVELOPMENT PROTEIN 2 (ODP2) genes can enable high transformation frequencies in numerous recalcitrant genotypes was an important milestone for maize transformation [30]. Morphogenic Regulator-Mediated Transformation (MRMT) vectors containing these morphogenetic genes can be introduced into Agrobacterium strains and used for immature embryo transformation. Through MRMT increased plant regeneration rates, recovery of transformed plants from recalcitrant genotypes, and a shortening in time needed for transformation by avoiding the callus culture step have been obtained [31]. However, since the constitutive expression of MRs can have a negative pleiotropic effect on important developmental traits, its expression has to be restricted to the embryogenesis induction step, either by excision of the MR expression cassette through a recombination system or driving the expression of MRs using specific promoters [31].

The first maize transgenic hybrids were commercialized more than 20 years ago, and till now more than 150 different events of transgenic maize have been approved for commercial cultivation or food/feed use. Most of the released events concerns simple traits, such as herbicide tolerance, insect resistance, modified product quality, pollination control system and abiotic stress tolerance [32]. It is a matter of fact that applying transgenic approaches for the improvement of complex traits is difficult, because these traits are controlled by numerous genomic loci with a small effect and are strongly influenced by the environment. Evidently this represents a limitation for maize improvement because the limitation concerns the integration of biotechnology and traditional breeding in the improvement of complex traits [33]. One more limitation of transgenic approaches is that foreign DNA integrates into random sites of the host genome. Random integration of transgene might affect the transgene expression, although some recent observations did not prove the assumption of this risk [34]. Moreover, from the first transgenic hybrid commercialization new techniques were developed, new regulations were adopted, and despite their significant beneficial impact on modern agriculture, public perception is still controversial about transgenic crops. The high costs necessary for the deregulation of genetically modified commercial maize plants can be afford only by the largest agricultural biotechnology companies, with a consequent increasing concentration of maize seed providers [35].

4 Mutagenesis in Maize

Traditionally in maize, mutagenesis has been an impressive useful tool for both broadening genetic variation and understanding gene function. Numerous strategies were developed for creating mutations and identifying genes based on phenotypes

(forward genetics). The same strategies have also been used to assignee a phenotype to target coding sequences (reverse genetics). While in early mutagenesis experiments UV and X rays [36, 37], ethyl methanesulfonate (EMS) and other chemical mutagens were used, the main strategies for comprehensive mutagenesis of maize genes comprise TILLING (Targeting Induced Local Lesions IN Genomes) [38], RNAi [39] and transposons mutagenesis. The discovery and characterization of transposon families as well as their massive presence into maize genome facilitated the development of transposable element systems, particularly useful for two maize inbreds, namely B73 and W22. In recent years these systems based on Class II "cut and paste" maize transposons were implemented for creating based genetic resources, such as Ac/Ds families and UniformMu available through MaizeGDB (https://www.maizegdb.org/) and Maize Genetics Cooperation Stock Center (http://maizecoop.crops ci.uiuc.edu/) respectively. In the genomic era maize transposons have been the premier method for gene discovery and phenotypic-related characterization in a whole -organism context [40].

The sequencing of the maize genome and the development of advanced genomic tools provided the biologists with the theoretical information necessary to attempt the genome modification at the pre-intended genomic loci in a more precise way than random mutation breeding, which can be time consuming and expensive for the large screens needed. Targeted mutagenesis initial attempts were rarely successful in maize, due to the very low frequency of homologous recombination (HR) events involving either endogenous target or exogenous donor DNA [41]. However, the adaptation to maize of Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) two novel technologies successfully applied in mammalian cells and in a few model plants, such as Arabidopsis and tobacco, allowed the first genome editing events on maize endogenous target genes. Targeted genome editing tools use nucleases to induce DNA double-strand breaks (DSBs). In plant cells, DSBs can be repaired by two main pathways, nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). The NHEJ pathway usually generates in/dels at the repair sites. Differently, when a template DNA is provided HDR can be adopted for precise sequence replacement or insertion [42]. The ZFNs are chimeric proteins with two domains: the N-terminal domain is a synthetic zinc finger-based domain that recognizes a 3-base-pair (bp) target sequence and binds to DNA; the C-terminal domain is a non-specific DNA cleavage domain using Fok1 a type IIS class of restriction endonucleases [43]. Because FokI functions as a dimer, ZFNs are designed as two ZFN monomers bound to an 18- or 24-bp sequence with a 5–7-nucleotide spacer. This spacing is a critical part of ZFN design as it allows Fok1 monomer to dimerise and create a DSB in the target sequence. A pair of Zinc finger arrays (ZFAs) binds to respective sequences targeted and get aligned in reverse fashion with each other. In 2009, Schukla et al. [44] reported the use of designed (ZFNs) that induced a double-stranded break to modify a target endogenous locus in maize. The simultaneous expression of ZFNs and delivery of a simple heterologous donor molecule allowed the targeted addition of an herbicide-tolerance gene, one of the phytic acid biosynthesis genes, namely inositol-1,3,4,5,6-pentakisphosphate kinase 1 (IPK1). ZFN-modified maize plants

could pass these genetic changes to their progeny: in developing maize seeds the targeted cleavage of IPK1 gives the characteristics of both herbicide tolerance and desired alteration of the inositol phosphate [44]. About five years later, the TALEN technology was successful applied for targeted gene mutagenesis in a proof-of-concept study in maize [45]. Similarly to ZFNs, TALENs are fusion proteins of native or artificial TAL effector DNA-binding domains and the DNA-cleavage domain of FokI. The modular TAL effector repeats can be custom-tailored into DNA recognizing domains for virtually any sequence in a genome [46]. When expressed in plant cells, the paired TALENs recognize and bind to two adjacent, opposite subsites, enabling the FokI domains (homo- or heterodimeric) to dimerize to an approximately 50–60-bp target sequence within a 14–18-bp spacer, which is necessary for its function. In maize, TALENs were employed to generate heritable mutations at the glossy2 (gl2) locus. Hi-II transgenic lines containing mono- or di-allelic mutations were produced at a frequency of about 10%. Three modified alleles were functionally tested in progeny seedlings, demonstrating that they conferred the glossy phenotype. The authors reported that the integrated TALEN T-DNA segregated independently from the loss of function gl2 alleles in most of the events, generating mutated null-segregant progeny in T1 generation [45].

These results confirmed ZFNs and TALENs as new strategies for maize genome editing in basic science, with potential in breeding applications. However, owing to construction complexity, high off target rate of ZFNs and high costs of result screenings they had limited applications in maize till now.

5 The CRISPR Technology Application in Maize

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system, comprising CRISPR repeat-spacer arrays and Cas proteins, is an RNA- mediated adaptive immune system in bacteria and archaea. It provides defense against invasive genetic elements by cleaving their nucleic acid genome [47]. The detailed description of the system and its classification in classes and types based on Cas genes are provided in other chapters of this book. Here we will focus on the CRISPR system that has been developed and improved for genome editing in maize, which is generally based on RNA-guided interference with DNA. Briefly, the CRISPR/Cas9 approach uses an RNA-guided endonuclease to generate DNA DSBs at the target sites of the plant genome. The type II CRISPR/Cas9 system adopted in plants requires the hetero-duplex RNA of CRISPR RNA (crRNA) and auxiliary trans-activating crRNA (tracrRNA) to guide the non-specific nuclease, Cas9, for DNA cleavage. In Cas9 the nuclease domains RuvC and HNH cleave the complementary DNA strands [48]. The crRNA and tracrRNA can be further replaced by a single chimeric guide RNA (sgRNA) [47] that can recognize any genomic locus that is followed by a 5′-NGG protospacer-adjacent motif (PAM), and a 20-nt sequence preceding the PAM directs the Cas9 and cleave the target sequence by complementary base pairing. Differently from ZFNs and TALENs that

require sophisticated protein engineering to define precise target site recognition, Cas9-sgRNA components are simple to design and clone. Additionally, the fewer limitations of potential target sites in plant genomes make the CRISPR/Cas system widely applicable. When using this technology, the NHEJ repair pathway is the preferred way to disrupt genes by producing small indels at specific sites in target genes. Moreover, NHEJ can also be exploited to produce insertions of donor DNA sequences in a homology-independent manner [49]. An important choice to make for the application of this technology in maize is the system of delivery to cells of editing reagents, which include DNA, RNA, and ribonucleoproteins (RNPs). Protoplast transfection is commonly uses for transient expression, while Agrobacterium-mediated transfer DNA (T-DNA) transformation, or particle bombardment are the delivery methods of choice to produce edited plants.

In 2014 Xing et al. reported the development of a toolkit to facilitate transient or stable expression of the CRISPR/Cas9 system and generate mutants bearing multiple gene mutations in a variety of plant species, which comprised maize [50]. This system was composed by a CRISPR/Cas9-based binary vector set and a sgRNA module vector set and was validated using maize protoplasts and maize transgenic lines for the targeting of the same maize genomic DNA site (ZmHKT1). In the same year, a comparison between two systems of targeted mutagenesis TALENs and the CRISPR/Cas was conducted by Liang and colleagues [51]. Five TALENs targeting the genes ZmPDS, ZmIPK1A, ZmIPK, ZmMRP4 and two sgRNAs targeting the ZmIPK gene were constructed. The efficiency in inducing targeted mutations was similar for the two systems: 13.1% CRISPR/Cas and 9.1% TALENs, respectively, in maize protoplasts.

The first detailed report in a scientific journal of Cas9-gRNA genome editing technology application in maize, with direct delivery of sgRNA in the form of RNA molecules, was published by Svitashev et al. in 2015 [52]. For targeting five different maize genomic regions, the biolistic transformation of maize immature embryos was conducted with DNA vectors expressing a maize codon optimized Cas9 endonuclease and sgRNAs, with or without DNA repair templates. The genomic regions were located upstream of the liguleless1 (LIG1) gene, at male fertility genes (Ms26 and Ms45) and acetolactate synthase (ALS) genes (ALS1 and ALS2). Following transformations, the authors could identify mutations at all sites targeted, as well as plants containing biallelic multiplex mutations at LIG1, Ms26, and Ms45. When immature embryo cells containing pre-integrated Cas9 were directly used for biolistic delivery of sgRNAs (as RNA molecules) targeted mutations could also be detected. The same authors also reported the editing of ALS2 gene and the recovery of chlorsulfuron-resistant plants, using either single-stranded oligonucleotides or double-stranded DNA vectors as repair templates yielded. Moreover, RNA-guided Cas9 endonuclease- generated double-strand breaks at a site near LIG1 stimulated insertion of a trait gene by homology-directed repair. In all cases of genes knockouts, edits, and insertions, T1 plants genetic analysis showed that the mutations followed a Mendelian segregation in subsequent generations.

CRISPR/Cas9 has been also applied for the targeted knockout of the endogenous ZmPSY1 gene in maize T0 transgenic plants [53]. Interestingly in this work, the

authors configured and optimized the CRISPR-Cas9 system for targeted genome editing in maize and accurately tested the activity of their customized system. Appropriate vectors to express Cas9 and sgRNAs for maize were generated. Cas9 open reading frame was maize codon-optimized and driven by maize Ubiquitin2 promoter. Additionally, to the N terminus of Cas9 a monopartite SV40 nuclear localization signal (NLS) was added and, to further facilitate nuclear localization, a bipartite nucleoplasmin nuclear localization signal (BiNLS) was added to the Cas9 C terminus. Similarly, the authors accurately studied the maize promoter that was used to transcribe the short noncoding sgRNA. A reasonably high mutation rate was firstly established in maize protoplasts. Subsequently, the mutations occurred in germ cells and were transmitted to the next generation with high efficiency. No off-target effect could be detected at the computationally predicted putative off-target loci and no significant difference between the transcriptomes of the Cas9 expressed and non-expressed lines was reported [53].

In maize, multiplex gene editing was achieved by expressing Cas9 together with multiple gRNAs, each targeting different sites and using conventional delivery methods. In principle, single construct containing more than one guide RNA can accelerate and improve the transformation procedures. Two procedures have been developed: the first was based on a multi-guide RNA activated by a single promoter and processed by tRNA motif-mediated self-cleavage into several sgRNAs [54] the second based on tandem repeats of different U3 and U6 promoters each controlling one guide RNA [55]. In both cases, the mutations resulting from targeted mutagenesis were mainly deletions or insertions of a few nucleotides probably due to NHEJ.

In the works described above, to produce edited maize plants, transformation mediated by Agrobacterium or particle bombardment was used for delivering CRISPR/Cas DNA and a selectable marker gene into recipient maize cells and these DNA constructs were stably integrated into the plant genome. However, this strategy of stable integration might increase off-target changes, gene disruption and plant mosaicism, as well as limit commercial applications. Transgene-free edited plants can be selected through genetic segregation by selfing and crossing, which is time consuming in maize hybrid breeding. To avoid these negative effects, transgenic maize plants with pre-integrated Cas9 nuclease have been generated and used for delivery of sgRNAs in the form of RNA molecules. However, this strategy requires the specific development and characterization of Cas9 pre-integrated lines. Transient gene expression of DNA constructs in protoplasts could represent an alternative approach for achieving transgene-free editing in plant. Till now, maize protoplast transient transformation experiments serve mainly for the evaluation of the efficiency of different Cas9 and sgRNA designs, due to the lack of an efficient protocol for the regeneration of maize plants from protoplasts. In 2016 Svitashev [56] and colleagues reported for the first time the biolistic delivery of in vitro assembled Cas9–sgRNA ribonucleoproteins (RNPs) into maize embryo cells and regeneration of plants with both mutated and edited alleles. Purified Cas9 protein pre-assembled with in vitro transcribed gRNAs were delivered into maize immature embryo cells. The previously studied four genomic regions, liguleless1 (LIG), acetolactate synthase (ALS2) and two male fertility genes (MS26 and MS45) [52] were

targeted. Using this method of delivery, they produced DNA- and selectable marker-free maize plants with mutated alleles at high frequencies [56]. As observed in other systems, delivery of RNP complexes led to a significantly decreased undesired mutation frequencies in comparison to DNA vectors. While the frequency of off-site mutations was about 50% for MS45 target site for both immature embryos and mature plants when Cas9 and sgRNA were delivered on DNA vectors, off-site mutations were not detected in regenerated plants when RNPs were used. These results demonstrated that Cas9–gRNA delivered as RNP complex has a significant advantage over DNA vector delivery by promoting high mutation frequencies in a more precise manner also in maize, as already observed for other organisms and plant species [57, 58].

Although involving genes and loci of agronomic interest, many of the initial works using CRISPR/Cas9 were proof-of-principle studies to test genome editing different strategies and efficiency for applications in precision breeding. For an efficient targeted genome editing in maize, the promoters for driving both Cas9 and sgRNA expression were proven to be an essential factor. In early works, the maize ubiquitin gene promoter was used in construct containing a maize codon optimized Cas9. In the same experiments the rice U3 or wheat U3 promoters were used for driving sgRNAs [50, 51]. Different promoter combinations, such as maize ubiquitin1 and U6 promoters, CaMV35S and maize U3, and maize ubiquitin1 gene promoter and two rice U6 promoters, were utilized for Cas9 and sgRNAs in other studies [53, 55, 59] providing additional evidence that the optimization of the promoters used for the CRISPR/Cas9 system is an essential step for efficient targeted genome editing procedures. Furthermore, the mutation efficiency of CRISPR/Cas9 system appeared largely depending upon both the expressions of Cas9 and sgRNAs. Feng and colleagues observed that when the 35S promoter was used for driving the expression of a human codon optimized Cas9 a low mutation rate was obtained and most of the regenerated T0 plants were mosaic [59]. For increasing the mutation efficiency and concomitantly avoiding mosaicism, in a subsequent work they used the promoter of dmc1 gene. DMC1 was thought to be expressed specifically in meiocytes and was used for expressing the Cas9, combined with the U3 promoter for driving the sgRNA expression. The authors reasoned that utilizing these constructs gametes could be mutated, and T1 homozygous or bi-allelic mutants could have been recovered at high frequency at the three loci selected for targeting in the maize genome. However, during their transformation experiments, they realized that the dmc1 promoter could drive the Cas9 to be highly expressed also in maize callus. This observation was confirmed by expression analysis of the endogenous dmc1 gene in different maize tissues including callus. The dmc1 gene was found to have the highest expression level in tassel but was also highly expressed in callus. Using this transformation approach, the T0 plants regenerated were highly efficiently edited at the target sites with homozygous or bi-allelic mutants accounting for about 66%; mutations could be stably transmitted to the T1 generation, while no off-target mutations could be detected in the predicted loci with sequence similarity to the targeted site [60].

After having established the CRISPR/Cas9 technology platform and confirmed its efficiency for single and multiple GT in maize, subsequent studies focused on exploitation of targeted editing for hybrid-breeding technique improvement. In maize, male-sterile maternal lines are an essential prerequisite for generating high-quality commercial hybrid varieties. ZmTMS5 gene controls a thermosensitive male-sterility trait in maize and is the orthologue of the previously characterized and edited TMS5 rice gene [61]. The targeted knockout of the ZmTMS5 gene using immature Hi-II embryos transformation by particle bombardment for delivering CRISPR/Cas9 elements, produced T1 tms5 mutant plants, male-sterile at 32 °C, but male-fertile at 24 °C. T1 plants were Cas9-free through segregation and carried only the desired tms5 mutation. They provide a useful germplasm that can potentially be used to simplify hybrid maize seed production [62].

A potential application of the genome-editing technology concerns the reduction of so-called linkage drag during back-cross breeding. Direct genome-editing technology provides the opportunity of stacking favorable genes without introgression breeding. An experimental proof-of-concept to validate this strategy was provided by the work of Li and coworkers [63]. They established an RNA-guided endonuclease (RGEN) system as an in vivo desired-target mutator (DTM) in maize, to reduce the linkage drag during breeding procedure, using the LIGULELESS1 (LG1) locus as target. The RNA-guided Cas9 system showed 51.5–91.2% mutation frequency in T0 transgenic plants. The T1 plants stably expressing DTM were crossed with six diverse recipient maize lines producing 11.79–28.71% of mutants. Furthermore, the analysis of F2 plants showed that the mutations induced by the DTM effect were heritable. The results were confirmed by the phenotypical characterization of the mutant plants in the field [63].

A further major technical limitation of utilizing gene targeting technologies resides on the recalcitrant nature of most elite maize inbred lines for genetic transformation. To facilitate GT techniques, initials functional tests and transformations are usually done in maize lines with relatively high transformation efficiencies, such as Hi-II and B104. Subsequently, the selected and desired transformation events must be introgressed into elite commercial inbred lines, through at least six backcrossing for achieving more than 99% background purity, requiring additional work and time.

Doubled haploid (DH) technology based on in vivo haploid induction (HI) is an important modern approach for maize breeding. Therefore, there is the need to identify candidate genes underlying HI in maize genome for their detailed functional characterization. Several genes involved in HI were recently validated through CRISPR–Cas9 system [64–66].

In 2018 Wang and colleagues reported the validation of a new strategy for the development of a haploid-inducer mediated genome editing system (IMGE) for accelerating maize breeding. This system uses a maize haploid inducer line carrying a CRISPR/Cas9 cassette targeting for a desired agronomic trait to pollinate an elite maize inbred line. The pollination can generate genome edited haploid maize plants in the elite background. During the process, HI genome is degraded, and no editing

tools are present in edited plants [67]. Within two generations homozygous pure DH lines improved for the desired trait could be obtained, avoiding repeated crossing and backcrossing used in traditional breeding for introgressing a desirable trait into elite commercial backgrounds [67]. Similarly, Kelliher and colleagues co-opted the aberrant reproductive process of haploid induction (HI) to induce edits in nascent seeds in maize and other monocot and dicot species. Their method, named HI-Edit, made direct genomic modification of commercial crop varieties possible, and was tested in field and sweet corn using a native haploid-inducer line. Also in this case, edited haploid plants lack both the haploid-inducer parental DNA and the editing machinery and could be used in trait testing and directly integrated into commercial variety development [68].

5.1 Novel Approaches for Maize Trait Improvement

Based on experimental proof-of-concept reported above, the application of CRISPR/Cas technology not only allows to modify the agronomic traits of interest through the insertion or deletion of single or few nucleotides, but it can also facilitate maize breeding by inserting new alleles in the genome without any linkage drag. Additionally, precise gene modifications can generate novel allelic variants by knock-ins and replacements, thus having great value for crop trait improvement Moreover, knock-in can be used to alter multiple elite traits by stacking genes in a single variety. CRISPR/Cas- systems are currently being applied for enhancing yield, product quality, resistance to diseases and abiotic stress [69].

Novel allelic variants for breeding drought-tolerant plants have been generated in maize [70]. Starting from the observation that maize transgenic plants constitutively overexpressing ARGOS8, which is a negative regulator of ethylene responses, have reduced ethylene sensitivity and improved grain yield under drought stress conditions [70], new variants of ARGOS8 were generated employing CRISPR-Cas technology. Precise genomic DNA modification at the ARGOS8 locus was produced by inserting the native maize GOS2 promoter into the 5′-untranslated region of the native ARGOS8 gene or was used to replace the promoter of ARGOS8. The modified ARGOS8 variants was highly expressed in all plant tissues and increased grain yield under flowering stress conditions when plants were grown in the field [71].

CRISPR–Cas9 can be used for editing of cis-elements, such as in promoters, alternatively to the generation of weak alleles by targeting coding regions. The fine-tuning of gene expression by editing of cis-regulatory elements can lead to quantitative trait variation. Weak promoter alleles of CLV3/EMBRYO-SURROUNDING REGION (ZmCLE) and ZmFON2-LIKE CLE PROTEIN1 (ZmFCP1) were engineered in maize with the aim to create quantitative variation for yield-related traits [72]. For CLE and FCP1 promoter mutagenesis, Cas9 with nine sgRNAs targeting promoter in accessible chromatin regions in developing ears were designed. Multiple maize grain-yield-related traits were successfully increased by using this strategy.

In the same work, Liu and colleagues demonstrated in an elegant way that, in addition to weak allele promoter editing, exploitation of compensation among paralogs can be used for improving maize traits through genome editing [72].

The construction of whole-genome-scale mutant libraries is a modern approach for both functional genomic studies and pre-breeding improvement. Commonly in plants mutant libraries are based on random mutations induced by different mutagenesis procedures like irradiation, T-DNA insertions, (EMS), and transposons. In all these cases many generations to stabilize loss-of-function mutations are required, and additionally the process for determining the relationship between phenotype and genotype in all mutants is a long and critical process.

Liu and co-workers [72] reported the development of a CRISPR/Cas9-based editing platform adapted to high-throughput gene targeting in maize. Li and colleagues established a low-cost optimized and quality-controlled pipelines that includes the design of guide RNAs (sgRNAs) up to the identification of targeted genes and edited sequences. They selected knowledge-driven candidate genes and screened a large number of mutants up to T1 or follow-up generations, showing that their platform allowed functional gene cloning and validation [63].

6 Prospects

In 2016, company researchers from Iowa–based DuPont Pioneer (currently Corteva) using the gene-editing tool CRISPR-Cas9, knocked out the endogenous maize waxy gene Wx1, which encodes the endosperm's granule-bound starch synthase responsible for producing amylose. Engineered maize contains starch composed exclusively of the branched polysaccharide amylopectin and not amylose. DuPont Pioneer expected the CRISPR-edited variety to have improved yields than conventional waxy maize and to commercialize the improved variety within five years [73]. Due to the high costs associated with the deregulation of genetically modified commercial maize hybrids only the largest agricultural biotechnology companies can afford these costs, with a consequent increasing concentration of maize seed providers [35]. It has been suggested that this scenario could be modified by both the benefits of a wider application of gene-editing technologies and reduced regulatory oversight of CRISPR-derived varieties in comparison to transgenic GM breeding technologies [74].

Using maize as a model species and CRISPR/Cas9 technology, a very recent interesting European initiative has developed a pipeline called BREEDIT to generate a collection of multiplex gene-edited plants [75]. BREEDIT combines multiplex genome editing of whole gene families with crossing schemes to improve maize quantitative traits. The researchers were able to knock out 48 growth-related maize genes and produced a collection of over 1000 gene-edited plants which displayed 5–10% increases in leaf length and up to 20% increases in leaf width compared with the controls. BREEDIT has the potential to generate diverse collections of mutants for the identification of allelic variants for use in breeding programs.

The tremendous advances in both basic plant research and crop breeding brought about by CRISPR/Cas systems from first applications to nowadays has made genome editing a powerful tool for precise maize improvement through multiple approaches, comprising point mutation, gene knock-out -in, allele replacement, fine-tuning of gene expression, and other modifications at any potential genome locus. It is expected that in the next years novel strategies will be designed to improve the specificity of Cas9-linked base editors, such as extending sgRNA guide sequences, and delivering base editors via RNP in many crops and in maize as well [58, 69].

Although many CRISPR-Cas-edited genes have been documented to improve maize traits of agronomic interest, only a few lines have been tested in field trials, with only CRISPR-waxy maize hybrids having had a limited diffusion in United States [76]. Thus, much additional work for determining potential breeding values of edited maize lines must be done in terms of field tests. For an efficient transfer of technologies from the bench to the field there is still the need to elucidate the genetic and regulatory architecture of important traits as well as to increase the efficiency of all steps of gene targeting and subsequent plant regeneration procedures. Finally, the integration of CRISPR-Cas technology in the breeding of new maize varieties also depends on existing and future regulatory policies that will be adopted worldwide.

References

1. Matsuoka, Y., Vigouroux, Y., Goodman, M.M., Sanchez, G.J., Buckler, E., Doebley, J.: A single domestication for maize shown by multilocus microsatellite genotyping. Proc. Natl. Acad. Sci. U.S.A. **99**(9), 6080–6084 (2002). https://doi.org/10.1073/pnas.052125199
2. Piperno, D.R., Ranere, A.J., Holst, I., Iriarte, J., Dickau, R.: Starch grain and phytolith evidence for early ninth millennium B.P. maize from the Central Balsas River Valley, Mexico. Proc. Natl. Acad. Sci. U.S.A. **106**(13), 5019–5024 (2009). https://doi.org/10.1073/pnas.0812525106
3. Stitzer, M.C., Ross-Ibarra, J.: Maize domestication and gene interaction. New Phytol. **220**(2), 395–408 (2018). https://doi.org/10.1111/nph.15350
4. Schnable, P.S., Ware, D., Fulton, R.S., et al.: The B73 maize genome: complexity, diversity, and dynamics. Science. **326**, 1112–1115 (2009)
5. Chia, J.M., Song, C., Bradbury, P.J., Costich, D., et al.: Maize HapMap2 identifies extant variation from a genome in flux. Nat. Genet. **3;44**(7), 803–807 (2012). https://doi.org/10.1038/ng.2313
6. Jiao, Y., Peluso, P., Shi, J., et al.: Improved maize reference genome with single-molecule technologies. Nature. **546**, 524–527 (2017)
7. Andorf, C., Beavis, W.D., Hufford, M., et al.: Technological advances in maize breeding: past, present and future. Theor. Appl. Genet. **132**, 817–849 (2019). https://doi.org/10.1007/s00122-019-03306-3
8. Portwood, J.L., Woodhouse, M.R., Cannon, E.K., et al.: MaizeGDB 2018: the maize multi-genome genetics and genomics database. Nucl. Ac. Res. **47**, D1146–D1154 (2019)
9. Gui, S., Yang, L., Li, J., et al.: ZEAMAP, a comprehensive database adapted to the maize multi-omics era. iScience. **23**, 101241 (2020)

10. Kadam, D.C., Lorenz, A.J.: Toward redesigning hybrid maize breeding through genomics-assisted breeding. In: Bennetzen, J., Flint-Garcia, S., Hirsch, C., Tuberosa, R. (eds.) The Maize Genome. Compendium of Plant Genomes. Springer, Cham (2018). https://doi.org/10.1007/978-3-319-97427-9_21
11. Shull, G.H.: The composition of a field of maize. Rep. Am. Breed. Ass. **4**, 296–301 (1908)
12. Duvick, D.N.: The contribution of breeding to yield advances in maize (Zea mays L.). Adv. Agron. **86**, 83–145 (2005)
13. Ren, J., et al.: Novel technologies in doubled haploid line development. Plant Biotechnol. J. **15**, 1361–1370 (2017)
14. Prigge, V., Xiaowei, X., Liang, L., et al.: New insights into the genetics of in vivo induction of maternal haploids, the backbone of doubled haploid technology in maize. Genetics. **190**(2), 781–793 (2012). https://doi.org/10.1534/genetics.111.133066
15. Smith, J.S., Gardner, C.A., Costich, D.E.: Ensuring the genetic diversity of maize and its wild relatives. In: Watson, D. (ed.) Achieving Sustainable Cultivation of Maize. Burleigh Dodds, Cambridge (2017)
16. Lu, X., Liu, J., Ren, W., et al.: Gene-indexed mutations in maize. Mol. Plant. **5;11**(3), 496–504 (2018). https://doi.org/10.1016/j.molp.2017.11.013
17. Shi, G., Chavas, J.P., Lauer, J.: Commercialized transgenic traits, maize productivity and yield risk. Nat. Biotechnol. **31**(2), 111–114 (2013). https://doi.org/10.1038/nbt.2496
18. Ishida, Y., Hiei, Y., Komari, T.: Agrobacterium-mediated transformation of maize. Nat. Protoc. **2**(7), 1614–1621 (2007). https://doi.org/10.1038/nprot.2007.241
19. Fromm, M.E., Taylor, L.P., Walbot, V.: Stable transformation of maize after gene transfer by electroporation. Nature. **319**, 791–793 (1986)
20. Grimsley, N., Hohn, T., Davies, J.W., Hohn, B.: Agrobacterium mediated delivery of infectious maize streak virus into maize plants. Nature. **325**, 177–179 (1987)
21. Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D., Detmer, J.J.: Genetically transformed maize plants from protoplasts. Science. **240**, 204–207 (1988)
22. Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., et al.: Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell. **2**, 603–618 (1990)
23. Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., Kumashiro, T.: High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. Nat. Biotechnol. **14**, 745–750 (1996). https://doi.org/10.1038/nbt0696-745
24. Wang, A.S., Evans, R.A., Altendorf, P.R., et al.: A mannose selection system for production of fertile transgenic maize plants from protoplasts. Plant Cell Rep. **19**, 654–660 (2000)
25. Zhao, Z.Y., Gu, W., Cai, T., Tagliani, L., et al.: High throughput genetic transformation mediated by Agrobacterium tumefaciens in maize. Mol. Breed. **8**, 323–333 (2001)
26. Frame, B.R., Shou, H., Chikwamba, R.K., Zhang, Z., et al.: Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. **129**, 13–22 (2002)
27. Armstrong, C.L.: The first decade of maize transformation: a review and future perspective. Maydica. **44**, 101–109 (1999)
28. Hansen, G., Wright, M.S.: Recent advances in transformation of plants. Trends Plant Sci. **4**, 226–231 (1999)
29. Torney, F., Moeller, L., Scarpa, A., Wang, K.: Genetic engineering approaches to improve bioethanol production from maize. Curr. Opin. Biotechnol. **18**, 193–199 (2007)
30. Lowe, K., Wu, E., Wang, N., et al.: Morphogenic regulators baby boom and Wuschel improve monocot transformation. Plant Cell. **28**, 1, 98-2015 (2016). https://doi.org/10.1105/tpc.16.00124
31. Yassitepe, J.E.C.T., da Silva, V.C.H., Hernandes-Lopes, J., et al.: From plant material to the release of genetically modified and edited varieties. Front. Plant Sci. **14;12**, 766702 (2021). https://doi.org/10.3389/fpls.2021.766702
32. ISAAA Database 2023 https://www.isaaa.org

33. Simmons, C.R., Lafitte, H.R., Reimann, K.S., et al.: Successes and insights of an industry biotech program to enhance maize agronomic traits. Plant Sci. **307**, 110899 (2021). https://doi.org/10.1016/j.plantsci.2021.110899
34. Betts, S., Basu, S., Bolar, J., Booth, R., Chang, S., Cigan, A.M., et al.: Uniform expression and relatively small position effects characterize sister transformants in maize and soybean. Front. Plant Sci. **10**, 1209 (2019). https://doi.org/10.3389/fpls.2019.01209
35. Deconinck, K.: New evidence on concentration in seed markets. Glob. Food Sec. **23**, 135–138 (2019). https://doi.org/10.1016/j.gfs.2019.05.001
36. Dollinger, E.J.: Studies on induced mutation in maize. Genetics. **39**, 750–766 (1954)
37. Stadler, L.J., Sprague, G.F.: Genetic effects of ultra-violet radiation in maize. II. Filtered radiations. Proc. Natl. Acad. Sci. U.S.A. **22**, 579–583 (1936)
38. Till, B.J., Reynolds, S.H., Weil, C., et al.: Discovery of induced point mutations in maize genes by TILLING. BMC Plant Biol. **4**, 12 (2004)
39. McGinnis, K., Murphy, N., Carlson, A.R., et al.: Assessing the efficiency of RNA interference for maize functional genomics. Plant Physiol. **143**(4), 1441–1451 (2007). https://doi.org/10.1104/pp.106.094334
40. McCarty, D.R., Meeley, R.B.: Transposon resources for forward and reverse genetics in maize. In: Bennetzen, J.L., Hake, S. (eds.) Handbook of Maize, pp. 561–584. Springer, New York (2009)
41. Cotsaftis O. and Guiderdoni E. Transgenic Research **14** (1), 1–14 (2005)
42. Puchta, H., Fauser, F.: Gene targeting in plants: 25 years later. Int. J. Dev. Biol. **57**, 629–637 (2013). https://doi.org/10.1387/ijdb.130194hp
43. Kim, H., Kim, J.: A guide to genome engineering with programmable nucleases. Nat. Rev. Genet. **15**, 321–334 (2014). https://doi.org/10.1038/nrg3686
44. Shukla, V.K., Doyon, Y., Miller, J.C., et al.: Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature. **459**, 437–441 (2009)
45. Char, S.N., Unger-Wallace, E., Frame, B., et al.: Heritable site-specific mutagenesis using TALENs in maize. Plant Biotechnol. J. **13**(7), 1002–1010 (2015). https://doi.org/10.1111/pbi.12344
46. Boch, J., Scholze, H., Schornack, S., et al.: Bonas breaking the code of DNA binding specificity of TAL-type III effectors. Science. **326**, 1509–1512 (2009)
47. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E.: A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. **337**, 816–821 (2012)
48. Gasiunas, G., Barrangou, R., Horvath, P., Siksnys, V.: Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc. Nat. Acad. Sci. U.S.A. **25;109**(39), E2579–E2586 (2012). https://doi.org/10.1073/pnas.1208507109
49. Bortesi, L., Fischer, R.: The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. **33**, 41–52 (2015)
50. Xing, H.L., Dong, L., Wang, Z.P., et al.: A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol. **14**, 327 (2014)
51. Liang, Z., Zhang, K., Chen, K., Gao, C.: Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. J. Genet. Gen. **20;41**(2), 63–68 (2014). https://doi.org/10.1016/j.jgg.2013.12.001
52. Svitashev, S., Young, J.K., Schwartz, C., et al.: Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant Physiol. **169**, 931–945 (2015)
53. Zhu, J., Song, N., Sun, S., et al.: Efficiency and inheritance of targeted mutagenesis in maize using CRISPR-Cas9. J. Genet. Genom. **43**, 5–36 (2016)
54. Qi, W., Zhu, T., Tian, Z., et al.: High-efficiency CRISPR/Cas9 multiplex gene editing using the glycine tRNA-processing system-based strategy in maize. BMC Biotechnol. **16**, 58 (2016). https://doi.org/10.1186/s12896-016-0289-2

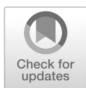
55. Char, S.N., Neelakandan, A.K., Nahampun, H., et al.: An agrobacterium-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. Plant Biotechnol. J. **15**(2), 257–268 (2017). https://doi.org/10.1111/pbi.12611
56. Svitashev, S., Schwartz, C., Lenderts, B., et al.: Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat. Commun. **7**, 13274 (2016). https://doi.org/10.1038/ncomms13274
57. Luo, M., Gilbert, B., Ayliffe, M.: Applications of CRISPR/Cas9 technology for targeted mutagenesis, gene replacement and stacking of genes in higher plants. Plant Cell Rep. **35**, 1439–1450 (2016)
58. Chen, K., Wang, Y., Zhang, R., Zhang, H., Gao, C.: CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu. Rev. Plant Biol. **70**, 667–697 (2019)
59. Feng, C., Yuan, J., Wang, R., et al.: Efficient targeted genome modification in maize using CRISPR/Cas9 system. J. Genet. Genom. **43**, 37–43 (2016)
60. Feng, C., et al.: High-efficiency genome editing using admc1 promoter-controlled CRISPR/Cas9 system in maize. Plant Biotechnol. J. **16**, 1848–1857. 6181213 (2018). https://doi.org/10.1111/pbi.12920
61. Zhou, H., He, M., Li, J., Chen, L., Huang, Z., et al.: Development of commercial thermo-sensitive genic male sterile rice accelerates hybrid rice breeding using the CRISPR/Cas9-mediated TMS5editing system. Sci. Rep. **6**, 37395 (2016)
62. Li, J., Zhang, H., Si, X., Tian, Y., Chen, K., et al.: Generation of thermosensitive male-sterile maize by targeted knockout of the ZmTMS5 gene. J. Genet. Genom. **44**, 465–468 (2017)
63. Li, C., Liu, C., Qi, X., Wu, Y., Fei, X., et al.: RNA-guided Cas9 as an in vivo desired-target mutator in maize. Plant Biotechnol. J. **15**(12), 1566–1576 (2017). https://doi.org/10.1111/pbi.12739
64. Liu, C.X., Li, X., Meng, D.X., et al.: A 4-bp insertion at ZmPLA1 encoding a putative phospholipase a generates haploid induction in maize. Mol. Plant. **10**(3), 520–522 (2017). https://doi.org/10.1016/j.molp.2017.01.011
65. Zhong, Y., Liu, C., Qi, X., et al.: Mutation of ZmDMP enhances haploid induction in maize. Nat. Plant. **5**, 575–580 (2019). https://doi.org/10.1038/s41477-019-0443-7
66. Li, Y., Lin, Z., Yue, Y., et al.: Loss-of-function alleles of ZmPLD3 cause haploid induction in maize. Nat. Plant. **7**, 1579–1588 (2021). https://doi.org/10.1038/s41477-021-01037-2
67. Wang, B., Regulski, M., Tseng, E., et al.: A comparative transcriptional landscape of maize and sorghum obtained by single-molecule sequencing. Genome Res. **28**(6), 921–932 (2018). https://doi.org/10.1101/gr.227462.117
68. Kelliher, T., Starr, D., Su, X., Tang, G., et al.: One-step genome editing of elite crop germplasm during haploid induction. Nat. Biotechnol. **37**(3), 287–292 (2019). https://doi.org/10.1038/s41587-019-0038-x
69. Wang, Y., Tang, Q., Pu, L., Zhang, H., Li, X.: CRISPR-Cas technology opens a new era for the creation of novel maize germplasms. Front. Plant Sci. **16;13**, 1049803 (2022). https://doi.org/10.3389/fpls.2022.1049803
70. Shi, J., Gao, H., Wang, H., Lafitte, H., et al.: ARGOS 8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol. J. **15**, 207–216 (2017)
71. Shi, J., Habben, J.E., Archibald, R.L., et al.: Overexpression of ARGOS genes modifies plant sensitivity to ethylene, leading to improved drought tolerance in both Arabidopsis and maize. Plant Physiol. **169**, 266–282 (2015)
72. Liu, H.J., Jian, L., Xu, J., et al.: High-throughput CRISPR/Cas9 mutagenesis streamlines trait gene identification in maize. Plant Cell. **32**(5), 1397–1413 (2020). https://doi.org/10.1105/tpc.19.00934
73. Waltz, E.: CRISPR-edited crops free to enter market, skip regulation. Nat. Biotechnol. **34**, 582 (2016). https://doi.org/10.1038/nbt0616-582

74. Jorasch, P.: Potential, challenges, and threats for the application of new breeding techniques by the private plant breeding sector in the EU. Front. Plant Sci. **25;11**, 582011 (2020). https://doi.org/10.3389/fpls.2020.582011
75. Lorenzo, C.D., Debray, K., Herwegh, D., et al.: BREEDIT: a multiplex genome editing strategy to improve complex quantitative traits in maize. Plant Cell. **2;35**(1), 218–238 (2023). https://doi.org/10.1093/plcell/koac243
76. Gao, H., Gadlage, M.J., Lafitte, H.R., et al.: Superior field performance of waxy corn engineered using CRISPR-Cas9. Nat. Biotechnol. **38**(5), 579–581 (2020). https://doi.org/10.1038/s41587-020-0444-0

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 12
Using Gene Editing Strategies for Wheat Improvement



Domenica Nigro, Mark A. Smedley, Francesco Camerlengo, and Sadiye Hayta

Abstract Despite wheat's global importance, it has trailed behind the other major cereals regarding genomic tools and resources as well as gene transformation. As each gene usually exists as two copies in the tetraploid durum wheat or as three in hexaploid bread wheat, it is very difficult assess gene function and improve important agronomic traits in polyploid wheat with traditional breeding methods. Recent advances allow researchers to use gene editing technologies in wheat which facilitates the opportunity to knockout or modify one, two or all three gene homoeologs simultaneously, which is important to clarify the function and contribution of gene copies in a specific phenotype or trait. CRISPR-Cas technology is now being used routinely for gene knockout. Technological advancement has been rapid within the field, and recently more advance and precise methods have been deployed such as cytidine base editing, adenosine base editing, and prime editing in wheat. Here we summarised gene editing strategies that are presently being applied for wheat improvement.

1 Introduction

Modern domesticated wheats are derivatives from ancient hybridisation events between ancestral progenitor species. The two most extensively cultivated wheats are the tetraploid durum or pasta wheat (*Triticum turgidum* ssp. *durum* L.) and hexaploid bread wheat (*Triticum aestivum* L.) Matsuoka and Nasuda [1]. Bread wheat is

D. Nigro
Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, Bari, Italy

M. A. Smedley · S. Hayta (✉)
John Innes Centre, Department of Crop Genetics, Norwich Research Park, Norwich, Norfolk, UK
e-mail: sadiye.hayta@jic.ac.uk

F. Camerlengo
Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Bologna, Italy

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_12

the major cultivated wheat species, while durum wheat accounts for about 5% of the total wheat production [2]. Both species have large genomes, durum ~12 and hexaploidy ~16 Gbp consisting mostly of repetitive elements. Within these polyploid species each gene usually exists as two copies in the tetraploid durum wheat or as three in hexaploid bread wheat. Homoeologous gene copies are usually highly conserved in gene structure and sequence among the subgenomes >95% [3]. Due to the polyploid nature of wheat, functional redundancy between homoeologs often occurs [4]. The traits controlled by recessive genes are particularly difficult to observe due to their multiple homeologs. This means that it may be necessary to manipulate all homoeologs and paralogs simultaneously to measure a phenotypic effect and this is very difficult to do it with traditional breeding methods [3]. A very low probability exists of the simultaneous mutation of genes in the A, B, and D genomes by natural processes or induced mutagenesis. Gene editing approaches gives the opportunity to knockout one, two or all three of the homoeologs of a gene, which is important to clarify the function and contribution of each homoeolog to a specific phenotype or trait.

Unrivalled in its geographic range of cultivation, wheat accounts for ~20% of the calorific value and ~25% of the daily protein intake of the world's population [5, 6]. In the 2020/2021 cropping season, over 770 million tons of wheat grain was harvested from over 220 million ha of arable land [7]. One of the three major cereal crops, along with maize (*Zea mays*) and rice (*Oryza sativa*), wheat has more influence on global food security than any other crop [5, 8]. Notwithstanding wheat's global importance, however, it has, until recently, trailed behind the other major cereals in regard to the development of genomic tools and resources for its improvement [2, 3]. One such tool is wheat transformation, a prerequisite for many CRISPR-Cas gene editing applications, which until lately had been languishing with low transformation efficiency ~5% and suffered with genotype dependence. Developments in open access robust transformation protocols and the use of morphological genes to improve regeneration, transformation efficiency and reduce genotype dependence have recently made outstanding improvements [9–11].

High quality DNA reference sequences of target genes are required for CRISPR gene editing, recent advances in sequencing technologies and bioinformatic tools have expediated wheat gene editing studies. Researchers are able to target multiple homoeoalleles simultaneously by CRISPR-Cas which enables the production of targeted mutations in all copies of a gene; therefore, the system holds great promise in the characterisation of genes endowing important agronomic traits in polyploid wheat. Furthermore, gene editing has been used to modify multiple genes simultaneously controlling different agronomic traits [12].

In wheat, CRISPR-Cas is being used for yield enhancement, improvement of grain quality, biofortification, development of resistance against diseases, and tolerance against abiotic factors (Table 12.1). The promising outcomes of the CRISPR-based multiplexing approach circumvent the constraint of targeting merely one gene at a time. Moreover, deployment of CRISPR-Cas variant systems such as cytidine base editing, adenosine base editing, and prime editing in wheat has been used to induce precise point mutations. The combination of these novel technologies

Table 12.1 Some agronomic traits improved by *CRISPR*/*Cas* in wheat

Target gene	Trait/Gene function	Editing type	Transformation method	Variety	References
TaLOX2	Expressed during grain development	Knockout	Particle bombardment	Bobwhite Kenong 199	[13]
Alpha-gliadin gene	Gluten protein	Knockout	Particle bombardment	BW208 and THA53, Don Pedro	[14]
TaGW2	Negative regulators of grain size and thousand grain weight	Knockout	Particle bombardment	Bobwhite Kenong 199	[15, 16]
TaGW7	Affects grain shape and weight	Knockout	Particle bombardment	Bobwhite	[17]
TaSPL	Affects grain size and number	Knockout	Particle bombardment	Fielder	[18]
TaARE1	Defective in N assimilation	Knockout	Particle bombardment	Zhengmai 7698	[19]
TaALS	A key enzyme in the biosynthesis of branched-chain amino acids and is known as an ideal herbicide tolerance	Knockout	Particle bombardment	Kenong 199 Kenong 9204	[20]
TaZip4-B2	Supress the level of homoeologous crossing over	Knockout	*A. tumefaciens*	Fielder Kronos	[21, 22]
TaCENH3α	*Paternal haploid induction Zygotic centromere formation*	Knockout	Particle bombardment	Fielder	[23]
TaSBEIIa	Increased amylose, resistant starch	Knockout	Particle bombardment	Zhengmai 7698 (ZM) and a spring wheat cv Bobwhite	[24]
TaASN2	Asparagine synthase gene	Knockout	Particle bombardment	Cadenza	[25]
WTAI-CM3 and WTAI-CM16	Involved in the onset of wheat allergies (bakers' asthma) and probably Non-Coeliac Wheat Sensitivity (NCWS)	Multiple knockout	Particle bombardment	Svevo	[26]
TaXip	Controlling the protein fractions, grain protein content, starch synthase, grain hardness, etc	Knockout	*A. tumefaciens*	Fielder	[27]

(continued)

Table 12.1 (continued)

Target gene	Trait/Gene function	Editing type	Transformation method	Variety	References
TaMLO	Durable and broad-spectrum resistance to powdery mildew	Knockout (RNP) Knockout	Particle bombardment	Bobwhite Kenong 199	[28, 29]
TaEDR1	Negatively regulates powdery mildew resistance in wheat	Knockout	Particle bombardment	Kenong 199	[30]
TaNFXL1	Represses trichothecene-induced defence responses and bacterial resistance	Knockout	Particle bombardment?	Fielder	[31]
TaHRC	Encodes a nuclear protein conferring FHB susceptibility	Knockout	Particle bombardment	Bobwhite	[32]
TaNFXL1	Represses trichothecene-induced defence responses and bacterial resistance	Knockout	Particle bombardment	Fielder	[31]
TaDREB2, TaDREB2	Stress-responsive transcription factor genes	Knockout	Protoplast	Chinese spring	[33]

addresses some of the most important limiting factors for sustainable and climate-smart wheat that should lead to the second "Green Revolution" for global food security.

Here we summarised advanced gene-editing tools to facilitate sustainable wheat production and use of these tools for the improvement of genetic traits related to the agronomic performance.

2 CRISPR-Cas Gene Editing

During the last two decades, there has been rapid development of genome-editing strategies that make it possible to directly target regions of genes in a DNA sequence-specific manner. Site-directed nucleases (SDNs)-based gene editing technologies considerably enhance the precision of gene modification in plants [34]. This set of tools, comprising zinc finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeats associated protein (CRISPR-Cas) [35, 36], allow to repress or activate gene expression, modification of gene function, or create gene knockouts, mediating the targeted manipulation of DNA sequences [37]. Gene editing involves the introduction of targeted DNA double-strand breaks (DSBs) at the specific targeted site within a gene by using an engineered nuclease, which induces cellular DNA repair

mechanisms. Once a DSB is induced repair pathways, such as, the non-homologous end-joining (NHEJ) mechanism or homologous recombination (HR) pathway can repair the induced DSBs introducing simultaneously the desired modifications at the target locus [38]. Due to its easiness, accuracy and effectiveness, along with its ability to produce transgene-free, gene edited crops, CRISPR-Cas rapidly diffused as the most used site-directed nucleases (SDNs)-based gene editing technology. The CRISPR-Cas system is usually introduced into plants as transgenes, however, in the following generation the transgene can be segregated away, leaving a transgene-free plant containing the desired mutations. Zhang et al. [13] developed a highly efficient transient expression-based gene-editing system for producing transgene-free and homozygous wheat mutants in the T0 generation. Liang et al. [40] reported an efficient gene edited method to produce transgene-free plants by CRISPR/Cas9 ribonucleoproteins (RNPs) in wheat [30, 39, 40].

In nature, CRISPR-Cas exists as a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements such as bacterial phage. Biotechnologists have harnessed and modified this system to enable gene editing applications in multiple species, including plants. There are over 30 different CRISPR-Cas systems naturally occurring that have been reported [41], however, the majority of gene editing applications are performed by CRISPR-Cas9 based systems, followed closely by CRISPR-Cas12a, previously known as CpfI. The Cas9 nuclease was first thought to make a blunt DSB of DNA, three base pairs upstream of the PAM, however, evidence strongly suggests that Cas9 leaves a single nucleotide 5′ overhang [42]. Cas12a on the other hand leaves 5 bp staggered overhang at the opposite end of the PAM motif making it more favoured for applications such as gene targeting [43] or recently reported disruption of *cis*-regulatory elements within promoter regions [44].

Since the first report of CRISPR-Cas technology being used for gene editing, technological advancement has been rapid within the field. There are experimentally derived protocols for the selection of sgRNA targets, construct assembly, and screening analysis for genome editing in hexaploid wheat such as Smedley et al. [45]. Although, the majority of reports are for gene knockout, researchers are now able to perform targeted base changes through base editing, rewrite small length of DNA using prime editing, insertion of DNA via gene targeting, upregulation or suppression of gene expression as well as multi-gene knockouts.

3 Multiplex Gene Editing

CRISPR-Cas based gene editing technologies enable the easy modification of two or more specific DNA loci in a genome with high precision. These tools have greatly increased the feasibility of introducing desired changes in specific but different genes, resulting in the development of new plant genotypes carrying multiple mutations in a single generation. There are three main strategies to produce multiple single-guide RNAs (sgRNAs), the conventional multiplex system with separate U3

or U6 promoters driving individual sgRNAs, the tRNA-processing system [46] and the ribozyme-processing system [47]. The polycistronic tRNA-sgRNA system consists of the sgRNAs linked together by tRNA sequences, the guides are transcribed in a single transcript. The tRNA sequences are then recognized and processed by endogenous RNases that excise the individual sgRNAs from the transcript [46]. The ribozyme system consists of a single transcript of multiple sgRNA, where individual sgRNAs are flanked by self-cleaving ribozyme sequences such as the hammerhead ribozyme (HH) and the hepatitis delta virus (HDV) ribozyme, [47, 48]. Transcripts are cleaved by the cis-acting ribozymes post-transcriptionally. Wang et al. [49] deployed a multiplexing gene editing approach based on the tRNA system in wheat to target *TaGW2*, *TaLpx-1,* and *TaMLO* genes, simultaneously. A similar tRNA approach was used in durum wheat cultivar Svevo by Camerlengo et al. [26] to edit the α-amylase/trypsin inhibitor subunits WTAI-CM3 and WTAI-CM16 in the grain to reduce allergen proteins. Abdallah et al. [50] created *TaSal1* mutants using this multiplex system to address drought tolerance in wheat. The three main multiplex CRISPR-Cas systems for simultaneous gene editing at 8 target sites in bread wheat were tested by Li et al. [51]. The ribozyme and tRNA systems were found to be more effective at gene editing than the conventional multiplex system with individual promoter driving individual guides [51].

4 Base Editing

Base editing enables the generation of targeted point mutations without DSBs, DNA donor templates, or the reliance of the homologous repair (HR) pathway [52, 53]. Base editors consist of a DNA deaminase fused to a catalytically impaired Cas nuclease such as deactivated Cas9 (dCas9) or a Cas9 nickase (nCas9). The Cas-deaminase fusion is guided to the target site by the guide RNA, where a single stranded DNA R-loop is formed allowing access for the DNA deaminase [54]. Where the deaminase is fused to SpCas9 variants, the 'activity window' for base editing spans approximately protospacer positions 4–8 (position 1 being the first nucleotide of the protospacer, the PAM being at positions 21–23). There are two main classes of base editors which have been developed so far: cytosine base editors (CBEs), which catalyse the conversion of C/G base pairs to T/A base pairs; and adenine base editors (ABEs), which catalyse A/T-to-G/C conversions [52, 53]. Therefore, CBEs and ABEs can facilitate four possible transition mutations (C → T, A → G, T → C, G → A). Improvements in base editor efficiency such as the inclusion of uracil glycosylase inhibitor proteins (UGI) typically CBEs such as BE3 [53] and optimisation of linker sequences Komor et al. [55] have substantially increased base editing yield, extensively reviewed in Anzalone et al. [54]. Zong et al. [56] used a CBE in both protoplasts and regenerated rice, wheat, and maize plants at frequencies of up to 43.48 in rice%. Li et al. [57], from same research group, described optimisation of an ABE for application in plant systems, demonstrating its high

efficiency in creating targeted point mutations at multiple endogenous loci in rice and wheat. Han et al. [58] compared two ABEs, ABE7.1 and the new ABE8e containing a high-activity adenosine deaminase TadA8e, both were codon optimised for wheat. To aid nuclear localisation Bipartite-SV40-Nuclear-Localization-Signals (bpNLS) were added at the N-terminus of TadA* and also a bpNLS followed by a nucleoplasmin NLS (npNLS) at the C-terminus of SpCas9n. Calling them wheat high-efficiency ABEs (WhieABE7.1 and WhieABE8e), it was found in the study that WhieABE8e out performed WhieABE7.1 when targeting 5 wheat tubulin alleles.

5 Prime Editing

Prime-editing technology enables targeted insertions, deletions and all 12 types of point mutation without requiring double-strand breaks or donor DNA templates. It expands the scope and capabilities of directly targeting and modifying genomic sequences. Prime editing is achieved by the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) fused to the C-terminus of Cas9 H840A nickase (nCas9) together with engineered prime editing guide RNA (pegRNA). Primer Editors (PEs) directly copy the desired genetic information from the pegRNA into the target genomic locus [59]. The pegRNA contains two parts: a primer binding site (PBS) and an RT template (RTT) and guides the nickase to the target site by homology to a genomic DNA locus. nCas9 recognizes and nicks the nontarget DNA strand of the target site and releases a single-strand DNA, and the PBS then hybridises with the released DNA and serves as a primer for reverse transcription. The desired edits encoded by the RTT are then reverse transcribed and transferred to the nontarget DNA strand, generating a DNA flap that is subsequently incorporated into the target site by DNA repair [60].

Initially, PE1, PE2, PE3 and PE3b were characterized by Anzalone et al. [54] who developed the technology in mammalian cells. PE1 is a fusion of Cas9 nickase to the wild-type M-MLV RT. PE2 substitutes for the wild-type M-MLV RT an engineered pentamutant M-MLV RT. PE3 combines the PE2 fusion protein and pegRNA with an additional sgRNA that targets the non-edited strand for nicking. A variant of the PE3 system called PE3b uses a nicking sgRNA that targets only the edited sequence, resulting in decreased levels of indel products by preventing nicking of the non-edited DNA strand until the other strand has been converted to the edited sequence [54].

Lin et al. [61] compared PE2, PE3, and PE3b in wheat protoplasts and rice, they produced a wide variety of edits at genomic sites in rice and wheat. Prime-edited efficiencies up to 21.8% were reported for regenerated rice plants [61]. An N-terminal RT–Cas9 nickase fusion PE and multiple synonymous nucleotide substitutions introduced into the RT template increased the average efficiency of prime editing in rice to 24.3% Xu et al. [59]. An engineered plant prime editor (ePPE) was

developed by removing the ribonuclease H domain from M-MLV RT and incorporating a viral nucleocapsid protein with nucleic acid chaperone activity. This enhanced the frequency of various targeted modifications, including base substitutions, insertions (34 bp) and deletions (90 bp) by an average of 5.8-fold in rice and wheat protoplast compared to PE2 [62]. An online design tool (PlantPegDesigner) was developed for designing efficient strategies for PE in plants, including dual-pegRNA designs [60]. The pegRNA primer-binding site length, RT template length and nicking sgRNA position effects editing frequencies in plants [60, 61].

6 Other Strategies

Strategies have been developed to deliver CRISPR system components into plant germline or meristematic cells that achieves genotype-independent editing. Plant RNA virus-based vector systems can deliver gene editing reagents into plant leaves. Based on this approach an engineered *Barley stripe mosaic virus*–based sgRNA delivery vector (BSMV-sg) was used by Li et al. [63] to perform heritable gene editing in Cas9 expressing transgenic wheat plants. The progeny in the next generation had editing at frequencies ranging from 12.9% to 100% in three different wheat varieties, and 53.8–100% of edited plants were virus free. The group achieved multiplex editing in the progeny using a pool of BSMV-sg vectors harbouring different sgRNAs and were able to generate Cas9-free wheat mutants by crossing BSMV-infected Cas9-transgenic wheat pollen with wild-type wheat.

Other delivery methods were developed in wheat. Nanomaterials have emerged as a promising candidate for delivery of genetic cargoes to intact plant cells. Functionalised high-aspect-ratio carbon nanotube (CNT) nanoparticles (NPs) have been successfully used in wheat leaves for efficient DNA delivery [64]. The use of nanomaterials for GE studies has yet to be reported.

Liu et al. [65] developed an *in planta* particle bombardment (iPB) method which has increased process efficiency since no culture steps are required to create stably genome-edited wheat plants. The biolistic delivery of gold particles coated with plasmids expressing CRISPR-Cas9 components designed to target *TaQsd1* were bombarded into the embryos of imbibed seeds with their shoot apical meristem (SAM) exposed. A total of 2.51% of the bombarded plants (cv. “Haruyokoi,” spring type) carried mutant alleles in the tissue. The method utilised transient expression of CRISPR-Cas and no detectable transgene integration was identified. Kumagai et al. [66] reported an iPB-ribonucleoprotein (RNP) method which represents an alternative approach for creating genome-edited wheat varieties with an editing efficiency comparable to the iPB-DNA method. Since no DNA is used, and therefore no transgene integration occurs, the iPB-RNP method has the potential for use in modern agricultural applications and commercialisation.

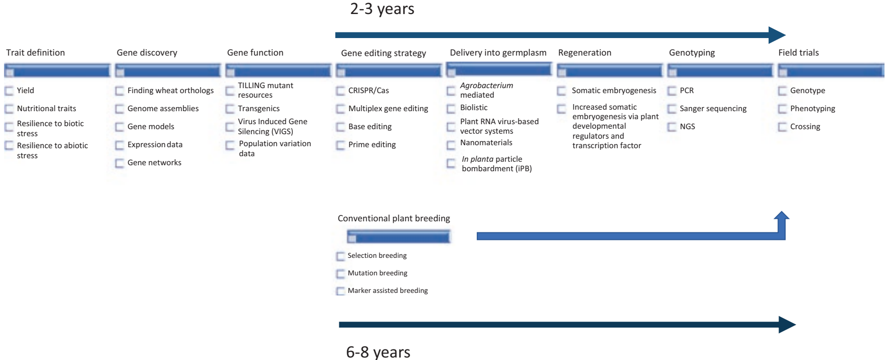


Fig. 12.1 Pathway for the improvement of genetic traits in wheat by gene-editing and conventional plant breeding

7 Recent Applications of Gene Editing in Wheat

Since the domestication of cereal crops, farmers selected plants with favorable agronomic traits that led to increased crop yield and performance. The introduction of semi-dwarf varieties and agrochemicals, starting from the second half of the last century, led to a leap forward in determining substantial increases in grain yield. In the modern agrifood context, crop yield remains one of the major traits to be improved to meet growing demands for food production and climate change effects. More recently, breeders and scientists also focused on the quality values of wheat grains to improve technological end-use quality of wheat flour and the nutritional value of derived foods in addition to the aim to produce safer genotypes with reduced toxic, immunogenic and antinutritional compounds.

To date, gene-editing tools have been demonstrated to extensively contribute to the study and improvement of genetic traits related to the agronomic performance and end-use quality of wheat, decreasing the time for the generation of new genotypes harboring precise mutations that can enhance grain yield and quality (Fig. 12.1).

8 Gene Editing for Grain Quality Improvement

Grain quality refers to the technological behavior of flours and doughs obtained from wheat as well as to internal quality factors such as nutrients and bioactive compounds. In recent years aspects related to nutritional and healthy values have gained more prominence since several disorders have been associated with wheat derived food consumption. In this respect, gene editing tools provide the ability to fine regulate the chemical composition of wheat kernels modulating biosynthetic pathways of major components such as protein, starch and lipids as well as offer the

opportunity to produce wheat with improved nutritional value, in which minor components play a predominant role.

Starch modifications in wheat were widely explored in the last two decades leading to wheat genotypes with diverse amylose/amylopectin ratio that influence not only the rheological behavior of doughs but also the nutritional value of derived foods. In general, starch contains two major glucose polymers, amylose and amylopectin, which differ in the degree of polymerization (DP) of glucan chains and in the frequency of branches. In wheat endosperm, starch consists of approximately 70–80% amylopectin and 20–30% amylose. Increasing the amount of amylose at the expense of amylopectin, the fraction of starch not digested and absorbed in the gastrointestinal trait (referred to as resistant starch) can be enhanced with a consequent beneficial effect on human health, correlated to blood glycemic index after wheat derived food consumption. Li et al. [24], using CRISPR-Cas9 in bread wheat, generated a series of transgene-free mutant lines with partial or triple-null *TaSBEIIa* alleles, an isoform of starch-branching enzymes. The triple-null lines (aabbdd) showed significantly increased amylose content, resulting in higher content of resistant starch, protein and soluble pentosan whereas a slight decrease of total starch was observed. On the other hand, they observed a series of pleiotropic effects related to plant growth and grain morphology traits besides negative effects on baking qualities of derived flours. Decreased plant height and tiller number, lower grain length, width and lower grain number associated with reduced thousand grain weight were observed in mutant lines compared to the control. In addition, the decrease in amylopectin content, generally recognized to be related to good end-use quality, strongly influenced the viscosity parameters and negatively affected the rheological proprieties of doughs.

Dough stability time (ST) and SDS-sedimentation values (SV) are two of the major quality parameters used to determines the final quality of bread wheat flours. These two parameters are mostly influenced by protein content and composition and are positively correlated to dough rheological properties. Sun et al. [27] reported for the first time the effect of proteinaceous inhibitors of endo-xylanases. In particular, they found a QTL for dough stability time and SDS-sedimentation value on chromosome 6A (*QSt/Sv-6A-2851*) and identified the *xylanase inhibitory protein* (*TaXip*) gene as the principal genetic component of the QTL producing variation in the above-mentioned parameters. They validated all the three homeoalleles (*TaXip-6A*, *TaXip-6B*, and *TaXip-6D*) producing CRISPR-Cas9 knock-outs, albeit they produced only two mutant genotypes (aaBBDD and AAbbdd). The SDS-sedimentation value was significantly higher in both mutant lines compared to the control whereas the stability time value was significantly higher only for aaBBDD genotype compared to the control. Based on these results and on the observation of homoeologous gene expression in the grain at the later stage of grain development, the authors claim that *TaXip-6A* has a greater effect on quality parameters compared to *TaXip-6B* and *TaXip-6D.*

Zhang et al. [15] observed an increase in the SDS sedimentation volume of bread wheat lines in which they edited *TaGW2* homoeologous genes. Grain protein

content and flour protein content resulted considerably elevated in all the mutant lines, particularly in AAbbDD and AAbbdd genotypes. Glutenins and gliadins were also increased. Otherwise, they observed that the grains of double and triple mutants were morphologically wrinkled compared to the control plants and single mutants.

Grain quality defects such as late maturity amylase (LMA) and pre-harvest sprouting (PHS) are correlated with low Hagberg falling number (FN), which is considered an indicator of the amount of sprout damage caused by enzyme activity (α-amylase) producing the decrease of dough viscosity. PHS determines the germination of grains while they are still on the spike causing significant decrease of grain quality. Abe et al. [67] produced loss-of function mutations of *TaQsd1* gene that controls seed dormancy in wheat and barley resulting in longer seed dormancy. They generated all triple homozygous transgene-free genotypes (AABBDD, aaBBDD, AAbbDD, AABBdd, aabbDD, aaBBdd, AAbbdd, and aabbdd) but only the triple mutant (aabbdd) showed significantly different germination rates and reduced PHS.

The consumption of wheat derived foods is associated to the increasing incidence of wheat related pathologies such as Coeliac Disease (CD), allergies and Non-Coeliac Wheat Sensitivity (NCWS) but also food processing of wheat flour can lead to the formation of antinutritional and toxic compounds.

Free asparagine is converted to acrylamide, a carcinogenic contaminant, during high-temperature processing of food made from wheat flour. Raffan et al. [25] knocked out the asparagine synthase gene, *TaASN2,* in bread wheat genotypes to reduce the concentration of free asparagine in the grain. They observed an almost total reduction of free asparagine concentrations in the grain of triple-null mutants genotypes (aabbdd). In contrast, an increase in free glutamine, glutamate and aspartate was found in all the edited lines. Raffan et al. [68] used the low asparagine edited lines for the first field trial of genome-edited wheat lines in Europe.

The main triggering factors of CD are prolamins, glutenins and gliadins that are proteins contained in wheat grain endosperm and responsible for the gluten matrix formation. Sánchez-León et al. [14] used the CRISPR/Cas9 technology to reduce the content of α-gliadins in wheat kernels. Since α-gliadins represent a large protein family with high sequence homology among members, they produced multiplex editing of gliadins targeting conserved regions among the gene family members. Although pleiotropic effects on the other gliadins classes (ω- and γ-) were observed, the mutant lines could be used to produce low-gluten wheat derived foods.

Other endosperm protein families such as a-amylase/trypsin inhibitors (ATI), which are structural and metabolic proteins involved in plant defense mechanisms, can trigger the onset of wheat allergies and NCWS. Camerlengo et al. [26] used a multiplexing strategy to edit the ATI subunits WTAI-CM3 and WTAI-CM16 in durum wheat producing transgene-free wheat lines with a reduced amount of ATI. The mutant lines completely lacked target ATI subunits resulting in a decrease of their allergenic potential.

9 Gene Editing for Grain Yield

One of the major goals of wheat breeding remains the improvement of yield. It is related to both the agronomic performance of crops as well as to environmental conditions. The constitution of semi-dwarf wheat varieties along with the introduction of chemicals and modern agronomic practice lead to a substantial increase of grain yield starting from the second half of last century.

Nowadays, a second "Green-Revolution" is required to face the global food demand due to the rapid increase of world population and to the worsening of climate change.

Grain morphology traits such as grain weight (GW), grain width (GWH) and grain length (GL) constitute a breeding target to enhance grain productivity. The knock-out of *TaGW2* homoeologous genes in two bread wheat cultivars had higher GWH and GL values and resulted in increased thousand grain weight (TGW) values of mutant lines compared to the control [15, 16]. Mutations in the homoeologous *TaGW2* genes had dosage-dependent effects on phenotypes in both bread wheat cultivar; although each homoeologous gene had different effect in the two genotypes, significant changes of TGW were associated with changes in gene dosage rather than with specific combination of mutated alleles.

TaGW7 encodes a TONNEAU1-recruiting motif (TRM) protein that affects grain morphology and weight in wheat and other cereal species. Wang et al. [17] demonstrated, by editing *TaGW7* in bread wheat, that mutations in the homoeologous genes of the B and D genomes increased the GWH and TGW but reduced the GL. They produced single (AABBdd) and double mutants (AAbbdd) with wider and shorter grains compared to the control, and *TaGW7-D1* seems to contribute at a greater extent to the phenotypic effects affecting grain size. Similar to *TaGW2*, *TaGW7* had dosage-dependent effects on phenotypes.

Another important target for improving grain yield is the *SQUAMOSA promoter-binding protein-like* (SPL) genes encoding for transcription factors that regulates a plethora of plant developmental and yield-related traits. The *SPL* family members are often negatively regulated by micro-RNA 156/157 (miR156). Gupta et al. [18] identified the microRNA 156 recognition elements (MRE) in the 3′-untraslated region of the *TaSPL13* gene and, using CRISPR-Cas9, they generated mutations in the three homoeologous genes in bread wheat. Mutations in MRE led to a higher expression of *TaSPL13* which produced a decrease in flowering time, tiller number and plant height but increased grain size and number.

To improve grain yield farmers are used to applying nitrogen fertilizers, but this type of agriculture practice leads to aggravating environmental pollution and ecological deterioration. Furthermore, most of the modern wheat varieties show low Nitrogen Use Efficiency (NUE) and absorb less than 40% of the supplied nitrogen. Zhang et al. [19] isolated and characterized the *abnormal cytokinin response1 repressor1* (*TaARE1*) gene in a Chinese winter wheat cultivar and then used CRISPR-Cas9 to generate a series of transgene-free mutant lines either with partial or triple-null *TaARE1* alleles. Loss of function mutations in this gene result in

delayed senescence, enhanced NUE and increased grain yield under normal field conditions. All the edited lines showed enhanced tolerance to N starvation with the AABBdd and aabbDD genotypes exhibiting significantly improved NUE without growth penalties compared to the control that results in increased TWG.

W eed competition is related to yield loss in wheat cultivation. Zhang et al. [20] generated transgene-free wheat germplasm by base editing the *acetolactate synthase* (*ALS*) and *acetylcoenzyme A carboxylase* genes. TaALS is a key enzyme in the biosynthesis of branched-chain amino acids and is known as an ideal herbicide tolerance target in wheat. Edited wheat lines, harboring different point mutations, were evaluated for the tolerance to multiple herbicides; homozygous mutants with four or six edited alleles showed enhanced tolerance to herbicides and grew normally whereas control plants died in few weeks after herbicide application. This strategy could be directly applied to produce wheat varieties tolerant to herbicides but also can be exploited as selection marker in wheat transformation and in vitro regeneration.

A number of important agronomic traits have been targeted by gene editing in wheat, although obtaining new high-yielding cultivars also resistant to biotic and abiotic stresses remains the main objective of most plant breeding programs. In the next sections, gene editing system successfully used for gene editing in wheat are reported.

10 Gene Editing for Biotic Stress Resistance

Pest and disease, among biotic constraints, are estimated to determine 21.5% of current yield losses [69]. Modern agriculture relies on chemical compounds to avoid and prevent yield losses due to fungal diseases, but the extensive application of such chemicals severely affects both human health and the environment. So far, the development of fungus-resistant wheat cultivars has been a noteworthy goal in wheat breeding programs. Gene editing in wheat has targeted several genes for improvement of resistance against diseases caused by fungi, specifically *Blumeria graminis* and *Fusarium graminearum*.

The first successful experiment using the CRISPR-Cas system in wheat was reported by Shan et al. [70], who edited the *MLO* gene, which encodes for the MILDEW-RESISTANCE LOCUS (MLO) protein. The authors reported a mutation frequency of *TaMLO* in protoplasts of 28.5%. MLO has a negative resistance function, thereby causing susceptibility to powdery mildew in plants expressing this gene [71]. Powdery mildew diseases are caused by *Blumeria graminis* f. sp. *tritici* and result in significant wheat yield losses. As knockout of the *TaMLO* leads to disease resistance, this gene was an ideal target for RNA-guided Cas9 knockouts to improve powdery mildew tolerance. The approach was successfully demonstrated by Wang et al. [28], who simultaneously targeted the three homoeoalleles of *TaMLO* in hexaploid bread wheat with both CRISPR-Cas9 and TALEN technologies using particle bombardment, which resulted in powdery mildew resistant plants. The

mutation frequency of regenerated *TaMLO*-edited wheat (5.6%) was similar for both editing methods.

More recently, Zhang et al. [30] demonstrated an improved powdery mildew resistance in wheat by simultaneously modifying the three homoeologous of *TaEDR1* by CRISPR-Cas9 gene editing. The *enhanced disease resistance1* (*EDR1*) gene is a negative regulator of resistance to powdery mildew in Arabidopsis Frye et al. [72]. The authors targeted a highly conserved region within the coding sequence of *TaEDR1*, obtaining homoeologous stable *Taedr1* mutations inherited in the T1 generation with a transmission rate of 97–100%. *Taedr1* plants were resistant to powdery mildew and did not show mildew-induced cell death.

Several studies have also been focused on *Fusarium gramineaurm* resistance, a fungus causing one of the most detrimental diseases in wheat. The lipoxygenase genes, *TaLpx* and *TaLox*, have been found to be good targets for gene editing resistance to Fusarium. These enzymes play a key role in the jasmonic acid-mediated defense responses in plants by catalyzing the hydrolysis of polyunsaturated fatty acids and then activating oxylipins biosynthesis. Nalam et al. [73] found that *TaLpx-1* gene silencing resulted in resistance to *Fusarium graminearum* in wheat. Shan et al. [16, 70] respectively edited *TaLpx* and *TaLox* genes in protoplasts, detecting a mutation frequency of 9% and 45%. Further studies by Zhang et al. [13] allowed to obtain wheat plants with mutated *TaLOX* with a frequency of 9.5%, of which homozygous mutants accounted for 44.7%.

Fusarium head blight (FHB) resistance was also investigated by targeting three wheat genes including an ABC transporter (*TaABCC6*), the nuclear transcription factor X box-binding like1 (*TaNFXL1*) both associated with FHB susceptibility, and a gene encoding a nonspecific lipid transfer protein (nsLTP), *TansLTP9.4*, which correlates with FHB resistance. PCR amplicons from protoplasts transformed with editing constructs were sequenced, showing that the three genes had been successfully edited with efficiencies of up to 42.2% [74]. Another target for Fusarium resistance was reported by Su et al. [32], who identified HISTIDINE RICH CALCIUM-BINDING PROTEIN (*TaHRC*) as a quantitative trait locus (QTL) responsible for resistance to Fusarium head blight. By cloning and sequencing a candidate gene they found a single deletion on the B genome homeolog, which was sufficient to determine resistance to Fusarium head blight. This result was confirmed by CRISPR-Cas9 approach targeting the *TaHRC* homeolog from the B subgenome, which caused frameshift mutations resulting in more than 40% reduction in fusarium head blight disease in wheat. This study showed that it is not always necessarily to knocked out all three homeologs in wheat, and in some cases the inactivation of one single homeolog is enough to successfully induce disease resistance.

More recently, Brauer et al. [31] followed a different approach to generate mutants resistant to Fusarium head blight. They focused on the NUCLEAR TRANSCRIPTION FACTOR, X-BOX BINDING 1-LIKE (NFXL1), a transcription factor used by Fusarium to repress defense responses upon infection, which is present as two copies on each of the wheat subgenomes. The authors showed that NFXL1 downregulation via RNAi in barley confers partial resistance upon

infection. Wheat NFXL1 knockout mutants with edits in all six homoeologous in the T1 generation were then obtained by designing sgRNA pair targeting all six loci simultaneously (one homeolog remained in heterozygous state), thus showing an increased Fusarium head blight resistance, similarly to that obtained through RNAi.

11 Gene Editing for Abiotic Stress Resistance

Using CRISPR-Cas to design resilience to abiotic stress in an era of global warming and extreme weather events is a justifiable aim. Abiotic stresses such as drought, extreme temperatures and salinity affect plant growth, survival, reproducibility and production, furthermore, it was shown that abiotic stresses modulate epigenetic changes in plants [75].

Genome engineering represent an effective approach to increase plant growth in response to abiotic stress in wheat, especially considering that abiotic stress-associated genes involved in cellular and molecular responses can easily be targeted with the available gene editing tools. So far, only few studies have shown the application of gene editing to improve abiotic stress response. CRISPR-Cas9 gene editing was successfully used in transient transformation in wheat protoplast. Two important abiotic stress-responsive transcription factor genes were targeted: *TaDREB2*, (wheat dehydration responsive element binding protein 2) and *TaERF3*, a wheat ethylene responsive factor 3 [33]. The results suggested that CRISPR-Cas gene editing has huge potential for manipulation of wheat genome to improve stress tolerance and obtain better crop performances, despite the challenging ploidy level of wheat. Abdallah et al. [50] used a multiplex gene editing approach to knockout the five active homologous gene copies of *TaSal1* in wheat variety Giza 168. In the primary transgenic plants 34% showed editing, these edits were heritable, and, in the progeny, five lines were identified with all five copies of *TaSal1* edited. Young leaves of edited *TaSal1* lines showed closed stomata, increased stomata width and increase in the size of the bulliform cells. *TaSal1* edited seedlings germinated and grew better on media containing polyethylene glycol than wildtype seedlings [50].

Further research and breeding programs will still be necessary to better elucidate the genetic and physiological bases of metabolic and signaling pathways of stress tolerance mechanisms. The identification of gene regulatory networks involved in stress responses and their targeting by gene editing tools will allow the development of new stress tolerant and high yielding varieties.

12 Summary

Recent advances in the production of high-quality genome sequences and efficient genotype- independent transgenic methods, developments in CRISPR-Cas based gene editing tools bring functional genomics and rational design-based molecular

breeding of polyploid wheat to unlock its hidden potential. Gene edited transgene free wheat has a critical role in addressing environmental issues while promoting sustainable agriculture and global food security. Gene editing is just one of the methods advancing wheat breeding programs and supporting wheat biology, which can be used alongside more conventional commercial breeding methods for delivering sustainability.

References

1. Matsuoka, Y., Nasuda, S.: Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F1 hybrid with Aegilops tauschii Coss. Theor. Appl. Genet. **109**, 1710–1717 (2004)
2. Borrill, P., Harrington, S.A., Uauy, C.: Applying the latest advances in genomics and phenomics for trait discovery in polyploid wheat. Plant J. **97**, 56–72 (2019)
3. Adamski, N.M., et al.: A roadmap for gene functional characterisation in crops with large genomes: lessons from polyploid wheat. Elife. **9** (2020)
4. Borrill, P., Adamski, N., Uauy, C.: Genomics as the key to unlocking the polyploid potential of wheat. New Phytol. **208**, 1008–1022 (2015)
5. Erenstein, O., et al.: Wheat Improvement: Food Security in a Changing Climate. (M.P. Reynolds, H.-J. Braun eds.) pp. 47–66. Springer, Cham (2022)
6. Shewry, P.R.: Wheat. J. Exp. Bot. **60**, 1537–1553 (2009)
7. FAOSTAT: 2020–2021
8. Wingen, L.U., et al.: Wheat Landrace genome diversity. Genetics. **205**, 1657–1676 (2017)
9. Debernardi, J.M., et al.: A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat. Biotechnol. **38**, 1274–1279 (2020)
10. Johnson, K., et al.: Rapid and highly efficient morphogenic gene-mediated hexaploid wheat transformation. Front. Plant Sci. **14**, 1151762 (2023)
11. Hayta, S., et al.: An efficient and reproducible Agrobacterium-mediated transformation method for hexaploid wheat (Triticum aestivum L.). Plant Method. **15**, 121 (2019)
12. Li, J., Li, Y., Ma, L.: Recent advances in CRISPR/Cas9 and applications for wheat functional genomics and breeding. aBIOTECH. **2**, 375–385 (2021)
13. Zhang, Y., et al.: Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat. Commun. **7**, 12617 (2016)
14. Sánchez-León, S., et al.: Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol. J. **16**, 902–910 (2018)
15. Zhang, Y., et al.: Analysis of the functions of TaGW2 homoeologs in wheat grain weight and protein content traits. Plant J. **94**, 857–866 (2018)
16. Wang, W., et al.: Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. Theor. Appl. Genet. **131**, 2463–2475 (2018)
17. Wang, W., et al.: Gene editing of the wheat homologs of TONNEAU1-recruiting motif encoding gene affects grain shape and weight in wheat. Plant J. **100**, 251–264 (2019)
18. Gupta, A., Hua, L., Zhang, Z., Yang, B., Li, W.: CRISPR-induced miRNA156-recognition element mutations in TaSPL13 improve multiple agronomic traits in wheat. Plant Biotechnol. J. **21**, 536–548 (2023)
19. Zhang, J., et al.: Increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9. J. Integr. Plant Biol. **63**, 1649–1663 (2021)

20. Zhang, R., et al.: Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing. Nat. Plant. **5**, 480–485 (2019)
21. Rey, M.D., et al.: Magnesium increases homoeologous crossover frequency during meiosis in ZIP4 (Ph1 gene) mutant wheat-wild relative hybrids. Front. Plant Sci. **9**, 509 (2018)
22. Draeger, T.N., et al.: ZIP4 is required for normal progression of synapsis and for over 95% of crossovers in wheat meiosis. Front. Plant Sci. **14** (2023)
23. Lv, J., et al.: Generation of paternal haploids in wheat by genome editing of the centromeric histone CENH3. Nat. Biotechnol. **38**, 1397–1401 (2020)
24. Li, J., et al.: Modification of starch composition, structure and properties through editing of TaSBEIIa in both winter and spring wheat varieties by CRISPR/Cas9. Plant Biotechnol. J. **19**, 937–951 (2021)
25. Raffan, S., et al.: Wheat with greatly reduced accumulation of free asparagine in the grain, produced by CRISPR/Cas9 editing of asparagine synthetase gene TaASN2. Plant Biotechnol. J. **19**, 1602–1613 (2021)
26. Camerlengo, F., et al.: CRISPR-Cas9 multiplex editing of the α-Amylase/Trypsin inhibitor genes to reduce allergen proteins in durum wheat. Front. Sustain. Food Syst. **4** (2020)
27. Sun, Z., et al.: CRISPR/Cas9-mediated disruption of Xylanase inhibitor protein (XIP) gene improved the dough quality of common wheat. Front. Plant Sci. **13**, 811668 (2022)
28. Wang, Y., et al.: Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. **32**, 947–951 (2014)
29. Li, S., et al.: Genome-edited powdery mildew resistance in wheat without growth penalties. Nature. **602**, 455–460 (2022)
30. Zhang, Y., et al.: Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. Plant J. **91**, 714–724 (2017)
31. Brauer, E.K., et al.: Genome editing of a deoxynivalenol-induced transcription factor confers resistance to Fusarium graminearum in wheat. Mol. Plant-Microbe Interact. **33**, 553–560 (2020)
32. Su, Z., et al.: A deletion mutation in TaHRC confers Fhb1 resistance to Fusarium head blight in wheat. Nat. Genet. **51**, 1099–1105 (2019)
33. Kim, D., Alptekin, B., Budak, H.: CRISPR/Cas9 genome editing in wheat. Funct. Integr. Genomics. **18**, 31–41 (2018)
34. Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S., Venkataraman, G.: CRISPR for crop improvement: an update review. Front. Plant Sci. **9**, 985 (2018)
35. Lusser, M., Parisi, C., Plan, D., Rodríguez-Cerezo, E.: Deployment of new biotechnologies in plant breeding. Nat. Biotechnol. **30**, 231–239 (2012)
36. Zhu, C., et al.: Characteristics of genome editing mutations in cereal crops. Trends Plant Sci. **22**, 38–52 (2017)
37. Kamburova, V.S., et al.: Genome editing in plants: an overview of tools and applications. Int. J. Agron. **2017**, 7315351 (2017)
38. Shrivastav, M., De Haro, L.P., Nickoloff, J.A.: Regulation of DNA double-strand break repair pathway choice. Cell Res. **18**, 134–147 (2008)
39. Liang, Z., et al.: Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat. Commun. **8**, 14261 (2017)
40. Liang, Z., Chen, K., Yan, Y., Zhang, Y., Gao, C.: Genotyping genome-edited mutations in plants using CRISPR ribonucleoprotein complexes. Plant Biotechnol. J. **16**, 2053–2062 (2018)
41. Koonin, E.V., Makarova, K.S., Zhang, F.: Diversity, classification and evolution of CRISPR-Cas systems. Curr. Opin. Microbiol. **37**, 67–78 (2017)
42. Lemos, B.R., et al.: CRISPR/Cas9 cleavages in budding yeast reveal templated insertions and strand-specific insertion/deletion profiles. Proc. Natl. Acad. Sci. **115**, E2040–E2047 (2018)
43. Merker, L., Schindele, P., Huang, T.K., Wolter, F., Puchta, H.: Enhancing in planta gene targeting efficiencies in Arabidopsis using temperature-tolerant CRISPR/LbCas12a. Plant Biotechnol. J. **18**, 2382–2384 (2020)

44. Zhou, J., et al.: An efficient CRISPR–Cas12a promoter editing system for crop improvement. Nat. Plant. **9**, 588–604 (2023)
45. Smedley, M.A., Hayta, S., Clarke, M., Harwood, W.A.: CRISPR-Cas9 based genome editing in wheat. Curr. Protoc. **1**, e65 (2021)
46. Xie, K., Minkenberg, B., Yang, Y.: Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc. Natl. Acad. Sci. U.S.A. **112**, 3570–3575 (2015)
47. Gao, Y., Zhao, Y.: Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. J. Integr. Plant Biol. **56**, 343–349 (2014)
48. Tang, X., et al.: A single transcript CRISPR-Cas9 system for efficient genome editing in plants. Mol. Plant. **9**, 1088–1091 (2016)
49. Wang, W., et al.: Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. CRISPR J. **1**, 65–74 (2018)
50. Abdallah, N.A., et al.: Multiplex CRISPR/Cas9-mediated genome editing to address drought tolerance in wheat. GM Crops Food. **6**, 1–17 (2022)
51. Li, J., et al.: Efficient multiplex genome editing by CRISPR/Cas9 in common wheat. Plant Biotechnol. J. **19**, 427–429 (2021)
52. Gaudelli, N.M., et al.: Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature. **551**, 464–471 (2017)
53. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., Liu, D.R.: Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. **533**, 420–424 (2016)
54. Anzalone, A.V., Koblan, L.W., Liu, D.R.: Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors. Nat. Biotechnol. **38**, 824–844 (2020)
55. Komor, A.C., et al.: Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. Sci. Adv. **3**, eaao4774 (2017)
56. Zong, Y., et al.: Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. Nat. Biotechnol. **35**, 438–440 (2017)
57. Li, C., et al.: Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. Genome Biol. **19**, 59 (2018)
58. Han, H., et al.: Generation of a high-efficiency adenine base editor with TadA8e for developing wheat dinitroaniline-resistant germplasm. Crop J. **10**(2), 368–374 (2022)
59. Xu, W., et al.: A design optimized prime editor with expanded scope and capability in plants. Nat. Plant. **8**, 45–52 (2022)
60. Jin, S., Lin, Q., Gao, Q., Gao, C.: Optimized prime editing in monocot plants using PlantPegDesigner and engineered plant prime editors (ePPEs). Nat. Protoc. **18**, 831–853 (2023)
61. Lin, Q., et al.: Prime genome editing in rice and wheat. Nat. Biotechnol. **38**, 582–585 (2020)
62. Zong, Y., et al.: An engineered prime editor with enhanced editing efficiency in plants. Nat. Biotechnol. **40**, 1394–1402 (2022)
63. Li, T., et al.: Highly efficient heritable genome editing in wheat using an RNA virus and bypassing tissue culture. Mol. Plant. **14**, 1787–1798 (2021)
64. Demirer, G.S., Zhang, H., Goh, N.S., González-Grandío, E., Landry, M.P.: Carbon nanotube–mediated DNA delivery without transgene integration in intact plants. Nat. Protoc. **14**, 2954–2971 (2019)
65. Liu, Y., et al.: In planta genome editing in commercial wheat varieties. Front. Plant Sci. **12** (2021)
66. Kumagai, Y., et al.: Introduction of a second "green revolution" mutation into wheat via in planta CRISPR/Cas9 delivery. Plant Physiol. **188**, 1838–1842 (2021)
67. Abe, F., et al.: Genome-edited triple-recessive mutation alters seed dormancy in wheat. Cell Rep. **28**, 1362–1369.e1364 (2019)
68. Raffan, S., et al.: Field assessment of genome-edited, low asparagine wheat: Europe's first CRISPR wheat field trial. Plant Biotechnol. J. **21**, 1097–1099 (2023)

69. Savary, S., et al.: The global burden of pathogens and pests on major food crops. Nat. Ecol. Evol. **3**, 430–439 (2019)
70. Shan, Q., Wang, Y., Li, J., Gao, C.: Genome editing in rice and wheat using the CRISPR/Cas system. Nat. Protoc. **9**, 2395–2410 (2014)
71. Humphry, M., Consonni, C., Panstruga, R.: mlo-based powdery mildew immunity: silver bullet or simply non-host resistance? Mol. Plant Pathol. **7**, 605–610 (2006)
72. Frye, C.A., et al.: Negative regulation of defense responses in plants by a conserved MAPKK kinase. Proc. Natl. Acad. Sci. U.S.A. **98**(1), 373–378 (2001) JSTOR, http://www.jstor.org/stable/3054682. Accessed 10 Oct 2023
73. Nalam, V.J., et al.: Facilitation of Fusarium graminearum infection by 9-Lipoxygenases in Arabidopsis and wheat. Mol. Plant-Microbe Interact. **28**, 1142–1152 (2015)
74. Cui, X.: Targeted gene editing using CRISPR/Cas9 in a wheat protoplast system. Ottawa, MSc thesis, Master of Biological Science (2017)
75. Pikaard, C.S., Mittelsten Scheid, O.: Epigenetic regulation in plants. Cold Spring Harb. Perspect. Biol. **6**, a019315 (2014)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 13
Gene Editing of Wheat to Reduce Coeliac Disease Epitopes in Gluten

Marinus J. M. Smulders, Luud J. W. J. Gilissen, Martina Juranić, Jan G. Schaart, and Clemens C. M. van de Wiel

Abstract By using gene editing technologies such as CRISPR/Cas, precise modifications can be made in the genome. CRISPR/Cas is especially valuable for targeted mutagenesis in polyploids, as it can induce mutations of multiple alleles simultaneously, to obtain regenerants that are homozygous for the desired mutation. A range of gene-edited traits have been developed in hexaploid bread wheat, including various nutrition and health-related traits, plant architecture, pest and disease resistance, tolerance to abiotic stress, and traits that enable more efficient breeding. Wheat is also known as a cause of some human diseases, particularly coeliac disease (CD), with a prevalence of 1–2% of the population. In the EU alone, at least 4.5 million people suffer from it. CD is a chronic inflammation of the small intestine, induced and maintained in genetically predisposed individuals by the consumption of gluten proteins from wheat, barley and rye. As there is no cure, patients must follow a life-long gluten-free diet. The dominant epitopes in gluten proteins that trigger the disease, have been characterized, but they cannot be removed by classical breeding without affecting baking quality, as it concerns over 100 gluten genes that occur partly as blocks of genes in the genome of wheat. Using gene editing, two studies have shown that it is possible to modify the epitopes in several alpha- and gamma-gliadins simultaneously, while deleting some of the genes completely. In some lines more than 80% of the alpha-gliadin genes were modified. These proof-of-principle studies show that it is feasible to use gene editing, along with other breeding approaches, to completely remove the CD epitopes from bread wheat. Gene-edited coeliac-safe wheat will have economic, social and environmental impact on food security, nutrition and public health, but the realisation will (partially) depend on new European legislation for plants produced by gene editing.

M. J. M. Smulders (✉) · L. J. W. J. Gilissen · M. Juranić · J. G. Schaart
C. C. M. van de Wiel
Plant Breeding, Wageningen University & Research, Wageningen, The Netherlands
e-mail: rene.smulders@wur.nl

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_13

1 Introduction

Bread wheat (*Triticum aestivum*, a hexaploid wheat species with the AABBDD genome) originated as a hybridization between tetraploid *Triticum* and (the) diploid *Aegilops* species during early agriculture in the Fertile Crescent. *Triticum* and *Aegilops* are closely related: Triticum is considered the domesticated form of *Aegilops*. Interspecific hybrids are found in nature and may also be produced artificially. Long before the onset of agriculture, a diploid wheat (*T. urartu*) carrying an AA genome hybridized with a grass-like wheat with a BB genome into the tetraploid *T. turgidum* with the combined AABB genome constitution. Near the Caspian Sea area, in an ancient agricultural field, a cultivated free-threshing tetraploid wheat (AABB) hybridized with a local wild diploid *Aegilops tauschii* with its DD genome, which led to the new hexaploid species *T. aestivum* (AABBDD). Spelt wheat is considered the result of one of the most recent natural hybridizations in which *T. aestivum* and a tetraploid *T. turgidum* (an emmer wheat type) were involved. Presently, synthetic hexaploid wheat lines are under production through various artificial hybridization programs, to increase the diversity in the D genome in hexaploid germplasm [1–3].

Wheat is a self-pollinating crop. Farmers can cultivate it by sowing seed material saved from the preceding year. Initially, cultivation practice applied mixtures of tetraploid and hexaploid genotypes. Through conscious or unconscious selection of spontaneous mutants, landraces gradually adapted to local environmental conditions, but there is a limit to the improvement of end-use quality that can be achieved in that way. Since the early twentieth century, genetics has been used in professional wheat breeding, including pure line selection and targeted breeding. Breeders are always interested in new genetic variation. This can be achieved through introgression (trait transfer through hybridization followed by back-crossing) from other wheat species, but this process also introduces many undesired traits that subsequently must be selected against. Alternatively, genetic variation can be induced within a cultivar through the application of mutagenic chemicals or ionizing irradiation (mutation breeding).

Presently, next to the hexaploid bread wheat and spelt wheat also diploid einkorn and various tetraploid *T. turgidum* subspecies (including emmer and durum wheat) are still being cultivated. However, almost 95% of the world's annual volume of 700–750 million metric tons is bread wheat; durum wheat makes up only 5%, the other species have a minimal volume. Bread wheat and durum wheat are also referred to as soft wheat and hard wheat, respectively, due to the difference in hardness of the kernel and further technological qualities regarding milling and baking: bread, cakes and breakfast cereals are made from soft wheat, pasta from durum wheat.

Its three homoeologous genomes make bread wheat flexible and adaptive in cultivation and versatile in its applications, due to its high genetic and allelic redundancy. This versatility becomes apparent in the specific qualitative and quantitative protein composition of its water-insoluble gluten, as used in various food and non-food processing applications, and, together with the grain's starch, in dough and

derived baked foods. Remarkable is the interdependence of the gluten protein structure, the gluten quantity and the gluten applicability regarding genetic, environmental and processing factors [4–6].

In keeping with its wide cultivation and application, bread wheat is the species of choice for further improvement of agricultural and food technological traits through conventional breeding, using various breeding tools developed in the twentieth and twenty-first century, including marker-assisted breeding and speed breeding [7].

In wheat breeding, the main focus was traditionally directed to yield and quality trait improvements. This has culminated in the 1960s in the Green Revolution through the introduction of dwarf genes. The dwarf genes (actually, dwarfing mutations) reduce energy investment in stem growth and increase grain yield. These new varieties were highly recognized worldwide. Today's breeding aims still include yield (especially starch quantity) and gluten and starch quality (for improved milling and baking quality), but adaptations to the biotic and abiotic environment, such as disease resistance genes, are receiving more attention because of the spread of major diseases and the threats of climate change, e.g., increased drought.

Impacts on export markets appeared to have played a role in the lack of commercialized GM wheat. The first GM (herbicide-tolerant) wheat applied for approval in the USA was abandoned in 2004 [8]. HB4 wheat, a GM wheat variety with drought tolerance has been approved for cultivation in Argentina in 2020 and subsequently for import in Brazil in 2021, which was important as for commercialization of GM events Argentina takes the impact on export markets into account. Since then, import has also been approved in the USA, Australia, New Zealand, Colombia, and Nigeria. Recently, Brazil has also approved cultivation of HB4 wheat.

2 Gene Editing in Polyploid Crops

To facilitate the time-consuming breeding of polyploid crops, alternative methods for introgression breeding and mutation breeding are important for polyploid crop improvement. Genome editing is such an alternative method. By using gene editing technologies such as CRISPR/Cas, precise modifications can be made to specific genes in the genome. CRISPR/Cas is especially valuable for targeted mutagenesis in polyploids, as it can induce mutations of multiple alleles simultaneously, so that regenerants homozygous for the desired mutation may be obtained. Moreover, the intrinsic specificity of CRISPR/Cas allows for the precise targeting of specific homoeologous genes. This enables allele-specific modifications, where only one copy of a gene is edited while leaving the others unchanged, or where all homoeologous copies are modified simultaneously. This ultimately allows for the introduction of desirable traits or the removal of undesirable ones without the need for laborious backcrossing to eliminate unwanted genetic material. Several studies have demonstrated the effectiveness and robustness of gene editing for targeted mutagenesis in auto- and allopolyploids [9], including wheat ([10]; see below and Table 13.1).

Table 13.1 Overview of research and trials using gene-edited traits in wheat. This overview was compiled in April 2023, and it is not exhaustive

Trait	Gene(s)	Reference
Health		
Low asparagine to suppress acrylamide production during heating	*ASN2*	[20–22]
High-amylopectin & low-discolouring	Waxy *GBSS* & *PPO*, respectively	[23]
High-fiber wheat pipeline	?	Calyxt news releases
Coeliac Disease (CD) epitopes	Alpha-gliadins	[24]
Coeliac Disease (CD) epitopes	Alpha- and gamma-gliadins	[25–27]
Biofortification		
Carotenoids	*Psy1*	[23]
Polyploidy/Recombination (incl. homoeologous pairing)		
Higher homoeologous CO (crossover) frequency (to facilitate introgressions from wild genomes)	*Tazip4-B2*	[28]
Testing effects on CO	*SPO11–1*	[29]
Haploid induction		
Paternal haploid induction by hetero-allelic combinations of genome-edited TaCENH3α	*TaCENH3α*	[30]
Haploid induction combined with targeted mutagenesis by using maize pollen (sperm cells) transgenically expressing *cas9* and selected gRNAs	Tested using *BRI1* or *SD1* involved in dwarfing	[31]
Male sterility		
Male sterility	*TaDCL4*, *TaDCL5*, *TaRDR6*	[32]
Male sterility	*Ms1*	[33]
Maize (and rice) counterpart Ms45 encoding strictosidine synthase-like enzyme potentially enabling an SPT-like hybrid seed production in wheat	*Ms45*	[34]
Plant architecture		
Alternative semi-dwarfing allele	*Rht-B1b* combined with *tasd1*	[35]
Possibly reproducing semidwarf alleles not negatively affecting coleoptile length hampering deep sowing in dry environments, such as Rht18, e.g., recessive semi-dwarfing in rice by GA20ox2 knockout	*GA20ox2*	[36]
TaQ alleles involved in domestication, impacting plant height, spike architecture	AP2-like TFs *TaAQ* and/or *TaDQ*	[37]
Yield		
Grain size and number, plant architecture, flowering timing	miRNA156 site in *TaSPL13*	[38]
Higher flower bearing spikelet number & higher grain protein	miRNA165/166 site in *HB-2* (HD-ZIP III)	[39]

(continued)

Table 13.1 (continued)

Trait	Gene(s)	Reference
Spike shape (length)	miRNA172 site in *Q5A* or *Q5D*	[40]
Supernumerary spikelets & higher grain number per spike	*DUO-B1* AP2/ERF TF	[41]
Testing on spikelet number per spike & grain number per spike	*WAPO-A1*	[42]
Increasing grain size	*TaTGW6*	[43]
Dosage-dependent increase in grain width & weight (not length)	*TaGW7*	[44]
Grain weight & protein content	*GW2* homoeologues	[45, 46]
Multiplex editing, among which grain weight	*GW2* together with *Lpx1* (against Fusarium graminacearum) & *MLO* (against PM)	[47]
Increased grain number per spikelet	*TaCKX-D1* with 1160 bp deletion homozygous, among other grain yield genes tested *GLW7*, *GW2* & *GW8*	[48]
ABE base editing	*TaGW2* & *TaDEP1*	[49]
Biotic stress tolerance (Susceptibility (S) genes)		
Viruses WSSMV & WYMV	*Ta-elF4E* & *Ta-elF(iso)4E* (& *TaBAK1*)	[50]
Virus YMV	*TaPDIL5*	[51]
Fusarium and powdery mildew (multiplex editing)	*Lpx1* (against *Fusarium graminacearum*) together with *GW2* & *MLO* (against powdery mildew)	[47]
Fusarium head blight	*TaHRC* (*Fhb1*)	[52–54]
Powdery mildew	3 *MLO* homoeologues	[55]
Powdery mildew without yield penalty	*Tamlo-R32*	[56, 57]
Powdery mildew	*TaEDR1*	[58]
Stripe rust	*TaCIPK14*	[59]
Potentially increased resistance to yellow and stem rust	*TaBCAT1*	[60]
Abiotic stress tolerance		
Osmotic stress tolerance	*TaSal1*	[61]
Nitrogen use efficiency (delayed senescence and increased grain yield in field)	*ARE1*	[62]
Delayed seed germination (dormancy)	*Qsd1*	[63]
Herbicide tolerance (HT)		
IMI, through Zinc Finger Nucleases (ZFN, SDN-2)	*AHAS*	[64]
SU herbicides (e.g., nicosulfuron), HT through CBE base editing	*TaALS*	[65, 66]
Phenoxy herbicide (quizalofob), HT through CBE base editing	*ACCase*	[66]

3 Improving Regeneration of Wheat in Tissue Culture

Efficient protocols for plant transformation and regeneration in tissue culture are essential prerequisites for utilizing gene editing techniques in wheat. However, the process of regeneration in tissue culture presents difficulties, particularly when dealing with recalcitrant species and is often dependent on identifying genotypes with capacity to regenerate. Overcoming these hurdles is crucial for wheat tissue culture as well. Promising advancements have been made in this area, such as the utilization of the developmental regulators that stimulate somatic embryogenesis and de novo meristem formation. Notably, recent studies have demonstrated that overexpressing a *GRF4-GIF1* chimera construct and the *TaWOX5* gene can substantially increase the regeneration capacity of wheat tissue cultures, while reducing the dependence on specific genotypes [11, 12]. These findings open new avenues for improving the regeneration efficiency of wheat in tissue culture and pave the way for more accessible and genotype-independent regeneration protocols. Continued research and exploration of innovative strategies, along with the advancement of gene editing techniques, can contribute to further enhancing the regeneration potential of wheat and accelerating the development of gene-edited varieties.

4 Current Research and Trials Using Gene Edited Traits in Wheat

A range of gene edited traits have been developed in wheat, largely in the last 5 years (Table 13.1). These include various nutrition and health-related traits such as low asparagine content, high fibre content, reduced coeliac disease epitopes in gluten, and biofortification. Next to these, plant architecture, yield improvement, pest and disease resistance, tolerance to abiotic stress, and herbicide tolerance are also investigated. The studies used Cas9 and Cpf1 [13–16] nucleases or prime editing [17] for targeted mutagenesis, as well as some base editors (see Table 13.1). Luo et al. [18] worked towards homology-directed repair enabling the introduction of larger sequences such as complete genes or promoters.

A series of breeding-related traits are also subject of gene editing research, including recombination, crossing over, haploid induction, and male sterility. These are related on one hand to the need to introgress traits, such as disease resistances and quality traits, from wild relatives, on the other hand to the desire to develop hybrid wheat varieties. When compared to traditionally inbred varieties, hybrid wheat is estimated to have a potential yield increase of 10% [19]. Furthermore, hybrid wheat, as with other hybrid crops, offers enhanced resilience and provides greater yield stability, even in the presence of environmental stresses and the extreme impacts of climate change. However, the breeding and widespread adoption of hybrid wheat is lagging behind. One reason is a costly production of hybrid seeds due to wheat's inherent tendency for self-pollination and the production of only one

seed per flower [19]. Overcoming these barriers and establishing effective breeding techniques for large-scale hybrid wheat breeding requires substantial research and development efforts. For example, male sterility provides the method to block self-fertilization, while modifying flower architecture can facilitate pollination.

Below we will in detail describe why and how gene editing and other approaches that target wheat gluten are used to reduce or remove epitopes that cause coeliac disease in susceptible people.

5 Wheat Gluten

Flour obtained by milling of whole wheat grains can be separated into four major protein fractions: albumins plus globulins, gliadins, glutenins, and a group of residual proteins. The gliadins and the glutenins, both water-insoluble, can be further subdivided into subtypes (Table 13.2). Cysteine residues, although relatively low in number, are characteristic to most gluten molecules (except to the omega-gliadins) as they form intrachain bonds in the alpha- and gamma-gliadins, stabilizing their tertiary structure, and interchain bonds among LMW and HMW glutenins. Gluten polymerization, a unique characteristic of gluten from wheat, is crucial for dough-making and baking. The building of large gas-retaining gluten networks may even increase through opening of intrachain gliadin S-S bonds by heating, enabling their binding to glutenin polymers [5]. High hydrostatic pressure (extrusion) of isolated guten to increase further polymerization can be applied in the production of meat analogues [67].

Wheat flour plus water produces a viscoelastic mass (a starchy dough) in which the gliadins are especially responsible for viscosity, whereas the glutenins are responsible for elasticity of the network that retains the gas from the yeast or sourdough fermentation and makes the dough rise. The degree of viscosity relates to the ratio of gliadins to glutenins: a high ratio (around 3.0) results in a soft and less

Table 13.2 Classification of gluten proteins and their main characteristics

Gluten group	Gluten type	Chromosome (A, B, D genome)	S-S bonds	Mono/ polymeric	Mol Weight (kDa)	% of total gluten
Gliadins	Alpha	6 (short arm)	+ (intrachain)	Mono	32	35
	Gamma	1 (short arm)	+ (intrachain)	Mono	35	21
	Omega 1,2	1 (short arm)	–	Mono	44	6
	Omega 5	1 (short arm)	–	Mono	51	5
Glutenins	LMW-GS	1 (long arm)	+ (interchain)	Poly	32[a]	24
	HMW-GSx;y	1 (long arm)	+ (interchain)	Poly	87[a]; 69[a]	9

Adapted from Ref. [5]

[a]As monomer, but as (combined) oligo- and polymers in stable 3D structures with MWs from 700 to 10,000 kDa [70]

viscous dough; a low ratio (around 2.0) results in a highly viscous and strong dough [5]. Heating during baking makes the gluten rigid by the loss of its water to the starch, keeping the baked bread shape stable. The presence of glutenin in doughs is essential for baking of bread and cookies, and in batter for cakes. Gliadins may be exchanged for other proteins or even partly omitted without significant loss of baking quality [68]. Guzmán-López et al. [69] managed to bake bread from wheat lines in which most gliadins had been silenced by RNAi.

Removing the starch from unheated dough by repeated washings with water yields in a purified gluten mass, called vital wheat gluten (VWG), which is applied in the food (bakery) and non-food industry.

6 Health-Related Wheat Components in Food

Due to its food technological versatility, wheat and its isolated components (vital wheat gluten, wheat starch, and derivatives such as glucose syrups, maltodextrin, sorbitol) are applied in numerous food products. They appear in about 30% of the labelled and mostly highly processed food items [71]. Highly processed foods, including those with wheat-derived ingredients, are often classified as unhealthy as they often contain a too high content of non-wheat ingredients such as sugar, salt and fat, and a too low content of fibre. However, whole grain wheat foods are recognized as healthy for their proven contribution to the reduction of risk of several 'western lifestyle'-related chronic diseases including obesity and diabetes, heart and vascular diseases, immune-related diseases, and certain forms of cancer. Therefore, consumption of whole grain (wheat) foods is stimulated by governmental food authorities in many countries [72, 73].

7 Wheat-Related Human Diseases

Wheat is also known as a cause of some human diseases: wheat allergy with a prevalence of 0.25% is relatively rare; non-coeliac wheat sensitivity (NCWS) with a self-reported prevalence of 10% and a clinically estimated prevalence of 1% has mostly mild symptoms; and coeliac disease (CD), the most severe but also the best-known and well-documented disease, has a prevalence of 1–2% [74, 75]. CD is a chronic inflammation of the small intestine, induced and maintained in genetically predisposed individuals by the consumption of gluten proteins from wheat, barley and rye. As there is no cure, patients must follow a life-long gluten-free diet. In the EU alone, at least 4.5 million people suffer from CD.

The general daily intake of gluten from the consumption of bread and related foods is about 15 g [76], whereas the safe daily amount of gluten in foods for individuals with CD is estimated between 10 and 100 mg gluten [77]. To stay below that load, food products should not surmount the 20 ppm threshold as is defined

according to extensive research and established in EU regulation EC828/2014. Note the huge gap between current foods in a gluten containing and a gluten-free lifestyle, especially when considering the high number of food items in supermarkets and elsewhere processed with wheat and wheat-derived components. As a correct diagnosis of CD is difficult because of the great variety of occurring symptoms, it is estimated that 85% of the patients remain undiagnosed, which means that they daily consume gluten, unaware that their bad chronic health status is caused by this food ingredient.

8 Gluten-Free Lifestyle

During the last decades, a gradual but significant increase has been observed in the number of consumers that embrace a gluten-free or wheat-free diet. This gluten/wheat avoiding population varies globally according to geographic location between 3.7% and 17.2% [78]. The main incentive for this diet choice is the desire for better health. This trend creates a decreasing bread and wheat-product market with a negative impact on the whole wheat value chain on the one hand, and an increasing and diversifying gluten-free product market on the other hand, with an annual growth rate of 10.4% between 2014 and 2019 in Europe, and an estimated compound annual growth rate (CAGR) of 8,1%. It had a market value of 8.3 Billion US dollars in 2025 (https://www.statista.com/statistics/248467/global-gluten-free-food-market-size/). The global gluten-free packaged food market now represents about 3% of the total global packaged food market, reflecting the population share that is interested in gluten-free foods, which is 15–20 times higher than the diagnosed CD population.

The problem remains to the 85% undiagnosed CD population. This group would be really served with the development, global cultivation and processing of safe-gluten wheat varieties replacing current high CD-immunogenic wheat. Gluten safety of such varieties should be guaranteed quantitatively (elimination of all immunogenic gluten) or qualitatively (inactivation of CD-immunogenic fragments (epitopes) from individual gluten proteins). However, maintenance of the full food technological and health qualities combined with unimpaired agronomic quality in such varieties remains a prime prerequisite for a crop to be competitive on the (global) market.

9 Gluten Epitopes for Coeliac Disease

What makes gluten intolerable to certain genetically predisposed individuals developing CD? The high abundance of glutamine and proline amino acids in gluten proteins hinders degradation by the proteolytic enzymes in the digestive tract, leaving relatively long peptides in the small intestine. Several of these peptides contain

a core sequence of nine amino acids that can be recognized by antigen-presenting cells and presented to T cells. Upon recognition, these T cells become activated, resulting in inflammation of the mucosa followed by degeneration of the mucosal villi with serious consequences for uptake of nutrients and leading to a variety of symptoms. Several epitopes are recognized by T cells from many patients, called 'major' epitopes. A comprehensive list of epitopes was recently updated [79].

Gluten proteins are encoded by large gene families located at different sites on the wheat genome (Table 13.2) with the alpha-gliadin loci on the short arm of the homoeologous chromosomes 6A, 6B and 6D, and the gamma-gliadin genes on the short arm of 1A, 1B and 1D. A single bread wheat variety genome (e.g., from the variety Chinese Spring for which the genome has been sequenced [80]), may contain as many as ~100 different gluten genes of which about sixty are expressed into proteins, as was shown by mass spectrometry analysis [4, 81–83]. Comparing different wheat varieties, variation exists in the number of expressed gluten genes, in the sequences of the encoded proteins, and in the amount of protein produced per gene. Additional variation in the gluten composition (quantitatively and qualitatively) is induced by environmental factors in the field during the growing season, such as the temperature during certain stages of crop development, and nutrient availability from the soil, in particular nitrogen and sulphur [4].

Although both gliadins and glutenins harbour immunogenic epitopes that can trigger CD, the alpha-, gamma- and omega-gliadins contain by far the highest number and the most dominant (severe) epitopes. For alpha-gliadins the dominant epitopes are related to the genes from the D-genome, followed by the genes from the A-genome, whereas some genes from the B-genome are free from epitopes [84]. D-genome alpha-gliadins contain the so-called 33-mer peptide carrying up to six overlapping CD epitopes and is a strong binder and activator of human T cells; similarly, some gamma-gliadins have a highly immunogenic 26-mer peptide.

10 Breeding Methods for Coeliac-Safe Wheat

Due to its genetic flexibility, several advanced breeding techniques are being used in wheat [85]. Wide hybridizations may result in irregularities (translocations as well as deletions) in the genomes of embryos and their later plant gametes. Chromosomal deletion lines, with impacts regarding reduction of CD-immunogenic gliadin loci, have been found and tested [86]. Chromosomal translocations and deletions may be induced technologically (as was done in the varieties Chinese Spring and Paragon, amongst others) but they also occur naturally. They are interesting for genetic research, but often the deletions impact the performance of the plants in the field.

Synthetic hexaploids can be obtained from hybridizations of wheat varieties and species of different ploidy levels (e.g., a tetraploid durum female with AABB genome with a diploid *Aegilops* male with DD genome) requiring duplication of the originally allotriploid embryonic genome (ABD into AABBDD) to form a fertile

hexaploid hybrid. An *Aegilops* line selected from the very wide D-genome diversity with a favourable CD profile may be hybridized with a low-immunogenic durum mother line. The resulting new hexaploids can be tested for their epitope profile at the gene and protein level, and applied in further breeding programs if desired [3]. This would be one way to eliminate the 33-mer in the D-genome alpha-gliadins.

EMS mutation breeding can generate large numbers of random mutations and could be applied to mutate gliadin genes, but it would be very resource-intensive to trace and combine mutations in multiple genes, from many plants, into one single, coeliac-safe and well-performing wheat plant [87]. Using γ-irradiation, large chromosomal deletions have been induced in two mutant lines of the wheat variety Paragon. Both mutants revealed a lack of sequence coverage in a large part of the gluten loci [25]. However, a clear need for more sophisticated approaches remains.

11 RNA Interference

RNA interference (RNAi) is a system that may be used to interfere with the synthesis of gluten proteins through their RNA transcripts, while the DNA still contains the intact genes. With a single RNAi construct designed for a conserved region that is common for many gluten genes, Gil-Humanes et al. [88] achieved up to 92% reduction of the gliadins, and a 10–100 fold reduction of epitopes as detected in T-cell tests. Similarly, the expression of twenty α-gliadin genes was decreased, although the production of other storage proteins increased [89]. Some of the wheat lines with reduced immunogenicity but with the baking quality largely intact [90] were sufficiently low in gliadins for successful clinical trials with patients [69]. Unfortunately, as RNAi requires stable genetic modification (GM) of the construct into wheat to silence the gliadins, and no transgenic wheat has been commercialised yet, it is unlikely that this transgenic line will be brought to the market.

12 Gene Editing of Gluten in Wheat

Two successful proof-of-concept studies with gene editing have been carried out in wheat. Sánchez-León et al. [24] targeted conserved sites in alpha-gliadin genes of a single wheat line. In this study, 47 offspring gene-edited plants were genotyped, and they found smaller and larger deletions at the target site, and plants with varying numbers of genes edited. In one plant 35 of the 45 alpha-gliadin genes were mutated, and seeds of this plant showed 85% reduction of total gluten protein as measured with the R5 gluten quantification assay, which is the type I method for gluten detection in the Codex Alimentarius. Guzmán-López et al. [91] developed a bioinformatics pipeline to show that in some offspring lines alpha-gliadin genes were deleted.

In the other study, Jouanin et al. [27] simultaneously targeted multiple sites in alpha-gliadin as well as gamma-gliadin genes, and confirmed 117 gene-edited

plants, including plants with mutations in both gene families. Some gluten genes were deleted, as evidenced by droplet digital PCR (ddPCR) results for alpha-gliadins [27]. Evidence that in some regenerated plants blocks of genes had been deleted came from mapping of sequence data obtained with GlutEnSeq, a variant to RenSeq that targets gluten genes, to the Chinese Spring reference genome [25]. This is presumably the result of double-strand breaks in different gliadin genes that occur in tandem arrays in the wheat genome, leading to the deletion of tandem gene blocks in between the genes with double strand breaks.

As not all target sites in the gliadin genes will be mutated in a single progeny plant, gene editing will lead to populations of plants that each may contain a mosaic of unaffected, edited, and lost genes. The edits on different chromosomes will segregate in the next self-pollinated generations. Hence, it is useful to have a rigorous selection program aimed at maintaining only the few most promising plants (genotypes) for multiplication, cultivation and eventual application in coeliac-safe(r) food [92]. In this process, gene-edited loci may be combined with loci that are lost through smaller or large deletions.

13 Impacts of Gene-Edited Low CD-Immunogenic ('Low-Gluten') Wheat

Producing low CD-immunogenic (hypo-immunogenic or 'low-gluten') wheat is technically feasible with gene editing using CRISPR/Cas technology. Such wheat varieties and the food products made with them have the potential to make a significant contribution to food security and public health for a group of individuals that suffer from coeliac disease, and for others that prefer to consume gluten-free products for health or diet related reasons. Such products could fit well in the gluten-free product market of packaged foods, which currently is growing fast.

Gene editing with CRISPR/Cas initially requires foreign DNA or proteins to be inserted into the plant genome or plant cell, as a temporary step to perform editing (read: to induce a directed and desired mutation) of target genes. In many jurisdictions such plants are not regulated as GM, provided that no foreign DNA is present in the final plant. In Europe, however, the process-based approach is applied, and because CRISPR/Cas includes a temporary GM step, gene-edited wheat will fall under the strict GM regulation (EC Directive 2001/18), whereas plants and derived products from chemically and irradiation induced mutagenesis are exempted from GM regulation according to EC Directive 2001/18, Annex 1B [93]. Presently, in 2023, the European Commission is preparing a proposal on changing the legislation for plants derived from cisgenesis or targeted mutagenesis. The possible impacts that gene-edited low CD-immunogenic wheat, once on the market, may have on the economy, society and the environment, were reviewed by Sánchez et al. (in press) [94].

One aspect of low-gluten wheat is that it will require the establishment of a separate supply chain under Hazard Analysis and Critical Control Points (HACCP) conditions [92]. Cross-contamination with regular wheat and other gluten-containing cereals (rye, barley) should be strictly avoided, requiring continuous control throughout the entire production chain. Further, labelling should emphasize the unique properties of the derived food products, particularly to CD-patients and NCWS patients. Importantly, the current legislations (EU 828/2014 and EU 41/2009) on gluten-free labelling requiring a threshold at 20 ppm gluten would need to be re-evaluated for low-gluten, low CD-immunogenic wheat products, because these regulations are based on quantitative measurement of the total amount of gluten in 'gluten-free' products which should be below the threshold for coeliac safety of 20 ppm. Gene-edited low CD-immunogenic wheat still will retain a gluten content far above this threshold and would require a separate 'CD-safe gluten' label.

Adoption of gene-edited coeliac-safe wheat might generate many new business opportunities for the entire wheat value chain, leading to diversification of production and products. In all, healthier and more safe foods for the CD patient population and reduced health care costs to society can be envisioned when gene-edited low-gluten CD-safe wheat would become mainstream on the food market ([94, 95] and refs therein).

Acknowledgements The writing of this chapter was partially made possible by the Well on Wheat (WoW) project (https://www.um-eatwell.nl/wow/), an international research project under the umbrella of the Health Grain Forum and ICC-Vienna and partially funded by the Dutch Topsector AgriFood (project TKI 1601P01 to LJWJG), and partly by the Netherlands' Ministry of Agriculture, Nature and Food Quality (BO-43-121-001 to MJMS and CMMvdW).

References

1. Ogbonnaya, F.C., Abdalla, O., Mujeeb-Kazi, A., Kazi, A.G., Xu, S.S., Gosman, N., Lagudah, E.S., Bonnett, D., Sorrells, M.E., Tsujimoto, H.: Synthetic Hexaploids: harnessing species of the primary Gene Pool for wheat improvement. In: Janick, J. (ed.) Plant Breeding Reviews, pp. 35–122. Wiley, Hoboken (2013)
2. Rosyara, U., Kishii, M., Payne, T., Sansaloni, C.P., Singh, R.P., Braun, H.-J., Dreisigacker, S.: Genetic contribution of synthetic Hexaploid wheat to CIMMYT's spring bread wheat breeding germplasm. Sci. Rep. **9**, 12355 (2019). https://doi.org/10.1038/s41598-019-47936-5
3. Schaart, J.G., Salentijn, E.M.J., Goryunova, S.V., Chidzanga, C., Esselink, D.G., Gosman, N., Bentley, A.R., Gilissen, L.J.W.J., Smulders, M.J.M.: Exploring the alpha-gliadin locus: the 33-mer peptide with six overlapping coeliac disease epitopes in Triticum aestivum is derived from a subgroup of Aegilops tauschii. Plant J. **106**, 86–94 (2021). https://doi.org/10.1111/tpj.15147
4. Shewry, P.: What is gluten—why is it special? Front. Nutr. **6**, 101 (2019). https://doi.org/10.3389/fnut.2019.00101
5. Wieser, H., Koehler, P., Scherf, K.A.: Chemistry of wheat gluten proteins: qualitative composition. Cereal Chem. **100**, 23–35 (2023). https://doi.org/10.1002/cche.10572
6. Wieser, H., Koehler, P., Scherf, K.A.: Chemistry of wheat gluten proteins: quantitative composition. Cereal Chem. **100**, 36–55 (2023). https://doi.org/10.1002/cche.10553

7. Smulders, M.J.M.: Then and now: a scientific view on plant breeding and technological innovation. In: Institute on Science for Global Policy (ISGP) (ed.) Sustainable Agriculture: The Role of Plant Breeding Innovation. A program and conference organized, facilitated, moderated, and convened by the ISGP with support from the American Seed Trade Association and Euroseeds (Internet Format), Nov. 17—18, 2020. pp. 21–25. http://scienceforglobalpolicy.org/publication/sustainable-agriculture-the-role-of-plant-breeding-innovation-sa-pbi (2021)
8. Wulff, B.B.H., Dhugga, K.S.: Wheat—the cereal abandoned by GM. Science. **361**, 451–452 (2018). https://doi.org/10.1126/science.aat5119
9. Schaart, J.G., van de Wiel, C.C.M., Smulders, M.J.M.: Genome editing of polyploid crops: prospects, achievements and bottlenecks. Transgenic Res. **30**, 337–351 (2021). https://doi.org/10.1007/s11248-021-00251-0
10. Bansal, M., Jindal, S., Wani, S.H., Ganie, S.A., Singh, R.: Genome editing and trait improvement in wheat. In: Wani, S.H., Mohan, A., Singh, G.P. (eds.) Physiological, Molecular, and Genetic Perspectives of Wheat Improvement, pp. 263–283. Springer, Cham (2021)
11. Debernardi, J.M., Tricoli, D.M., Ercoli, M.F., Hayta, S., Ronald, P., Palatnik, J.F., Dubcovsky, J.: A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat. Biotechnol. **38**, 1274–1279 (2020). https://doi.org/10.1038/s41587-020-0703-0
12. Wang, K., Shi, L., Liang, X., Zhao, P., Wang, W., Liu, J., Chang, Y., Hiei, Y., Yanagihara, C., Du, L., Ishida, Y., Ye, X.: The gene TaWOX5 overcomes genotype dependency in wheat genetic transformation. Nat. Plant. **8**, 110–117 (2022). https://doi.org/10.1038/s41477-021-01085-8
13. Kumar, R., Kaur, A., Pandey, A., Mamrutha, H.M., Singh, G.P.: CRISPR-based genome editing in wheat: a comprehensive review and future prospects. Mol. Biol. Rep. **46**, 3557–3569 (2019). https://doi.org/10.1007/s11033-019-04761-3
14. Kim, D., Hager, M., Brant, E., Budak, H.: Efficient genome editing in wheat using Cas9 and Cpf1 (AsCpf1 and LbCpf1) nucleases. Funct. Integr. Genomics. **21**, 355–366 (2021). https://doi.org/10.1007/s10142-021-00782-z
15. Li, S., Zhang, C., Li, J., Yan, L., Wang, N., Xia, L.: Present and future prospects for wheat improvement through genome editing and advanced technologies. Plant Commun. **2**, 100211 (2021). https://doi.org/10.1016/j.xplc.2021.100211
16. Smedley, M.A., Hayta, S., Clarke, M., Harwood, W.A.: CRISPR-Cas9 based genome editing in wheat. Curr. Protoc. **1**, e65 (2021). https://doi.org/10.1002/cpz1.65
17. Lin, Q., Zong, Y., Xue, C., Wang, S., Jin, S., Zhu, Z., Wang, Y., Anzalone, A.V., Raguram, A., Doman, J.L., Liu, D.R., Gao, C.: Prime genome editing in rice and wheat. Nat. Biotechnol. **38**, 582–585 (2020). https://doi.org/10.1038/s41587-020-0455-x
18. Luo, W., Suzuki, R., Imai, R.: Precise in planta genome editing via homology-directed repair in wheat. Plant Biotechnol. J. **21**, 668–670 (2022). https://doi.org/10.1111/pbi.13984
19. ter Steeg, E.M.S., Struik, P.C., Visser, R.G.F., Lindhout, P.: Crucial factors for the feasibility of commercial hybrid breeding in food crops. Nat. Plant. **8**, 463–473 (2022). https://doi.org/10.1038/s41477-022-01142-w
20. Raffan, S., Oddy, J., Mead, A., Barker, G., Curtis, T., Usher, S., Burt, C., Halford, N.G.: Field assessment of genome-edited, low asparagine wheat: Europe's first CRISPR wheat field trial. Plant Biotechnol. J. **21**, 1097–1099 (2023). https://doi.org/10.1111/pbi.14026
21. Raffan, S., Sparks, C., Huttly, A., Hyde, L., Martignago, D., Mead, A., Hanley, S.J., Wilkinson, P.A., Barker, G., Edwards, K.J., Curtis, T.Y., Usher, S., Kosik, O., Halford, N.G.: Wheat with greatly reduced accumulation of free asparagine in the grain, produced by CRISPR/Cas9 editing of asparagine synthetase gene TaASN2. Plant Biotechnol. J. **19**, 1602–1613 (2021). https://doi.org/10.1111/pbi.13573
22. Anonymous: UK trials first CRISPR-edited wheat in Europe. Nat. Biotechnol. **39**, 1174 (2021). https://doi.org/10.1038/s41587-021-01098-w
23. Zhang, S., Zhang, R., Gao, J., Song, G., Li, J., Li, W., Qi, Y., Li, Y., Li, G.: CRISPR/Cas9-mediated genome editing for wheat grain quality improvement. Plant Biotechnol. J. **19**, 1684–1686 (2021). https://doi.org/10.1111/pbi.13647

24. Sánchez-León, S., Gil-Humanes, J., Ozuna, C.V., Giménez, M.J., Sousa, C., Voytas, D.F., Barro, F.: Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol. J. **16**, 902–910 (2018). https://doi.org/10.1111/pbi.12837
25. Jouanin, A., Borm, T., Boyd, L.A., Cockram, J., Leigh, F., Santos, B.A.C.M., Visser, R.G.F., Smulders, M.J.M.: Development of the GlutEnSeq capture system for sequencing gluten gene families in hexaploid bread wheat with deletions or mutations induced by γ-irradiation or CRISPR/Cas9. J. Cereal Sci. **88**, 157–166 (2019). https://doi.org/10.1016/j.jcs.2019.04.008
26. Jouanin, A., Schaart, J.G., Boyd, L.A., Cockram, J., Leigh, F.J., Bates, R., Wallington, E.J., Visser, R.G.F., Smulders, M.J.M.: Outlook for coeliac disease patients: towards bread wheat with hypoimmunogenic gluten by gene editing of α- and γ-gliadin gene families. BMC Plant Biol. **19**, 333 (2019). https://doi.org/10.1186/s12870-019-1889-5
27. Jouanin, A., Tenorio-Berrio, R., Schaart, J.G., Leigh, F., Visser, R.G.F., Smulders, M.J.M.: Optimisation of droplet digital PCR for determining copy number variation of α-gliadin genes in mutant and gene-edited polyploid bread wheat. J. Cereal Sci. **92**, 102903 (2020). https://doi.org/10.1016/j.jcs.2019.102903
28. Rey, M.-D., Martín, A.C., Smedley, M., Hayta, S., Harwood, W., Shaw, P., Moore, G.: Magnesium increases Homoeologous crossover frequency during meiosis in ZIP4 (Ph1 gene) mutant wheat-wild relative hybrids. Front. Plant Sci. **9**, 509 (2018). https://doi.org/10.3389/fpls.2018.00509
29. Hyde, L., Osman, K., Winfield, M., Sanchez-Moran, E., Higgins, J.D., Henderson, I.R., Sparks, C., Franklin, F.C.H., Edwards, K.J.: Identification, characterization, and rescue of CRISPR/Cas9 generated wheat SPO11-1 mutants. Plant Biotechnol. J. **21**, 405–418 (2023). https://doi.org/10.1111/pbi.13961
30. Lv, J., Yu, K., Wei, J., Gui, H., Liu, C., Liang, D., Wang, Y., Zhou, H., Carlin, R., Rich, R., Lu, T., Que, Q., Wang, W.C., Zhang, X., Kelliher, T.: Generation of paternal haploids in wheat by genome editing of the centromeric histone CENH3. Nat. Biotechnol. **38**, 1397–1401 (2020). https://doi.org/10.1038/s41587-020-0728-4
31. Budhagatapalli, N., Halbach, T., Hiekel, S., Büchner, H., Müller, A.E., Kumlehn, J.: Site-directed mutagenesis in bread and durum wheat via pollination by cas9/guide RNA-transgenic maize used as haploidy inducer. Plant Biotechnol. J. **18**, 2376–2378 (2020). https://doi.org/10.1111/pbi.13415
32. Zhang, R., Zhang, S., Li, J., Gao, J., Song, G., Li, W., Geng, S., Liu, C., Lin, Y., Li, Y., Li, G.: CRISPR/Cas9-targeted mutagenesis of TaDCL4, TaDCL5 and TaRDR6 induces male sterility in common wheat. Plant Biotechnol. J. **21**, 839–853 (2023). https://doi.org/10.1111/pbi.14000
33. Okada, A., Arndell, T., Borisjuk, N., Sharma, N., Watson-Haigh, N.S., Tucker, E.J., Baumann, U., Langridge, P., Whitford, R.: CRISPR/Cas9-mediated knockout of Ms1 enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. Plant Biotechnol. J. **17**, 1905–1913 (2019). https://doi.org/10.1111/pbi.13106
34. Singh, M., Kumar, M., Albertsen, M.C., Young, J.K., Cigan, A.M.: Concurrent modifications in the three homeologs of Ms45 gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (Triticum aestivum L.). Plant Mol. Biol. **97**, 371–383 (2018). https://doi.org/10.1007/s11103-018-0749-2
35. Kumagai, Y., Liu, Y., Hamada, H., Luo, W., Zhu, J., Kuroki, M., Nagira, Y., Taoka, N., Katoh, E., Imai, R.: Introduction of a second "green revolution" mutation into wheat via in planta CRISPR/Cas9 delivery. Plant Physiol. **188**, 1838–1842 (2022). https://doi.org/10.1093/plphys/kiab570
36. Pearce, S.: Towards the replacement of wheat 'green revolution' genes. J. Exp. Bot. **72**, 157–160 (2021). https://doi.org/10.1093/jxb/eraa494
37. Liu, H., Wang, K., Tang, H., Gong, Q., Du, L., Pei, X., Ye, X.: CRISPR/Cas9 editing of wheat TaQ genes alters spike morphogenesis and grain threshability. J. Genet. Genomics. **47**, 563–575 (2020). https://doi.org/10.1016/j.jgg.2020.08.004

38. Gupta, A., Hua, L., Zhang, Z., Yang, B., Li, W.: CRISPR-induced miRNA156 -recognition element mutations in TaSPL13 improve multiple agronomic traits in wheat. Plant Biotechnol. J. **21**, 536–548 (2023). https://doi.org/10.1111/pbi.13969
39. Dixon, L.E., Pasquariello, M., Badgami, R., Levin, K.A., Poschet, G., Ng, P.Q., Orford, S., Chayut, N., Adamski, N.M., Brinton, J., Simmonds, J., Steuernagel, B., Searle, I.R., Uauy, C., Boden, S.A.: MicroRNA-resistant alleles of HOMEOBOX DOMAIN-2 modify inflorescence branching and increase grain protein content of wheat. Sci. Adv. **8**, 5907 (2022). https://doi.org/10.1126/sciadv.abn5907
40. Fan, M., Zhang, X., Nagarajan, R., Zhai, W., Rauf, Y., Jia, H., Ma, Z., Yan, L.: Natural variants and editing events provide insights into routes for spike architecture modification in common wheat. Crop J. **11**, 148–156 (2023). https://doi.org/10.1016/j.cj.2022.04.009
41. Wang, Y., Du, F., Wang, J., Wang, K., Tian, C., Qi, X., Lu, F., Liu, X., Ye, X., Jiao, Y.: Improving bread wheat yield through modulating an unselected AP2/ERF gene. Nat. Plant. **8**, 930–939 (2022). https://doi.org/10.1038/s41477-022-01197-9
42. Kuzay, S., Lin, H., Li, C., Chen, S., Woods, D.P., Zhang, J., Lan, T., von Korff, M., Dubcovsky, J.: WAPO-A1 is the causal gene of the 7AL QTL for spikelet number per spike in wheat. PLoS Genet. **18**, e1009747 (2022). https://doi.org/10.1371/journal.pgen.1009747
43. Kabir, M.R., Nonhebel, H.M.: Reinvestigation of THOUSAND-GRAIN WEIGHT 6 grain weight genes in wheat and rice indicates a role in pollen development rather than regulation of auxin content in grains. Theor. Appl. Genet. **134**, 2051–2062 (2021). https://doi.org/10.1007/s00122-021-03804-3
44. Wang, W., Pan, Q., Tian, B., He, F., Chen, Y., Bai, G., Akhunova, A., Trick, H.N., Akhunov, E.: Gene editing of the wheat homologs of TONNEAU1-recruiting motif encoding gene affects grain shape and weight in wheat. Plant J. **100**, 251–264 (2019). https://doi.org/10.1111/tpj.14440
45. Zhang, Y., Li, D., Zhang, D., Zhao, X., Cao, X., Dong, L., Liu, J., Chen, K., Zhang, H., Gao, C., Wang, D.: Analysis of the functions of TaGW2 homoeologs in wheat grain weight and protein content traits. Plant J. **94**, 857–866 (2018). https://doi.org/10.1111/tpj.13903
46. Wang, W., Simmonds, J., Pan, Q., Davidson, D., He, F., Battal, A., Akhunova, A., Trick, H.N., Uauy, C., Akhunov, E.: Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. Theor. Appl. Genet. **131**, 2463–2475 (2018). https://doi.org/10.1007/s00122-018-3166-7
47. Wang, W., Pan, Q.L., He, F., Akhunova, A., Chao, S.M., Trick, H., Akhunov, E.: Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. CRISPR J. **1**, 65–74 (2018). https://doi.org/10.1089/crispr.2017.0010
48. Zhang, Z., Hua, L., Gupta, A., Tricoli, D., Edwards, K.J., Yang, B., Li, W.: Development of an Agrobacterium -delivered CRISPR/Cas9 system for wheat genome editing. Plant Biotechnol. J. **17**, 1623–1635 (2019). https://doi.org/10.1111/pbi.13088
49. Li, C., Zong, Y., Wang, Y., Jin, S., Zhang, D., Song, Q., Zhang, R., Gao, C.: Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. Genome Biol. **19**, 59 (2018). https://doi.org/10.1186/s13059-018-1443-z
50. Hahn, F., Sanjurjo Loures, L., Sparks, C.A., Kanyuka, K., Nekrasov, V.: Efficient CRISPR/Cas-mediated targeted mutagenesis in spring and winter wheat varieties. Plan. Theory. **10**, 1481 (2021). https://doi.org/10.3390/plants10071481
51. Kan, J., Cai, Y., Cheng, C., Jiang, C., Jin, Y., Yang, P.: Simultaneous editing of host factor gene TaPDIL5-1 homoeoalleles confers wheat yellow mosaic virus resistance in hexaploid wheat. New Phytol. **234**, 340–344 (2022). https://doi.org/10.1111/nph.18002
52. Chen, H., Su, Z., Tian, B., Liu, Y., Pang, Y., Kavetskyi, V., Trick, H.N., Bai, G.: Development and optimization of a Barley stripe mosaic virus -mediated gene editing system to improve Fusarium head blight resistance in wheat. Plant Biotechnol. J. **20**, 1018–1020 (2022). https://doi.org/10.1111/pbi.13819

53. Li, G.Q., Zhou, J.Y., Jia, H.Y., Gao, Z.X., Fan, M., Luo, Y.J., Zhao, P.T., Xue, S.L., Li, N., Yuan, Y., Ma, S.W., Kong, Z.X., Jia, L., An, X., Jiang, G., Liu, W.X., Cao, W.J., Zhang, R.R., Fan, J.C., Xu, X.W., Liu, Y.F., Kong, Q.Q., Zheng, S.H., Wang, Y., Qin, B., Cao, S.Y., Ding, Y.X., Shi, J.X., Yan, H.S., Wang, X., Ran, C.F., Ma, Z.Q.: Mutation of a histidine-rich calcium-binding-protein gene in wheat confers resistance to Fusarium head blight. Nat. Genet. **51**, 1106–1112 (2019). https://doi.org/10.1038/s41588-019-0426-7
54. Su, Z.Q., Bernardo, A., Tian, B., Chen, H., Wang, S., Ma, H.X., Cai, S.B., Liu, D.T., Zhang, D.D., Li, T., Trick, H., St Amand, P., Yu, J.M., Zhang, Z.Y., Bai, G.H.: A deletion mutation in TaHRC confers Fhb1 resistance to Fusarium head blight in wheat. Nat. Genet. **51**, 1099–1105 (2019). https://doi.org/10.1038/s41588-019-0425-8
55. Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., Qiu, J.-L.: Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. **32**, 947–951 (2014). https://doi.org/10.1038/nbt.2969
56. Li, S., Lin, D., Zhang, Y., Deng, M., Chen, Y., Lv, B., Li, B., Lei, Y., Wang, Y., Zhao, L., Liang, Y., Liu, J., Chen, K., Liu, Z., Xiao, J., Qiu, J.-L., Gao, C.: Genome-edited powdery mildew resistance in wheat without growth penalties. Nature. **602**, 455–460 (2022). https://doi.org/10.1038/s41586-022-04395-9
57. Deng, X., Cao, X.: Reconciliation between high yield and disease resistance. Nat. Rev. Genet. **23**, 262–263 (2022). https://doi.org/10.1038/s41576-022-00471-y
58. Zhang, Y., Bai, Y., Wu, G., Zou, S., Chen, Y., Gao, C., Tang, D.: Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. Plant J. **91**, 714–724 (2017). https://doi.org/10.1111/tpj.13599
59. He, F., Wang, C., Sun, H., Tian, S., Zhao, G., Liu, C., Wan, C., Guo, J., Huang, X., Zhan, G., Yu, X., Kang, Z., Guo, J.: Simultaneous editing of three homoeologues of TaCIPK14 confers broad-spectrum resistance to stripe rust in wheat. Plant Biotechnol. J. **21**, 354–368 (2023). https://doi.org/10.1111/pbi.13956
60. Corredor-Moreno, P., Minter, F., Davey, P.E., Wegel, E., Kular, B., Brett, P., Lewis, C.M., Morgan, Y.M.L., Macías Pérez, L.A., Korolev, A.V., Hill, L., Saunders, D.G.O.: The branched-chain amino acid aminotransferase TaBCAT1 modulates amino acid metabolism and positively regulates wheat rust susceptibility. Plant Cell. **33**, 1728–1747 (2021). https://doi.org/10.1093/plcell/koab049
61. Abdallah, N.A., Elsharawy, H., Abulela, H.A., Thilmony, R., Abdelhadi, A.A., Elarabi, N.I.: Multiplex CRISPR/Cas9-mediated genome editing to address drought tolerance in wheat. GM Crops Food. (2022). https://doi.org/10.1080/21645698.2022.2120313
62. Zhang, J., Zhang, H., Li, S., Li, J., Yan, L., Xia, L.: Increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9. J. Integr. Plant Biol. **63**, 1649–1663 (2021). https://doi.org/10.1111/jipb.13151
63. Liu, Y., Luo, W., Linghu, Q., Abe, F., Hisano, H., Sato, K., Kamiya, Y., Kawaura, K., Onishi, K., Endo, M., Toki, S., Hamada, H., Nagira, Y., Taoka, N., Imai, R.: In planta genome editing in commercial wheat varieties. Front. Plant Sci. **12**, 648841 (2021). https://doi.org/10.3389/fpls.2021.648841
64. Ran, Y., Patron, N., Kay, P., Wong, D., Buchanan, M., Cao, Y.-Y., Sawbridge, T., Davies, J.P., Mason, J., Webb, S.R., Spangenberg, G., Ainley, W.M., Walsh, T.A., Hayden, M.J.: Zinc finger nuclease-mediated precision genome editing of an endogenous gene in hexaploid bread wheat (Triticum aestivum) using a DNA repair template. Plant Biotechnol. J. **16**, 2088–2101 (2018). https://doi.org/10.1111/pbi.12941
65. Qiu, F., Xing, S., Xue, C., Liu, J., Chen, K., Chai, T., Gao, C.: Transient expression of a TaGRF4-TaGIF1 complex stimulates wheat regeneration and improves genome editing. Sci. China Life Sci. **65**, 731–738 (2022). https://doi.org/10.1007/s11427-021-1949-9
66. Zhang, R., Liu, J., Chai, Z., Chen, S., Bai, Y., Zong, Y., Chen, K., Li, J., Jiang, L., Gao, C.: Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing. Nat. Plant. **5**, 480–485 (2019). https://doi.org/10.1038/s41477-019-0405-0

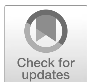
67. Pietsch, V.L., Werner, R., Karbstein, H.P., Emin, M.A.: High moisture extrusion of wheat gluten: relationship between process parameters, protein polymerization, and final product characteristics. J. Food Eng. **259**, 3–11 (2019). https://doi.org/10.1016/j.jfoodeng.2019.04.006
68. van den Broeck, H.C., Gilissen, L.J.W.J., Smulders, M.J.M., van der Meer, I.M., Hamer, R.J.: Dough quality of bread wheat lacking α-gliadins with celiac disease epitopes and addition of celiac-safe avenins to improve dough quality. J. Cereal Sci. **53**, 206–216 (2011). https://doi.org/10.1016/j.jcs.2010.12.004
69. Guzmán-López, M.H., Sánchez-León, S., Marín-Sanz, M., Comino, I., Segura, V., Vaquero, L., Rivero-Lezcano, O.M., Pastor, J., Sousa, C., Vivas, S., Barro, F.: Oral consumption of bread from an RNAi wheat line with strongly silenced gliadins elicits no immunogenic response in a pilot study with celiac disease patients. Nutrients. **13**, 4548 (2021). https://doi.org/10.3390/nu13124548
70. Köhler, P., Belitz, H.-D., Wieser, H.: Disulphide bonds in wheat gluten: further cystine peptides from high molecular weight (HMW) and low molecular weight (LMW) subunits of glutenin and from γ-gliadins. Z. Lebensm. Unters. Forsch. **196**, 239–247 (1993). https://doi.org/10.1007/BF01202740
71. Atchison, J., Head, L., Gates, A.: Wheat as food, wheat as industrial substance; comparative geographies of transformation and mobility. Geoforum. **41**, 236–246 (2010). https://doi.org/10.1016/j.geoforum.2009.09.006
72. Kromhout, D., Spaaij, C.J.K., de Goede, J., Weggemans, R.M.: The 2015 Dutch food-based dietary guidelines. Eur. J. Clin. Nutr. **70**, 869–878 (2016). https://doi.org/10.1038/ejcn.2016.52
73. Gilissen, L.J.W.J., van den Broeck, H.C.: Breeding for healthier wheat. Cereal Foods World. **63**, 132–136 (2018). https://doi.org/10.1094/CFW-63-4-0132
74. Gilissen, L.J.W.J., van der Meer, I.M., Smulders, M.J.M.: Reducing the incidence of allergy and intolerance to cereals. J. Cereal Sci. **59**, 337–353 (2014). https://doi.org/10.1016/j.jcs.2014.01.005
75. Rustgi, S., Kashyap, S., Ankrah, N., von Wettstein, D.: Use of microspore-derived Calli as explants for biolistic transformation of common wheat. In: Rustgi, S., Luo, H. (eds.) Biolistic DNA Delivery in Plants. Methods and Protocols. Methods in Molecular Biology, pp. 263–279. Humana Press Inc., New York (2020)
76. Kasarda, D.D.: Can an increase in celiac disease be attributed to an increase in the gluten content of wheat as a consequence of wheat breeding? J. Agric. Food Chem. **61**, 1155–1159 (2013). https://doi.org/10.1021/jf305122s
77. Hischenhuber, C., Crevel, R., Jarry, B., Maki, M., Moneret-Vautrin, D.A., Romano, A., Troncone, R., Ward, R.: Review article: safe amounts of gluten for patients with wheat allergy or coeliac disease. Aliment. Pharmacol. Ther. **23**, 559–575 (2006). https://doi.org/10.1111/j.1365-2036.2006.02768.x
78. Perrin, L., Allès, B., Buscail, C., Ravel, C., Hercberg, S., Julia, C., Kesse-Guyot, E.: Gluten-free diet in French adults without coeliac disease: sociodemographic characteristics, motives and dietary profile. Br. J. Nutr. **122**, 231–239 (2019). https://doi.org/10.1017/S0007114519001053
79. Sollid, L.M., Tye-Din, J.A., Qiao, S.-W., Anderson, R.P., Gianfrani, C., Koning, F.: Update 2020: nomenclature and listing of celiac disease–relevant gluten epitopes recognized by CD4+ T cells. Immunogenetics. **72**, 85–88 (2020). https://doi.org/10.1007/s00251-019-01141-w
80. International Wheat Genome Sequencing Consortium (IWGSC): Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science. **361**, eaar7191 (2018). https://doi.org/10.1126/science.aar7191
81. Huo, N., Zhang, S., Zhu, T., Dong, L., Wang, Y., Mohr, T., Hu, T., Liu, Z., Dvorak, J., Luo, M.-C., Wang, D., Lee, J.-Y., Altenbach, S., Gu, Y.Q.: Gene duplication and evolution dynamics in the Homeologous regions harboring multiple Prolamin and resistance gene families in Hexaploid wheat. Front. Plant Sci. **9**, 673 (2018). https://doi.org/10.3389/fpls.2018.00673
82. Huo, N., Zhu, T., Altenbach, S., Dong, L., Wang, Y., Mohr, T., Liu, Z., Dvorak, J., Luo, M.-C., Gu, Y.Q.: Dynamic evolution of α-Gliadin Prolamin gene family in Homeologous genomes of Hexaploid wheat. Sci. Rep. **8**, 5181 (2018). https://doi.org/10.1038/s41598-018-23570-5

83. Altenbach, S.B., Chang, H.-C., Simon-Buss, A., Mohr, T., Huo, N., Gu, Y.Q.: Exploiting the reference genome sequence of hexaploid wheat: a proteomic study of flour proteins from the cultivar Chinese Spring. Funct. Integr. Genomics. **20**, 1–16 (2020). https://doi.org/10.1007/s10142-019-00694-z
84. van Herpen, T.W., Goryunova, S.V., van der Schoot, J., Mitreva, M., Salentijn, E., Vorst, O., Schenk, M.F., van Veelen, P.A., Koning, F., van Soest, L.J., Vosman, B., Bosch, D., Hamer, R.J., Gilissen, L.J., Smulders, M.J.: Alpha-gliadin genes from the a, B, and D genomes of wheat contain different sets of celiac disease epitopes. BMC Genomics. **7**, 1 (2006). https://doi.org/10.1186/1471-2164-7-1
85. Gilissen, L.J.W.J., Smulders, M.J.M.: Low gluten and coeliac-safe wheat through gene editing. In: Ricroch, A., Chopra, S., Kuntz, M. (eds.) Plant Biotechnology, pp. 231–248. Springer, Cham (2021)
86. van den Broeck, H.C., van Herpen, T.W., Schuit, C., Salentijn, E.M., Dekking, L., Bosch, D., Hamer, R.J., Smulders, M.J., Gilissen, L.J., van der Meer, I.M.: Removing celiac disease-related gluten proteins from bread wheat while retaining technological properties: a study with Chinese Spring deletion lines. BMC Plant Biol. **9**, 41 (2009). https://doi.org/10.1186/1471-2229-9-41
87. Jouanin, A., Gilissen, L.J.W.J., Boyd, L.A., Cockram, J., Leigh, F.J., Wallington, E.J., Van den Broeck, H.C., Van der Meer, I.M., Schaart, J.G., Visser, R.G.F., Smulders, M.J.M.: Food processing and breeding strategies for coeliac-safe and healthy wheat products. Food Res. Int. **110**, 11–21 (2018). https://doi.org/10.1016/j.foodres.2017.04.025
88. Gil-Humanes, J., Pistón, F., Tollefsen, S., Sollid, L.M., Barro, F.: Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. Proc. Natl. Acad. Sci. **107**, 17023–17028 (2010). https://doi.org/10.1073/pnas.1007773107
89. Becker, D., Wieser, H., Koehler, P., Folck, A., Mühling, K.H., Zörb, C.: Protein composition and techno-functional properties of transgenic wheat with reduced α-gliadin content obtained by RNA interference. J. Appl. Bot. Food Qual. **85**, 23–33 (2012). https://ojs.openagrar.de/index.php/JABFQ/article/view/1995
90. Gil-Humanes, J., Pistón, F., Altamirano-Fortoul, R., Real, A., Comino, I., Sousa, C., Rosell, C.M., Barro, F.: Reduced-gliadin wheat bread: an alternative to the gluten-free diet for consumers suffering gluten-related pathologies. PLoS One. **9**, e90898 (2014). https://doi.org/10.1371/journal.pone.0090898
91. Guzmán-López, M.H., Marín-Sanz, M., Sánchez-León, S., Barro, F.: A bioinformatic workflow for InDel analysis in the wheat multi-copy α-gliadin gene family engineered with CRISPR/Cas9. Int. J. Mol. Sci. **22**, 13076 (2021). https://doi.org/10.3390/ijms222313076
92. Jouanin, A., Gilissen, L.J.W.J., Schaart, J.G., Leigh, F.J., Cockram, J., Wallington, E.J., Boyd, L.A., van den Broeck, H.C., van der Meer, I.M., America, A.H.P., Visser, R.G.F., Smulders, M.J.M.: CRISPR/Cas9 gene editing of gluten in wheat to reduce gluten content and exposure—reviewing methods to screen for coeliac safety. Front. Nutr. **7**, 51 (2020). https://doi.org/10.3389/fnut.2020.00051
93. Jouanin, A., Boyd, L., Visser, R.G.F., Smulders, M.J.M.: Development of wheat with hypoimmunogenic gluten obstructed by the gene editing policy in Europe. Front. Plant Sci. **9**, 1523 (2018). https://doi.org/10.3389/fpls.2018.01523
94. Sánchez, B., Barro, F., Smulders, M.J.M., et al.: Economic and social impacts of low-gluten coeliac-safe wheat developed by gene editing. JRC Tech. Rep. (in press) (2023)
95. Maaß, O., Consmüller, N., Kehlenbeck, H.: Socioeconomic impact of genome editing on agricultural value chains: the case of fungal-resistant and coeliac-safe wheat. Sustainability. **11**, 6421 (2019). https://doi.org/10.3390/su11226421

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 14
Genome Editing in Horticultural Plants: Present Applications and Future Perspective



Nasser Mahna and Shahnoush Nayeri

Abstract This chapter considers the genome editing technologies that have been utilized for breeding horticultural plants. Many examples of the successful application of genome editing technologies including ZFN, TALEN, and especially CRISPR/Cas systems in improving diverse characteristics of horticultural plants are mentioned and discussed. Based on the literature review, CRISPR/Cas technology has proved its potential in altering many genes of interest in horticultural plants including fruits, vegetables, and ornamental plants for improving agronomically important traits and attributes such as growth rate, seed size, flowering time, flower color, storage time, resistance to biotic stresses, tolerance to abiotic stresses, herbicide tolerance, metabolism, fruit color, fruit ripening, and so forth. This advanced technology paves the way for more favorable and precise manipulation of plant genomes to improve crop performance.

1 Introduction

Horticulture goes back to ancient times as an important sector within agriculture and has improved a lot during human civilization. Horticultural crops are typically known as vegetables, fruits, and ornamental plants which are planted and harvested for food, medical, and cosmetic purposes [1]. Nevertheless, horticultural crop cultivation continues to face a variety of challenges, such as serious environmental concerns, the spread of viruses and pests, climate change, and increasing population growth [2]. Conventional breeding, molecular markers, and genetic modification

N. Mahna (✉)
Department of Horticultural Sciences, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
e-mail: mahna@tabrizu.ac.ir

S. Nayeri
Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_14

have been used for improving traits in horticultural plants. However, faster development of better varieties requires more precise and safe techniques such as genome editing systems.

2 Non-CRISPR/Cas Genome Editing Systems and Their Applications in Horticultural Plants

Before the discovery of CRISPR/Cas, other genome editing techniques were used to modify traits in plants, but not so frequently in horticultural crops. Strictly speaking, there is no report on the application of meganucleases [3], the first developed genome editing tool, in horticultural crops. Later, after the rise of Zinc Finger Nuclease (ZFN) technology, two groups applied ZFN in the functional analysis and characterization of genes in apple and fig [4] as well as tomato [5] plants. Thereafter, TALEN-based gene editing technology was developed and effectively employed in some economically important horticultural crops such as rapeseed (*Brassica oleracea*) [6], potato (*Solanum tuberosum*) [7–12] and tomato (*Solanum lycopersicum*) [13, 14]. The first instance of genome editing in a horticulture crop was accomplished in 2013 using TALEN in *Brassica oleracea* [6]. Later, in potato, TALEN was used for silencing the vacuolar invertase gene to improve the processing and cold storage of potato tubers [12].

3 CRISPR/Cas Systems in Horticultural Plants

The CRISPR/Cas genome editing technology was developed almost two years later than the discovery of TALEN proteins [15]. The CRISPR/Cas technology is expected to open up novel opportunities for the improvement of horticultural plants. This technology has already been used widely in horticultural plant breeding projects (see Table 14.1). For example, CRISPR/Cas9 was employed in the genome editing of sweet orange and tomato hairy roots [16, 17]. Several works have endeavored to enhance floricultural characteristics in ornamental plants, such as flower size, shape, color, aroma, shelf life, stress tolerance, etc. Through knocking down the *Argonaute7*, CRISPR/Cas9 was exploited to develop the first targeted mutation in the needle-leaf of tomato [18]. Since then, some research have been published on the potential uses of CRISPR/Cas in improving fruit quality, plant architecture, and shelf life as well as protecting plants from biotic and abiotic stresses [19]. The technique is now being exploited for a variety of fruits and vegetable crops, including watermelon, mustard, tomato, potato, and cabbage, etc. Table 14.1 presents a wide variety of genome-edited horticultural plants including vegetables, ornamentals, and fruit crops for different types of traits. Until now, just a handful number of teams have utilized CRISPR/Cas-mediated genome-editing in ornamental plants,

Table 14.1 The List of the mutated genes responsible for specific trait categories using CRISPR/Cas9 gene/genome editing system in horticultural plants [21–24]

Crop species	Scientific name	Plant type	Gene name	Trait modification	Classification
Apple	*Malus pumila*	Fruit	*PDS*	Albino phenotype	Reporter
			TFL1.1	Early flowering	Development
			DIPM	Blight resistance	Stress response
			CNGC2	Resistance to *Botryosphaeria dothidea*	Stress response
Banana	*Musa acuminata*	Fruit	*PDS*	Photobleaching, albinism	Reporter
			ACO1	Fruit ripening delay, extended shelf life	Agronomic characteristics
	Musa acuminata 'Gros Michel'		*GA20ox2*	Semi-dwarf phenotype	Development
	Musa balbisiana		*BSOLV*	Banana streak virus resistance	Stress response
	Musa spp.		*LCYε*	Sixfold enhancement of β-carotene content in fruits	Metabolism
			DMR6	Banana *Xanthomonas* wilt resistance	Stress response
	Cavendish cv. Williams		*PDS*	Photobleaching, albinism, dwarfing	Reporter
Blueberry	*Vaccinum corymbosum*	Fruit	*CEN*	Dwarfism, lack of precocious flowering	Flowering
Cacao	*Theobroma cacao*	Fruit	*NPR3*	Resistance to *Ph. tropicalis*	Stress response
Camelina	*Camelina sativa*	Vegetable	*FAE1*	Reduced long-chain fatty acids	Metabolism
			DGAT1	Altered fatty acid composition	
			PDAT1	Reduced oil content	
			FAD2	Reduced levels of polyunsaturated fatty acids	
				Decreased polyunsaturated fatty acids	
Carrizo citrange	*Citrus sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.	Fruit	*ALS*	Herbicide resistance	Stress response
Coffee	*Coffea canephora*	Fruit	*PDS*	Photobleaching, albinism	Reporter
Cucumber	*Cucumis sativus*	Vegetable	*eIF4E*	Enhanced viral resistance	Stress response
			WIP1	Gynoecious phenotype	Development

(continued)

Table 14.1 (continued)

Crop species	Scientific name	Plant type	Gene name	Trait modification	Classification
Dendrobium	*Dendrobium officinale*	Medicinal herb (flower)	*C3H*	Lignocellulose biosynthesis	Metabolism
			C4H		
			4CL		
			CCR		
			IRX		
Ethiopian mustard	*Brassica carinata*	Vegetable	*Fascilin-like arabinogalactan*	Regulation of root hairs under phosphorus stress	Development, stress response
				Root hair development	Development
Field mustard	*Brassica oleracea and B. rapa*	Vegetable	*PDS*	Albino phenotype	Reporter
			FRI	Flowering	Development
			AP2a	Sepal to carpal modification	
			AP2b		
Kiwifruit	*Actinidia chinensis*	Fruit	*Cen4*, *SyGl*	Rapid flowering	Flowering
	Actinidia chinensis		*Cen4*	Compact growth, terminal flowering	
	Actinidia deliciosa		*PDS*	Albino phenotype	Reporter
Grape Vine	*Vitis vinifera*	Fruit	*PDS*	Albino phenotype	Reporter
			IdnDH	Biosynthesis of tartaric acid	Metabolism
			WRKY52	Increased resistance to *Botrytis cinerea*	Stress response
			PDS	Albino phenotype	Reporter
			MLO-7	Powdery mildew resistance	Stress response
			IdnDH	Biosynthesis of tartaric acid	Metabolism
			MLO3	Resistance to powdery mildew	Stress response
			MLO4		
			PR4b		
			CCD8	Highly branched phenotype	Agronomic characteristics

Grapefruit	*Citrus paradisi*	Fruit	*PDS*	Albino phenotype	Reporter
			Cs2g12470		
			Cs7g03360		
			LOB1	Canker resistance	Stress response
Hybrid strawberry	*Fragaria × ananassa*	Fruit	*PDS*	Albino phenotype	Reporter
	Fragaria vesca, Fragaria × ananassa		*MYB10*	Anthocyanin biosynthesis	Metabolism
			CHS		
Japanese gentians	*Gentiana scabra × G. triflora*	Flower	*5GT*	Flower color change	Flowering
			3′GT		
			5/3′AT		
			GST1		
			EPH1	Flower longevity	
Japanese morning glory	*Ipomoea nil*	Flower	*DFR-B*	Anthocyanin biosynthesis and white flowers	Metabolism
			CCD4	Altered petal color	Development
			EPH1	Flower longevity	Flowering
Kumquat	*Fortunella hindsii*	Fruit	*PDS*	Photobleaching, albinism	Reporter
			CCD4b	No mutant phenotype	Development
			DUO1		
			NZZ	Leaf curling, longer pedicel length	
Lettuce	*Lettuce sativa*	Vegetable	*BIN2*	Impaired brassinosteroid response	Development
			NCED4	Thermo-inhibition of seed germination	
Lilium	*Lilium longiflorum,*	Flower	*PDS*	Photobleaching, albinism	Reporter
	Lilium pumilum				
Melon	*Cucumis melo*	Fruit	*PDS*	Photobleaching, albinism	Reporter
			NAC-NOR	Shelf life	Agronomic characteristics
			CTR1-like, ROS1		

(continued)

Table 14.1 (continued)

Crop species	Scientific name	Plant type	Gene name	Trait modification	Classification
Mum	*Chrysanthemum morifolium*	Flower	*YGFP*	Targeted editing of the YGFP reporter gene	Reporter
Orchid	*Phalaenopsis equestris*	Flower	*MADS8*	Long juvenile period	Flowering
			MADS36		
			MADS44		
Papaya	*Carica papaya* L.	Fruit	*PDS*	Photobleaching, albinism	Reporter
Pear	*Pyrus bretschneideri*	Fruit	*ALS*	Herbicide resistance	Stress response
			PAT14	Dwarf yellowing phenotype	Development
	Pyrus communis L.		*TFL1*	Early flowering	Flowering
Petunia	*Petunia hybrida*	Flower	*PDS*	Albino phenotype	Reporter
			NR	Flower longevity	Flowering
			ACO1	Absence of corolla tube venation	Metabolism
			AN4	Self-incompatibility	Development
	Petunia inflata		*SSK1*	Self-incompatibility	Development
Plantain (Wild banana)	*Musa balbisiana*	Fruit	*eBSV*	Control of virus pathogenesis	Stress response
Poinsettia	*Euphorbia pulcherrima*	Flower	*F3′H*	Change of the bract color from red to reddish orange	Agronomic characteristics
Pomegranate	*Punica granatum* L.	Fruit	*UGT84A23*	Change of phenolic metabolites	Metabolism
			PgUGT84A24		
Potato	*Solanum tuberosum*	Vegetable	*16DOX*	Steroidal glycoalkaloids metabolism	Metabolism
			GBSS genes	Starch biosynthesis	
			S-RNase	Self-incompatibility	Development
			Coilin gene	Enhanced resistance to biotic and abiotic components	Stress response
			ALS1	Enhanced herbicide resistance	
			ALS2	Enhanced herbicide resistance	
			GBSS1	Starch biosynthesis	Metabolism

Potato	*Solanum tuberosum*	Vegetable	*Coilin gene*	Enhanced resistance to biotic and abiotic agents	Stress response
			MYB44	Phosphorus homeostasis	
			GBSS	Starch metabolism and tuber quality	Metabolism
			IAA2	Aux/IAA protein, shoot morphogenesis	Development
Rapeseed	*Brassica napus*	Vegetable	*LMI1*	Leaf lobe development	Development
			FAD2	Fatty acid metabolism	Metabolism
			WRKY11	Enhanced biotic resistance	Stress response
			WRKY70		
			SDG8	Histone lysine methyltransferase	Development
			CLV1	Regulate multilocular seeds	
			CLV2		
			CLV3		
			RGA	Multiple genes involved in plant development	
			DA1		
			DA2		
			FUL		
			ALC	Valve margin development, seed shattering	
Red raspberry	*Rubus idaeus* L.	Fruit	*F3′H*	No mutant phenotype	Metabolism
Red sage	*Salvia miltiorrhiza*	Medicinal plant	*CPS1*	Tanshinone biosynthesis	
Southern asia banana	*Musa acuminata*	Fruit	*PDS*	Albino phenotype	Reporter
Strawberry	*Fragaria × ananassa*	Fruit	*TM6*	Anther development	Development
Sweet orange	*Citrus sinensis*	Fruit	*PDS*	Albino phenotype	Reporter
			DMR6	Huanglongbin resistance	Stress response
			LOB1	Canker resistance	
			CsWRKY22	Delayed citrus canker symptoms	

(continued)

Table 14.1 (continued)

Crop species	Scientific name	Plant type	Gene name	Trait modification	Classification
Tomato	*Solanum lycopersicum*	Vegetable	*ACS4*	Ethylene response and fruit development	Development
			AGL6	Parthenocarpic	
			AP2a	Fruit development and ripening	
			BOP	Early flowering with simplified inflorescence architecture	Flowering
Tomato	*Solanum lycopersicum*	Vegetable	*CCD8*	Resistance against *Phelipanche aegytiaca*	Resistance to biotic stress
			FUL1/TDR4	Fruit development and ripening	Development
			GAD	γ-aminobutyric acid metabolism	Metabolism
			LCY-E	Lycopene content	Agronomic characteristics
			MAX1	Resistance against *Phelipanche aegytiaca*	Resistance to biotic stress
			NOR	Fruit development and ripening	Development
			PG	Chilling tolerance	Stress response
			PG2a	Pectin degradation control	Development
			PL	Cell wall gene, altered fruit color and firmness	
				Cell wall gene	
				Pectin degradation control	
			TBG4	Cell wall gene, altered fruit color and firmness	
			ACS2	Ethylene response and fruit development	
			AGL6	Production of parthenocarpic fruit under high temperature	
			AGO7	Leaf morphology	
			ALC	Long shelf-life	Metabolism
			ALS1	Enhanced herbicide resistance	Stress response
			ALS2	Herbicide resistance	
			ANT1	Anthocyanin biosynthesis	Metabolism
			ANT2	Purple-coloured tomato	Agronomic characteristics
			ARF2B	Ethylene response and fruit development	Development
			ARF7	Parthenocarpic	Development
			Blc	Increased lycopene content	Metabolism

Tomato	*Solanum lycopersicum*	Vegetable	*ARF7*	Parthenocarpic	Development
			Blc	Increased lycopene content	Metabolism
			BOP	Inflorescence structure	Development
			BZR1	Decrease in heat stress tolerance	Stress response
			Carotenoid isomerase	Carotenoid metabolism	Metabolism
			CAT9	γ-aminobutyric acid metabolism	
			CBF1	Decrease in chilling stress tolerance	Stress response
			CLV-WUS	Altered locule number	Development
			DMR6	Resistance against downy mildew	Stress response
			Mlo1		
			CLV3	Introduction of desirable traits with morphology, flower number, fruit size and number, and ascorbic acid synthesis	Flowering
			CrtR-b2	Carotenoid metabolism	Metabolism
			DDM1a	Decrease in DNA methylation	Development
			DDM1b		
			DELLA	Hormone response	
			DML2	Activation and inhibition of fruit ripening	Agronomic characteristics
			EIN2	Ethylene response and fruit development	Development
			ERFE1		
			ETR	Hormone response	
			FUL2/MBP7	Fruit development and ripening	
			GABA-TP1	γ-aminobutyric acid metabolism	Metabolism
				Increase in γ-aminobutyric acid (GABA) content	

(continued)

Table 14.1 (continued)

Crop species	Scientific name	Plant type	Gene name	Trait modification	Classification
Tomato	*Solanum lycopersicum*	Vegetable	*GABA-TP2*	γ-aminobutyric acid metabolism	Metabolism
			GABA-TP3	Increase in γ-aminobutyric acid (GABA) content	
			GAD2		
			GAD3		
			GAI	Gibberellin response and dwarfism	Development
Tomato	*Solanum lycopersicum*	Vegetable	*GGP1*	Plant architecture, day-length insensitivity, enlarged fruit size and vitamin C	Development, metabolism
			GRAS8	Ethylene response and fruit development	Development
			IAA9	IAA9	
				Parthenocarpic	
			JAZ2	Resistance to bacterial speck	Stress response
			L1L4	Involved in fruit metabolism during ripening	Metabolism
			LCY-B1	Lycopene content	
			LCY-B2		
			LCY-E	Increased lycopene content	
			lncRNA1459	Repressed fruit ripening, lycopene, ethylene and carotenoid biosynthesis	
			MAPK3	Resistance to *Botrytis cinerea*	Stress response
			MBP21	Jointless fruit stem	Agronomic characteristics
			MPK20	Repression of genes controlling sugar and auxin metabolism	Metabolism
			MYB12	Pink-colored tomato	Agronomic characteristics
			NADK2A	NAD Kinase 2A	Development
			NPR1	Reduced drought tolerance	Stress response
			NPTII	N.A.	Others

Tomato	*Solanum lycopersicum*	Vegetable	*PDS*	Albino phenotype	Reporter
				γ-aminobutyric acid metabolism	Metabolism
			PG2a	Cell wall gene, altered fruit color and firmness	Development
			PIF	Albino phenotype	Reporter
			PSY	Fruit color	Development
			Psy1	Carotenoid metabolism	Metabolism
				Yellow-coloured tomato	Agronomic characteristics
				Carotenoid metabolism	Metabolism
			Replicase from TYLCV	Obtained resistance to tomato yellow leaf curl virus	Stress response
			RIN	Ethylene production and fruit ripening	Development
				Fruit ripening	
				MADS-box transcription factor regulating fruit ripening	Agronomic characteristics
			SBPase	Leaf senescence (SBPase in primary metabolism)	Metabolism
			SGR1	Lycopene content	
Tomato	*Solanum lycopersicum*	Vegetable	*RIN*	Ethylene production and fruit ripening	Development
				Fruit ripening	
				MADS-box transcription factor regulating fruit ripening	Agronomic characteristics
			SBPase	Leaf senescence (SBPase in primary metabolism)	Metabolism
			SGR1	Lycopene content	
			SlAGO7	Wiry phenotype	Agronomic characteristics
			CLV3 promoter	Fruit size, inflorescence branching, and plant architecture	
			MAPK20	Aborted pollen development	Development
			MAPK3	Drought stress	Stress response
				Decrease in drought stress tolerance	

(continued)

Table 14.1 (continued)

Crop species	Scientific name	Plant type	Gene name	Trait modification	Classification
Tomato	*Solanum lycopersicum*	Vegetable	*ORRM4*	RNA editing and fruit ripening	Development
			WUS CarG element	Fruit size, inflorescence branching, and plant architecture	Agronomic characteristics
			SlyCAT9	Increase in γ-aminobutyric acid (GABA) content	Metabolism
			Solyc08g075770	*Fusarium* wilt susceptibility	Stress response
			Solyc12g038510	Jointless mutant, abscission	Development
				Jointless and branching	
			SP	Flower number, fruit size and number, and ascorbic acid synthesis	Agronomic characteristics
			SP5G	Flower number, fruit size and number, and ascorbic acid synthesis	Flowering
				Day-length-sensitive flowering	
			SSADH	Increase in γ-aminobutyric acid (GABA) content	Metabolism
			TBG4	Pectin degradation control	
			TypeII GRX 14	Redox regulation	
			TypeII GRX 15		
			TypeII GRX 17		
			TypeII GRX16		
Tomato	*Solanum pimpinellifolium*	Vegetable	*CyCb*	Plant and inflorescence architecture, fruit shape and lycopene biosynthesis	Development, metabolism
			FAS		
			FW2.2		
			OVUTE		
			SP		
			SP5	Plant architecture, day-length insensitivity, enlarged fruit size and vitamin C	
			WUS	Flower number, fruit size and number, and ascorbic acid synthesis	Metabolism

Walnut	*Juglans regia*	Fruit	*PDS*	Photobleaching, albinism	Reporter
			WOX11	Reduced adventitious root formation and vegetative growth	Agronomic characteristics
Watermelon	*Citrullus lanatus*	Fruit	*PDS*	Photobleaching, albinism	Reporter
			ALS	Herbicide resistance	Stress response
			PSK1	Resistance to *Fusarium*	
Watermelon	*Citrullus lanatus*	Fruit	*BG1*	Decreased seed size and promoted seed germination	Agronomic characteristics
			COMT1	Decreased melatonin content	Metabolism
			ALS	Increased herbicide resistance	Stress response
			PDS	Albino phenotype	Reporter
Wild cabbage	*Brassica oleracea*	Vegetable	*C.GA4.a*	GA response and dwarfism	Development
			PDS	Albino phenotype	Development
			MS1	Male sterility	
			SRK3	Self-incompatibility	
Wild carrot	*Daucus carota*	Vegetable	*PDS*	Albino phenotype	Reporter
			MYB113-like	Albino phenotype	
			F3′H	Altered anthocyanin biosynthesis	Metabolism
Wild strawberry	*Fragaria vesca*	Fruit	*PDS*	Photobleaching, albinism	Reporter
			TAA1	Auxin signaling	Development
			ARF8	Plant development	
			UF3GT,	Changes in anthocyanin synthesis	
			LDOX	Proanthocyanin biosynthesis	
			3-Sep	Alteration in flowers, abnormal berries	Reporter
			RAP	White berries	
Wishbone flower	*Torenia fournieri*	Flower	*RAD1*	Abnormal shape and color of flowers	Agronomic characteristics
			RAD2	Pale blue flowers	
			F3′H		

because it is challenging to edit genomes of the plants without any genome sequence information. Thus, more whole-genome sequencing data should be generated. In addition, for the improvement of polyploid plant species, such as tetraploid roses and hexaploid chrysanthemum plants, a very effective editing platform has yet to be developed. Eleven research papers concerning genetic manipulation through CRISPR/Cas in ornamental plants have been collected from previously published research articles (Table 14.1). Specialized application of CRISPR/Cas such as base-editing has been reported for tomato [20]; however, any research generating mutations in horticultural plants other than tomato have not been reported using either the base-editing or prime-editing approaches yet.

4 Making Horticultural Plants More Tolerant to Abiotic and Biotic Stresses

Pathogens including bacteria, fungi, and viruses can cause a variety of diseases in plants. This hinders plant growth and development, which can result in significant losses and hence increase agricultural production costs. Nevertheless, plant tolerance to biotic stresses can be significantly improved through the utilization of the CRISPR/Cas technology.

Two separate strategies are utilized to develop virus-resistant plants: editing the virus genome and modifying the plant genes responsible for susceptibility to viruses. Viruses typically employ the transcription and translation machinery found in the host plant. Using CRISPR/Cas tools, sensitivity (S) genes may have their expression altered to protect plants from viruses, for example by knocking off translation initiation factors. For example, producing bananas with endogenous banana streak virus resistance was made possible by the CRISPR/Cas9 technique [25] through introucing mutation in the integrative viral components that inhibited the transcription and translation of viral proteins in banana trees. An example of modifying plant genes to introduce more resistance is apple for which CRISPR/Cas9 RNPs were introduced into protoplasts to target the *DIPM-1, -2,* and *-4* genes which are fire blight resistance inhibitors [26]. The benefit of this transient expression was revealed through a decrease in undesirable mutations. The *MdDIPM-4* gene was also knocked out in apple plants by the other researchers. Surprisingly, the FLP/FRT recombination system was used to eliminate the T-DNA harboring the expression cassettes for CRISPR/Cas9 from the genome of the transformed plants [27]. Also Citrus (*Citrus sinensis* orange and *C*. *paradisi* grapefruit) mutants were created by genome editing showing significant tolerance to *Xanthomonas* causing citrus canker [28, 29]. Citrus plants have the *CsLOB1* gene, which makes them susceptible to *Xanthomonas citri* subsp. *citri* [30]. This gene's promoter region contains elements necessary for the pathogenicity factor *PthA4* of the bacterium to interact, which promotes the emergence of disease symptoms [31]. The PthA4 factor's binding sites were edited using CRISPR/Cas9, which reduced the bacteria's capacity to infect *Citrus sinensis* [29].

The Wanjincheng orange variety's *CsLOB1* gene's promoter region was altered using some vector constructs. The rate of identified mutations varies between 11.5% and 64.7% according to the structure. As a result, four canker-resistant citrus mutant lines were selected. Significant plant resistance was obtained when a complete deletion happened in the promoter region of *CsLOB1* where the *PthA4* effector binds. Both Cas9 and Cpf1 have been used in similar investigations [32]. The CRISPR/Cas9-mediated editing of the *CsWRKY22* gene, which codes for a different transcription factor, was another strategy to improve the bacterial canker resistance in Wanjincheng orange [33]. Genome editing also helped in the development of mutant bananas containing the *DMR6* gene that are resistant to the *Xanthomonas* bacteria-caused banana wilt [34]. Many plant diseases are brought on by fungal infections. The introduction of CRISPR/Cas9 technology has brought novel promises for breeding crops resistant to a variety of fungal diseases by altering pathogen-sensitivity genes. It is well known that plants' sensitivity genes make it easier for pathogens to invade and infect them. For example, CRISPR/Cas9 has made possible to knock out the *MLO-7* gene, which negatively regulates resistance to powdery mildew pathogen, *Erysiphe necator,* in grapevine [26]. RNPs were used to deliver sgRNA to plants, and the mutagenesis frequency was reported quite low (0.1–6.9%). The RNP-mediated editing approach has been developed in recent studies [35]. Three *MLO* gene mutations caused grapevine plants to be 77% less sensitive to powdery mildew [36]. Moreover, by the deletion of the WRKY52 gene, a jasmonic acid pathway's negative regulator, highly resistant grapevine plants to *Botrytis cinerea* have been produced [37, 38]. A noteworthy number of sgRNAs targeting various sequences within the first exon of the *WRKY52* gene, were generated, and it was discovered that mutations in two alleles of the gene were more protective against the pathogen than those in only one allele. It is helpful to employ genome editing to explain how certain genes contribute to bring resistance to a disease. For instance, through knocking out the *pathogenesis-related protein 4b* gene (*VvPR4b*), grapevines became less resistant to *Plasmopara viticola* [39]. The *VvPR4b* gene produces the chitinase II-like protein which is required to stop hyphae development in *P. viticola*.

The fungal pathogen *Botryosphaeria dothidea* infects apple plants, causing serious damage. Increased response to this pathogen has been observed in apple calli after *CNGC2* gene knockout [40]. Salicylic acid levels were found to be increasing at the same time as the expression of the gene encoding PR protein was still being inhibited. The gene *CNGC2* was selected for targeted mutagenesis, but this was not the right alternative because mutations in this gene can have negative influences, like significantly reduced fertility. In another research, transient expression of the CRISPR/Cas9 in *Theobroma cacao*, the cacao plant, resulted in more resistant embryos and leaves against *Phythophtora tropicalis* [41]. The *TcNPR3* gene was selected as the candidate for modification because it is an inhibitor of the defense mechanism. These findings support the outlook of cacao breeding for resistance to *P. tropicalis*.

It has been indicated that a *Clpsk1* gene mutation makes watermelon plants more resistant to the fungus *Fusarium oxysporum f. sp. niveum* [42]. With CRISPR/Cas9 technology, the alteration of the genes controlling the pathogen-sensitivity in plants seems to be an efficient and fast method to make resistant plants against viral, bacterial and fungal diseases.

A few studies report that horticultural plants had been improved for abiotic stress tolerance using genome editing. As an example, the watermelon acetolactate synthase (*ClALS*) gene knocked out by CRISPR/Cas9, promoted the development of watermelons with herbicide tolerance [43]. The *ALS* gene was employed as an indicator for CRISPR/Cas9-mediated base editing, resulting in the development of herbicide (chlorosulfuron) tolerant pear trees (*Pyrus communis* L.) [44]. Similar *CsALS* gene editing in orange (*Carrizo citrange*) led to the mutant varieties that were tolerant to the herbicide imazapyr [45].

Therefore, editing the genomes of horticultural plants by CRISPR/Cas9 can be advantageous for developing crops resistant to a variety of (a)biotic stresses. Nevertheless, it seems essential to obtain stable mutants and completely study how genome editing influences traits and metabolic processes in plants.

5 Editing of Phenotypic Characteristics of Horticultural Plants

There are reports that genome editing can alter a variety of plant characteristics, including plant growth and morphology, fruit maturation time, fruit color, metabolism, and shelf life. The semi-dwarf banana plant (*Musa acuminat*a "Gros Michel") was produced through CRISPR/Cas9-mediated editing of the *MaGA20ox2* genes regulating gibberellin biosynthesis [46]. The mutant plants displayed less growth than the wild-type plants, however thicker and deep-green leaves. The modified plants' cells were structurally different compared to the wild-type plants. The findings of these works are crucial for the screening of dwarf banana genotypes because tall trees are susceptible to strong winds, which causes significant harvest losses. In grapevine plants, the targeted mutagenesis of the *VvCCD8* gene resulted in more branches per shoot than in wild-type plants [47]. Plant phytohormones known as strigolactones prevent the development of axillary buds. The CRISPR/Cas9 editing system helped to functionally characterize the *VvCCD8* gene's role in the regulation of shoot branching. It is then planned to look into additional mechanisms for controlling the morphology and development of shoots in grapevines. In strawberry, it appeared feasible to produce white colored fruits using CRISPR/Cas9. The *RAP* (reduced anthocyanins in petioles) gene, which encodes a glutathione S-transferase enzyme, was targeted using CRISPR/Cas9 gene editing system [48]. This enzyme binds anthocyanins allowing them to move from the cytosol to the vacuole. It may be possible to produce strawberry types with popular white berries by editing the *RAP* gene.

CRISPR/Cas9-mediated genome editing is also considered as an efficient method for enhancing the nutritional value of fruits by editing their genomes. By mutating the lycopene epsilon-cyclase (*LCYε*) locus, it was possible to produce bananas with higher levels of β-carotene [49]. The amount of β-carotene rose six-fold in the fruit pulp of the mutant lines, whereas the α-carotene and lutein levels considerably diminished. Limited efforts have been made to apply CRISPR/Cas9 to create plants with improved phenotypes in red raspberry (*Rubus idaeus* L.). The flavone 3-hydrolase (F3′H), one of the important enzymes in flavonoid biosynthetic pathway, was knocked out in an investigation [50]. The gene *MYB-16*-like, functioning as a prickle formation regulator in raspberries, was another candidate for editing [51]. However, in both examples, it proved challenging for researchers to develop new seedlings from the generated raspberry calli.

Watermelon (*Citrullus lanatus*) seeds with mutations in the *ClBG1* (*β-glucosidase*) gene had smaller seeds but better emerging due to decreased abscisic acid levels [52]. This gene can influence seed size and germination, which is a crucial characteristic of watermelon breeding. In another research, the role of the genes involved in fruit ripening such as 1-aminocyclopropane-1-carboxylate oxidase 1 (*MaACO1*) was evaluated and its sequences altered using CRISPR/Cas9 to find a way to extend the shelf life of bananas [53]. The obtained mutants developed smaller fruits with a slower maturity (60 days in comparison with 21 days for control bananas), which improved the storage capacity. The modified banana fruits additionally contained a higher vitamin C content. The *CTR1-like*, *ROS1*, and *CmNAC-NOR* genes in the melon (*Cucumis melo* var. *cantalupensis*) were knocked out employing CRISPR/Cas9, resulting in the development of melons with prolonged maturation and a long shelf life [54–56]. Thus, the reviewed studies demonstrated the sustainability of utilizing CRISPR/Cas9 to modify numerous characteristics in horticultural plants, for instance to improve fruit color and flavor, alter ripening and storage times, and alter growth-related traits.

6 Modification of the Flowering Period and Lifetime

Some studies indicate that horticultural plants can have their flowering time, floral life span, and flower shape and color altered by employing the CRISPR/Cas9. The function of numerous genes in the formation of flowers and fruits has been successfully identified in both wild and cultivated strawberry plants using the CRISPR/Cas9 technology. Since auxins are essential to the development of strawberries, *FveARF8* and *FveTAA1* genes were modified resulting in homozygous *FveARF8* mutant plants with larger size and faster growth compared to the control plants [57]. Furthermore, mutations in *FaTM6* and *FveSEP3* strawberry genes resulted in parthenocarpy and an inaccurate fruit phenotype, as well as abnormal petals, anthers, and pollen grains [58, 59]. Thus, it has been demonstrated how these genes contribute to the growth of strawberry flowers and berries.

The flowering mechanisms of fruit plants can be manipulated with the use of genome editing. Growth traits have been manipulated in tomato to alter fruit development and flowering patterns. The transcriptional cofactors encoding genes such as *BLADE-ON-PETIOLE* (*BOP*) in tomato, can influence the inflorescences structure, and gene editing can be used to reduce the number of flowers per inflorescence by editing *SlBOP* genes [60]. Rapid flowering and early harvest are induced by CRISPR/Cas9-mediated targeted mutagenesis in the flowering inhibitor *SELF-PRUNING 5G* [61]. Editing the coding sequences of *SlDML2* [62], *SlORRM4* [63], the *RIN* locus [64], or the cis-regulatory domain of *SlCLV3* [65] also affects the fruit development and ripening. The characteristics of the wild tomato relative (*Solanum pimpinellifolium*) were significantly altered by multiplex targeting of many genes crucial for tomato domestication, resulting in mutants that resembled domesticated tomatoes [66, 67]. Apple and pear mutant plants were obtained via knocking out of the *TFL1* flowering repressor gene [68]. Only a few pear mutant plants (9% of all) but many apple mutant tree lines (93%) showed early flowering. Genome editing was used to clarify the significance of the *AcCen4*, *AcCen*, and *SyGl* genes role in delaying flowering in kiwifruit plants (*Actinidia chinensis*) [69, 70]. So there is a chance to get horticultural plants that flower earlier, which would decrease the period it takes for them to yield fruit. Likewise, in blueberry (*Vaccinium corymbosum* L.) plants, gene editing was performed on the gene *CENTRORADIALIS* (*CEN*) [71]. Based on the evidence for *TFL1/CEN*-like genes in kiwifruit, pear, and apple, the researchers hypothesized that the knocking out of this gene would result in early flowering [68–70]. However, attempts to use *CEN* gene editing to influence blueberry plant flowering were ineffective. Moreover, compared to the control plants, mutant plants grew far more slowly. Additional examination are yet to be done to explain the dwarf phenotype resulted in these mutant seedlings of *CEN*-edited blueberry plants [71].

Genes that control aging and corollas color in petunias, gentian, lily, ipomoea, chrysanthemum, and orchids have been studied by some researchers using genome editing [40, 72–89]. For instance, plants of the petunia cultivar "Mirage Rose" have been modified targeting the *PhACO1* gene, which regulates the biosynthesis of the hormone ethylene [86]. As a result, petunia plants with less ethylene synthesis and flowers that lasted longer were developed. In Japanese morning glory (*Ipomoea nil*, "Violet") plants, the *EPH1* gene, a regulator of petal aging, was also knocked out to decrease flower wilting [76]. The problem of altering the color of flower corollas in ornamentals has been the center of many investigations. The genes *carotenoid cleavage dioxygenase 4* (*CCD4*) and *dihydroflavonol-4-reductase* (*DFR*) were knocked out in *Ipomoea nil* plants [84, 85], which allowed for the modification of flower color. One of the key enzymes responsible for the production of flavonoids, *flavone 3-hydrolase* (*F3′H*), was knocked out by another study [74]. As a result, *Torenia fournieri*'s flower color converted from bright blue to white. In another research, the essential enzyme of carotenoid synthesis, *PDS*, was mutated, resulting in the creation of mutant *Lilium pumilum* and *L. longiflorum* with chimeric morphologies and different flower colors [87]. CRISPR/Cas9 gene editing has only

been used in a few orchid species [73, 83]. Hence, it has been demonstrated that it is possible to modify horticultural plants' flowering time, flower color, and flowering period using the CRISPR/Cas9 technology.

7 Conclusion

CRISPR/Cas9 technology has so far demonstrated its efficacy to modify horticultural plant genomes. The genomes of these plants have been edited to regulate the period of fruit ripening and flowering, to increase plant resistance to biotic and tolerance to abiotic stresses, and to enhance the characteristics related to plant growth and fruit flavor. Better editing and transcription-activation techniques as well as improved enzymes for more effective genome editing are now available for the development of novel types of horticultural plants.

References

1. Ravichandra, N., Ravichandra, N.: Horticulture and its role in the national economies. Hortic. Nematol. 1–3 (2014)
2. Mishra, R., Joshi, R.K., Zhao, K.: Genome editing in rice: recent advances, challenges, and future implications. Front. Plant Sci. **9**, 1361 (2018)
3. Voytas, D.F.: Plant genome engineering with sequence-specific nucleases. Annu. Rev. Plant Biol. **64**, 327–350 (2013)
4. Peer, R., Rivlin, G., Golobovitch, S., Lapidot, M., Gal-On, A., Vainstein, A., Tzfira, T., Flaishman, M.A.: Targeted mutagenesis using zinc-finger nucleases in perennial fruit trees. Planta. **241**, 941–951 (2015)
5. Hilioti, Z., Ganopoulos, I., Ajith, S., Bossis, I., Tsaftaris, A.: A novel arrangement of zinc finger nuclease system for in vivo targeted genome engineering: the tomato LEC1-LIKE4 gene case. Plant Cell Rep. **35**, 2241–2255 (2016)
6. Sun, Z., Li, N., Huang, G., Xu, J., Pan, Y., Wang, Z., Tang, Q., Song, M., Wang, X.: Site-specific gene targeting using transcription activator-like effector (TALE)-based nuclease in Brassica oleracea. J. Integr. Plant Biol. **55**, 1092–1103 (2013)
7. Sawai, S., Ohyama, K., Yasumoto, S., Seki, H., Sakuma, T., Yamamoto, T., Takebayashi, Y., Kojima, M., Sakakibara, H., Aoki, T., Muranaka, T., Saito, K., Umemoto, N.: Sterol side chain reductase 2 is a key enzyme in the biosynthesis of cholesterol, the common precursor of toxic steroidal glycoalkaloids in potato. Plant Cell. **26**, 3763–3774 (2014)
8. Ma, J., Xiang, H., Donnelly, D.J., Meng, F.-R., Xu, H., Durnford, D., Li, X.-Q.: Genome editing in potato plants by agrobacterium-mediated transient expression of transcription activator-like effector nucleases. Plant Biotechnol. Rep. **11**, 249–258 (2017)
9. Forsyth, A., Weeks, T., Richael, C., Duan, H.: Transcription activator-like effector nucleases (TALEN)-mediated targeted DNA insertion in potato plants. Front. Plant Sci. **7**, 1572 (2016)
10. Butler, N.M., Baltes, N.J., Voytas, D.F., Douches, D.S.: Geminivirus-mediated genome editing in potato (Solanum tuberosum L.) using sequence-specific nucleases. Front. Plant Sci. **7**, 1045 (2016)
11. Nicolia, A., Proux-Wéra, E., Åhman, I., Onkokesung, N., Andersson, M., Andreasson, E., Zhu, L.-H.: Targeted gene mutation in Tetraploid potato through transient TALEN expression in protoplasts. J. Biotechnol. **204**, 17–24 (2015)

12. Clasen, B.M., Stoddard, T.J., Luo, S., Demorest, Z.L., Li, J., Cedrone, F., Tibebu, R., Davison, S., Ray, E.E., Daulhac, A.: Improving cold storage and processing traits in potato through targeted gene knockout. Plant Biotechnol. J. **14**, 169–176 (2016)
13. Lor, V.S., Starker, C.G., Voytas, D.F., Weiss, D., Olszewski, N.E.: Targeted mutagenesis of the tomato Procera gene using transcription activator-like effector nucleases. Plant Physiol. **166**, 1288–1291 (2014)
14. Čermák, T., Baltes, N.J., Čegan, R., Zhang, Y., Voytas, D.F.: High-frequency, precise modification of the tomato genome. Genome Biol. **16**, 1–15 (2015)
15. Malzahn, A., Lowder, L., Qi, Y.: Plant genome editing with TALEN and CRISPR. Cell Biosci. **7**, 1–18 (2017)
16. Ron, M., Kajala, K., Pauluzzi, G., Wang, D., Reynoso, M.A., Zumstein, K., Garcha, J., Winte, S., Masson, H., Inagaki, S.: Hairy root transformation using agrobacterium rhizogenes as a tool for exploring cell type-specific gene expression and function using tomato as a model. Plant Physiol. **166**, 455–469 (2014)
17. Jia, H., Wang, N.: Targeted genome editing of sweet orange using Cas9/SGRNA. PLoS One. **9**, e93806 (2014)
18. Brooks, C., Nekrasov, V., Lippman, Z.B., Van Eck, J.: Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol. **166**, 1292–1297 (2014)
19. Kulus, D.: Genetic resources and selected conservation methods of tomato. J. Appl. Bot. Food Qual. **91**, 135–144 (2018)
20. Chen, L., Yang, D., Zhang, Y., Wu, L., Zhang, Y., Ye, L., Pan, C., He, Y., Huang, L., Ruan, Y.-L., Lu, G.: Evidence for a specific and critical role of mitogen-activated protein kinase 20 in uni-to-binucleate transition of microgametogenesis in tomato. New Phytol. **219**, 176–194 (2018)
21. Wan, L., Wang, Z., Tang, M., Hong, D., Sun, Y., Ren, J., Zhang, N., Zeng, H.: CRISPR-Cas9 gene editing for fruit and vegetable crops: strategies and prospects. Horticulturae. **7**, 193 (2021)
22. Rukavtsova, E.B., Zakharchenko, N.S., Lebedev, V.G., Shestibratov, K.A.: CRISPR-Cas genome editing for horticultural crops improvement: advantages and prospects. Horticulturae. **9**, 38 (2023)
23. Xiong, J.-S., Ding, J., Li, Y.: Genome-editing technologies and their potential application in horticultural crop breeding. Hortic. Res. **2**, 15019 (2015)
24. Xu, J., Hua, K., Lang, Z.: Genome editing for horticultural crop improvement. Hortic. Res. **6** (2019)
25. Tripathi, J.N., Ntui, V.O., Ron, M., Muiruri, S.K., Britt, A., Tripathi, L.: CRISPR/Cas9 editing of endogenous banana streak virus in the B genome of Musa spp. overcomes a major challenge in banana breeding. Commun. Biol. **2**, 46 (2019)
26. Malnoy, M., Viola, R., Jung, M.-H., Koo, O.-J., Kim, S., Kim, J.-S., Velasco, R., Nagamangala Kanchiswamy, C.: DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci. **7**, 1904 (2016)
27. Pompili, V., Dalla Costa, L., Piazza, S., Pindo, M., Malnoy, M.: Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system. Plant Biotechnol. J. **18**, 845–858 (2020)
28. Jia, H., Omar, A.A., Orbović, V., Wang, N.: Biallelic editing of the LOB1 promoter via CRISPR/Cas9 creates canker-resistant 'Duncan' grapefruit. Phytopathology. **112**, 308–314 (2022)
29. Peng, A., Chen, S., Lei, T., Xu, L., He, Y., Wu, L., Yao, L., Zou, X.: Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene CsLOB1 promoter in citrus. Plant Biotechnol. J. **15**, 1509–1519 (2017)
30. Gottwald, T.R., Graham, J.H., Schubert, T.S.: Citrus canker: the pathogen and its impact. Plant Health Prog. **3**, 15 (2002)
31. Hu, Y., Zhang, J., Jia, H., Sosso, D., Li, T., Frommer, W.B., Yang, B., White, F.F., Wang, N., Jones, J.B.: Lateral organ boundaries 1 is a disease susceptibility gene for citrus bacterial canker disease. Proc. Natl. Acad. Sci. **111**, E521–E529 (2014)

32. Jia, H., Orbović, V., Wang, N.: CRISPR-LbCas12a-mediated modification of citrus. Plant Biotechnol. J. **17**, 1928–1937 (2019)
33. Wang, L., Chen, S., Peng, A., Xie, Z., He, Y., Zou, X.: CRISPR/Cas9-mediated editing of CsWRKY22 reduces susceptibility to Xanthomonas citri subsp. Citri in Wanjincheng orange (Citrus sinensis (L.) Osbeck). Plant Biotechnol. Rep. **13**, 501–510 (2019)
34. Tripathi, J.N., Ntui, V.O., Shah, T., Tripathi, L.: CRISPR/Cas9-mediated editing of DMR6 orthologue in banana (Musa spp.) confers enhanced resistance to bacterial disease. Plant Biotechnol. J. **19**, 1291 (2021)
35. Osakabe, Y., Liang, Z., Ren, C., Nishitani, C., Osakabe, K., Wada, M., Komori, S., Malnoy, M., Velasco, R., Poli, M.: CRISPR–Cas9-mediated genome editing in apple and grapevine. Nat. Protoc. **13**, 2844–2863 (2018)
36. Pessina, S., Lenzi, L., Perazzolli, M., Campa, M., Dalla Costa, L., Urso, S., Valè, G., Salamini, F., Velasco, R., Malnoy, M.: Knockdown of MLO genes reduces susceptibility to powdery mildew in grapevine. Hortic. Res. **3** (2016)
37. Leisen, T., Bietz, F., Werner, J., Wegner, A., Schaffrath, U., Scheuring, D., Willmund, F., Mosbach, A., Scalliet, G., Hahn, M.: CRISPR/Cas with ribonucleoprotein complexes and transiently selected telomere vectors allows highly efficient marker-free and multiple genome editing in Botrytis cinerea. PLoS Pathog. **16**, e1008326 (2020)
38. Wang, X., Tu, M., Wang, D., Liu, J., Li, Y., Li, Z., Wang, Y., Wang, X.: CRISPR/cas9-mediated efficient targeted mutagenesis in Grape in the first generation. Plant Biotechnol. J. **16**, 844–855 (2018)
39. Wan, D.-Y., Guo, Y., Cheng, Y., Hu, Y., Xiao, S., Wang, Y., Wen, Y.-Q.: CRISPR/cas9-mediated mutagenesis of VvMLO3 results in enhanced resistance to powdery mildew in grapevine (Vitis vinifera). Hortic. Res. **7** (2020)
40. Zhou, H., Bai, S., Wang, N., Sun, X., Zhang, Y., Zhu, J., Dong, C.: CRISPR/Cas9-mediated mutagenesis of MdCNGC2 in Apple Callus and VIGS-mediated silencing of MdCNGC2 in fruits improve resistance to Botryosphaeria dothidea. Front. Plant Sci. **11**, 575477 (2020)
41. Fister, A.S., Landherr, L., Maximova, S.N., Guiltinan, M.J.: Transient expression of CRISPR/Cas9 machinery targeting TcNPR3 enhances defense response in Theobroma cacao. Front. Plant Sci. **9**, 268 (2018)
42. Zhang, M., Liu, Q., Yang, X., Xu, J., Liu, G., Yao, X., Ren, R., Xu, J., Lou, L.: CRISPR/Cas9-mediated mutagenesis of clpsk1 in watermelon to confer resistance to Fusarium oxysporum f. sp. niveum. Plant Cell Rep. **39**, 589–595 (2020)
43. Tian, S., Jiang, L., Cui, X., Zhang, J., Guo, S., Li, M., Zhang, H., Ren, Y., Gong, G., Zong, M.: Engineering herbicide-resistant watermelon variety through CRISPR/cas9-mediated base-editing. Plant Cell Rep. **37**, 1353–1356 (2018)
44. Malabarba, J., Chevreau, E., Dousset, N., Veillet, F., Moizan, J., Vergne, E.: New strategies to overcome present CRISPR/Cas9 limitations in apple and pear: efficient dechimerization and base editing. Int. J. Mol. Sci. **22**, 319 (2020)
45. Alquézar, B., Bennici, S., Carmona, L., Gentile, A., Peña, L.: Generation of transfer-DNA-free base-edited citrus plants. Front. Plant Sci. **13** (2022)
46. Shao, X., Wu, S., Dou, T., Zhu, H., Hu, C., Huo, H., He, W., Deng, G., Sheng, O., Bi, F.: Using CRISPR/Cas9 genome editing system to create MaGA20ox2 gene-modified semi-dwarf Banana. Plant Biotechnol. J. **18**, 17 (2020)
47. Ren, C., Guo, Y., Kong, J., Lecourieux, F., Dai, Z., Li, S., Liang, Z.: Knockout of VvCCD8 gene in grapevine affects shoot branching. BMC Plant Biol. **20**, 1–8 (2020)
48. Gao, Q., Luo, H., Li, Y., Liu, Z., Kang, C.: Genetic modulation of RAP alters fruit coloration in both wild and cultivated strawberry. Plant Biotechnol. J. **18**, 1550–1561 (2020)
49. Kaur, N., Alok, A., Kumar, P., Kaur, N., Awasthi, P., Chaturvedi, S., Pandey, P., Pandey, A., Pandey, A.K., Tiwari, S.: CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for β-carotene biosynthesis in Banana fruit. Metab. Eng. **59**, 76–86 (2020)

50. Miller, S.: Gene Editing of Red Raspberry (Rubus Idaeus L.) with CRISPR/Cas9 Knocking out F3′H. Norwegian University of Life Sciences, Ås (2019)
51. Khadgi, A.: Uncovering Genetic and Transcriptomic Regulation of Prickle Development in Red Raspberry. Cornell University (2020)
52. Wang, Y., Wang, J., Guo, S., Tian, S., Zhang, J., Ren, Y., Li, M., Gong, G., Zhang, H., Xu, Y.: CRISPR/Cas9-mediated mutagenesis of ClBG1 decreased seed size and promoted seed germination in Watermelon. Hortic. Res. **8** (2021)
53. Hu, C., Sheng, O., Deng, G., He, W., Dong, T., Yang, Q., Dou, T., Li, C., Gao, H., Liu, S.: CRISPR/Cas9-mediated genome editing of MaACO1 (aminocyclopropane-1-carboxylate oxidase 1) promotes the shelf life of Banana fruit. Plant Biotechnol. J. **19**, 654 (2021)
54. Giordano, A., Santo Domingo, M., Quadrana, L., Pujol, M., Martín-Hernández, A.M., Garcia-Mas, J.: CRISPR/cas9 gene editing uncovers the roles of constitutive triple response 1 and repressor of silencing 1 in melon fruit ripening and epigenetic regulation. J. Exp. Bot. **73**, 4022–4033 (2022)
55. Hooghvorst, I., López-Cristoffanini, C., Nogués, S.: Efficient knockout of phytoene desaturase gene using CRISPR/Cas9 in melon. Sci. Rep. **9**, 1–7 (2019)
56. Liu, B., Santo Domingo, M., Mayobre, C., Martín-Hernández, A.M., Pujol, M., Garcia-Mas, J.: Knock-out of CmNAC-NOR affects melon climacteric fruit ripening. Front. Plant Sci. **13** (2022)
57. Zhou, J., Wang, G., Liu, Z.: Efficient genome editing of wild strawberry genes, vector development and validation. Plant Biotechnol. J. **16**, 1868–1877 (2018)
58. Martín-Pizarro, C., Triviño, J.C., Posé, D.: Functional analysis of the TM6 MADS-box gene in the octoploid strawberry by CRISPR/Cas9-directed mutagenesis. J. Exp. Bot. **70**, 885–895 (2019)
59. Pi, M., Hu, S., Cheng, L., Zhong, R., Cai, Z., Liu, Z., Yao, J.-L., Kang, C.: The MADS-box gene FveSEP3 plays essential roles in flower organogenesis and fruit development in woodland strawberry. Hortic. Res. **8** (2021)
60. Xu, C., Park, S.J., Van Eck, J., Lippman, Z.B.: Control of inflorescence architecture in tomato by BTB/POZ transcriptional regulators. Genes Dev. **30**, 2048–2061 (2016)
61. Soyk, S., Müller, N.A., Park, S.J., Schmalenbach, I., Jiang, K., Hayama, R., Zhang, L., Van Eck, J., Jiménez-Gómez, J.M., Lippman, Z.B.: Variation in the flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in Tomato. Nat. Genet. **49**, 162–168 (2017)
62. Lang, Z., Wang, Y., Tang, K., Tang, D., Datsenka, T., Cheng, J., Zhang, Y., Handa, A.K., Zhu, J.-K.: Critical roles of DNA demethylation in the activation of ripening-induced genes and inhibition of ripening-repressed genes in Tomato fruit. Proc. Natl. Acad. Sci. **114**, E4511–E4519 (2017)
63. Yang, Y., Zhu, G., Li, R., Yan, S., Fu, D., Zhu, B., Tian, H., Luo, Y., Zhu, H.: The RNA editing factor SLORRM4 is required for normal fruit ripening in Tomato. Plant Physiol. **175**, 1690–1702 (2017)
64. Ito, Y., Nishizawa-Yokoi, A., Endo, M., Mikami, M., Shima, Y., Nakamura, N., Kotake-Nara, E., Kawasaki, S., Toki, S.: Re-evaluation of the rin mutation and the role of RIN in the induction of tomato ripening. Nat. Plant. **3**, 866–874 (2017)
65. Rodríguez-Leal, D., Lemmon, Z.H., Man, J., Bartlett, M.E., Lippman, Z.B.: Engineering quantitative trait variation for crop improvement by genome editing. Cell. **171**, 470–480. e478 (2017)
66. Li, T., Yang, X., Yu, Y., Si, X., Zhai, X., Zhang, H., Dong, W., Gao, C., Xu, C.: Domestication of wild tomato is accelerated by genome editing. Nat. Biotechnol. **36**, 1160–1163 (2018)
67. Zsögön, A., Čermák, T., Naves, E.R., Notini, M.M., Edel, K.H., Weinl, S., Freschi, L., Voytas, D.F., Kudla, J., Peres, L.E.P.: De novo domestication of wild tomato using genome editing. Nat. Biotechnol. **36**, 1211–1216 (2018)
68. Charrier, A., Vergne, E., Dousset, N., Richer, A., Petiteau, A., Chevreau, E.: Efficient targeted mutagenesis in apple and first time edition of pear using the CRISPR-cas9 system. Front. Plant Sci. **10**(Feb) (2019)

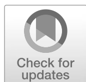
69. Varkonyi-Gasic, E., Wang, T., Cooney, J., Jeon, S., Voogd, C., Douglas, M.J., Pilkington, S.M., Akagi, T., Allan, A.C.: Shy girl, a kiwifruit suppressor of feminization, restricts gynoecium development via regulation of cytokinin metabolism and signalling. New Phytol. **230**(May), 1461–1475 (2021)
70. Varkonyi-Gasic, E., Wang, T., Voogd, C., Jeon, S., Drummond, R.S.M., Gleave, A.P., Allan, A.C.: Mutagenesis of kiwifruit CENTRORADIALIS-like genes transforms a climbing woody perennial with long juvenility and axillary flowering into a compact plant with rapid terminal flowering. Plant Biotechnol. J. **17**, 869–880 (2019)
71. Omori, M., Yamane, H., Osakabe, K., Osakabe, Y., Tao, R.: Targeted mutagenesis of CENTRORADIALIS using CRISPR/Cas9 system through the improvement of genetic transformation efficiency of tetraploid highbush blueberry. J. Hortic. Sci. Biotechnol. **96**, 153–161 (2021)
72. Kishi-Kaboshi, M., Aida, R., Sasaki, K.: Generation of gene-edited Chrysanthemum morifolium using multicopy transgenes as targets and markers. Plant Cell Physiol. **58**, 216–226 (2017)
73. Kui, L., Chen, H., Zhang, W., He, S., Xiong, Z., Zhang, Y., Yan, L., Zhong, C., He, F., Chen, J., Zeng, P., Zhang, G., Yang, S., Dong, Y., Wang, W., Cai, J.: Building a genetic manipulation tool box for Orchid biology: identification of constitutive promoters and application of CRISPR/Cas9 in the Orchid, Dendrobium officinale. Front. Plant Sci. **7**, 2036 (2016)
74. Nishihara, M., Higuchi, A., Watanabe, A., Tasaki, K.: Application of the CRISPR/Cas9 system for modification of flower color in Torenia fournieri. BMC Plant Biol. **18**, 331 (2018)
75. Nitarska, D., Boehm, R., Debener, T., Lucaciu, R.C., Halbwirth, H.: First genome edited poinsettias: targeted mutagenesis of flavonoid 3′-hydroxylase using CRISPR/Cas9 results in a colour shift. Plant Cell Tissue Organ Cult. **147**, 49–60 (2021)
76. Shibuya, K., Watanabe, K., Ono, M.: CRISPR/cas9-mediated mutagenesis of the EPHEMERAL1 locus that regulates petal senescence in Japanese morning glory. Plant Physiol. Biochem. **131**, 53–57 (2018)
77. Su, S., Xiao, W., Guo, W., Yao, X., Xiao, J., Ye, Z., Wang, N., Jiao, K., Lei, M., Peng, Q., Hu, X., Huang, X., Luo, D.: The CYCLOIDEA-RADIALIS module regulates petal shape and pigmentation, leading to bilateral corolla symmetry in Torenia fournieri (Linderniaceae). New Phytol. **215**, 1582–1593 (2017)
78. Subburaj, S., Chung, S.J., Lee, C., Ryu, S.-M., Kim, D.H., Kim, J.-S., Bae, S., Lee, G.-J.: Site-directed mutagenesis in petunia × hybrida protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. Plant Cell Rep. **35**, 1535–1544 (2016)
79. Sun, L., Kao, T.H.: CRISPR/cas9-mediated knockout of PiSSK1 reveals essential role of S-locus F-box protein-containing SCF complexes in recognition of non-self S-RNases during cross-compatible pollination in self-incompatible Petunia inflata. Plant Reprod. **31**, 129–143 (2018)
80. Takahashi, S., Yoshida, C., Takahashi, H., Nishihara, M.: Isolation and functional analysis of EPHEMERAL1-LIKE (EPH1L) genes involved in flower senescence in cultivated Japanese gentians. Int. J. Mol. Sci. **23**, 5608 (2022)
81. Tasaki, K., Higuchi, A., Watanabe, A., Sasaki, N., Nishihara, M.: Effects of knocking out three anthocyanin modification genes on the blue pigmentation of gentian flowers. Sci. Rep. **9**, 15831 (2019)
82. Tasaki, K., Yoshida, M., Nakajima, M., Higuchi, A., Watanabe, A., Nishihara, M.: Molecular characterization of an anthocyanin-related glutathione S-transferase gene in Japanese gentian with the CRISPR/Cas9 system. BMC Plant Biol. **20**, 370 (2020)
83. Tong, C.G., Wu, F.H., Yuan, Y.H., Chen, Y.R., Lin, C.S.: High-efficiency CRISPR/Cas-based editing of Phalaenopsis or chid mads genes. Plant Biotechnol. J. **18**, 889–891 (2020)
84. Watanabe, K., Kobayashi, A., Endo, M., Sage-Ono, K., Toki, S., Ono, M.: CRISPR/cas9-mediated mutagenesis of the dihydroflavonol-4-reductase-B (DFR-B) locus in the Japanese morning glory Ipomoea (Pharbitis) nil. Sci. Rep. **7**, 10028 (2017)
85. Watanabe, K., Oda-Yamamizo, C., Sage-Ono, K., Ohmiya, A., Ono, M.: Alteration of flower colour in ipomoea nil through CRISPR/Cas9-mediated mutagenesis of carotenoid cleavage dioxygenase 4. Transgenic Res. **27**, 25–38 (2018)

86. Xu, J., Kang, B.-C., Naing, A.H., Bae, S.-J., Kim, J.-S., Kim, H., Kim, C.K.: CRISPR/Cas9-mediated editing of 1-aminocyclopropane-1-carboxylate oxidase1 enhances petunia flower longevity. Plant Biotechnol. J. **18**, 287–297 (2020)
87. Yan, R., Wang, Z., Ren, Y., Li, H., Liu, N., Sun, H.: Establishment of efficient genetic transformation systems and application of CRISPR/Cas9 genome editing technology in Lilium pumilum dc. Fisch. and Lilium longiflorum White Heaven. Int. J. Mol. Sci. **20**, 2920 (2019)
88. Zhang, B., Xu, X., Huang, R., Yang, S., Li, M., Guo, Y.: CRISPR/Cas9-mediated targeted mutation reveals a role for AN4 rather than DPL in regulating venation formation in the corolla tube of Petunia Hybrida. Hortic. Res. **8** (2021)
89. Zhang, B., Yang, X., Yang, C., Li, M., Guo, Y.: Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in Petunia. Sci. Rep. **6**, 20315 (2016)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 15
Application of CRISPR/Cas-Mediated Genome Editing Techniques in Leguminous Crops



Debajit Das and Sumita Acharjee

Abstract Grain legumes are prized for their high protein content and abundance of phytochemicals, which are essential in the human diet. Scientists have made significant advancements in discovering novel genetic features in legumes, including, but not limited to, productivity, tolerance/resistance to various environmental stresses, and improved nutritive value. The contemporary surge in genetic resources of grain legumes has facilitated the integration of advanced molecular breeding techniques such as transgenic methodologies, genome modification, and genomic selection, to augment the crop's overall performance. This chapter discusses the application of CRISPR/Cas9-based genome editing tools for the improvement of grain legumes. Furthermore, it elaborates upon the latest developments in plant-specific genetic modification techniques, while also addressing the challenges and prospective benefits that come with enhancing grain legumes with significant agronomical attributes. Genome editing techniques have been proficiently employed in diverse legumes, encompassing model legumes such as Medicago, alfalfa, and lotus, alongside other widely cultivated legumes like soybean, cowpea, and chickpea. The advent of gene-editing methodologies in legume breeding has presented exciting opportunities for enhancing important agronomic characteristics.

1 Introduction

Legumes, comprising over 19,500 species and 751 genera, are the third-largest angiosperm family [1]. They encompass a diverse range of food crops that are crucial sources of plant-based proteins and essential amino acids. Grain legumes play a crucial role in agricultural sustainability by promoting soil fertility through symbiotic nitrogen fixation (SNF) and the discharge of abundant organic matter into the ground. Notwithstanding their environmental benefits and health advantages, poor

D. Das · S. Acharjee (✉)
Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam, India
e-mail: sumita.acharjee@aau.ac.in

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_15

productivity has impacts on legume agronomy. The implementation of innovative approaches like genomics-assisted selection (also known as marker-assisted selection or genomic selection) and genome editing can help address the challenging problem of boosting productivity and reliability.

Over the course of the past 10 years, significant advancements have been achieved in the sequencing of the genomes of leguminous crops. The draft genomes and transcriptome data of about 35 different legume species have been substantially assembled [2]. To select for intricate traits and generate better cultivars of grain legume crops, these genomic resources are proving invaluable [3]. The detailed assessment of the application of marker-assisted backcrossing (MAB), marker-assisted selection (MAS), and genomic selection (GS) techniques for improving the production of legume crops have been exhaustively investigated [3–5]. This chapter focuses on the latest developments in genome-editing technology and its efficacy as a precision breeding method for improving leguminous crops.

Genome editing involves making use of engineered nucleases and the inherent DNA repair mechanisms of cells to effectuate accurate and tailored modifications to an organism's genetic makeup. The conception of gene-editing techniques commenced approximately thirty years ago, following the important revelation that targeted double-stranded breaks can be instigated in chromosomes using a meganuclease, namely I-SceI [6]. The effective use of meganucleases in genome editing was constrained by the limited number of target regions in the majority of genes. With the arrival of customizable zinc finger nucleases (ZFNs) [7] and transcription activator-like effector nucleases (TALENs) [8], the development of effective genome editing technologies witnessed a major acceleration. The remarkable progress in this state-of-the-art methodology was attained by successfully incorporating RNA-guided Cas9 nuclease, which originates from the type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) adaptive defense mechanism, to facilitate genetic modification in eukaryotic organisms [9, 10].

2 Genome Editing Technology

The implementation of genome editing is a groundbreaking technological innovation that has the potential to significantly advance the field of plant biology and agricultural breeding [11]. This method relies on site-directed nucleases (SDNs) such as meganucleases, ZFNs, TALENs, and the CRISPR/Cas system [12, 13]. The CRISPR/Cas system's growing popularity in genome modification tool development can be attributed to its simplistic nature and facile manipulability [14]. The fundamental CRISPR/Cas mechanism necessitates the presence of two integral constituents: a Cas endonuclease, such as Cas9 or Cpf1, and a guide RNA (gRNA) [15, 16]. The gRNA may be directed to bind to the desired DNA sequence and then use the Cas nuclease to create a double-strand break (DSB) at that specific location. The predominant mechanism for DSB repair in plants is the non-homologous end

joining (NHEJ) pathway, which is known to be error-prone and often results in the insertion or deletion of bases, leading to genetic changes at the site of repair [16]. Thus far, a multitude of base and prime editor tools have been devised leveraging CRISPR/Cas technology to achieve greater precision in the editing process [17].

Many Cas9 variants have been synthesized that exhibit great fidelity, including those with paired nickase, point mutations, chimeric dCas9-FokI, etc. These modified Cas9 variants have cleavage activity with decreased off-target effects (refer to www.addgene.org). Thus choosing an appropriate Cas9 endonuclease variant is important yet difficult. With the use of these editing tools, breeders can manipulate target genes in the way they want to boost crop production and quality, endurance to biotic and abiotic stress, and herbicide resistance [11]. As a result, genome editing is seen as the breeding approach of the future.

The approval of the commercialization of genome-edited crops necessitates a framework of legislation and regulation [18]. Genome editing generates minor indels, base-pair changes, and targeted short sequence modifications by homology-directed repair (HDR), which are identical to those caused by natural mutations. As a result, in several countries, these sorts of mutants are not classified as genetically modified organisms (GMOs) and hence are excluded from GMO restrictions [19, 20].

3 Limitations in Genetic Modification of Legumes

The availability of a suitable genetic transformation protocol to introduce DNA into the plant is a prerequisite for achieving a fruitful plant transformation. Most of the grain legumes are grouped as recalcitrance to *in vitro* regeneration and transformation [21]. This phenomenon is further compounded by the intricate fact that only certain tissues (immature cotyledons, mature cotyledons with embryonic axis, embryonic axis or hypocotyl) within leguminous crops exhibit the ability to transform, while others possess the capacity for regeneration. It is noteworthy that these two distinct events do not invariably manifest within the same tissue. Achieving successful in vitro rooting can be a significant obstacle, particularly for legumes with large seeds. The impediments to large-scale transformation in legumes include method specificity and other factors. Traditional methods of genetic transformation are inadequate for achieving optimal results. Nevertheless, regeneration procedures for several legumes have not been successful, mostly because of poor *in vitro* roots during regeneration. It has been posited that conventional breeding methods are insufficient in tackling these obstacles [22, 23].

The development of efficient transformation techniques is crucial for validating the role of genes in targeted crops [24]. Various methodologies, including sonication-assisted *Agrobacterium* transformation, have garnered attention as a means to augment the genetic transformation procedure in leguminous plants. Improving the pace of genetic transformation in leguminous crops can be achieved through significant approaches such as optimizing explant, improving the affinity of host plant

interaction, and refining culture media additives [25]. Additional research is necessary to explore the barriers to legume transformation and potential remedies. Advancements in the field of molecular science are expected to generate novel ideas and provide insights into the rapid enhancement of legume transformation rates.

The *Agrobacterium*-mediated transformation method is the primary means of genetic modification for the majority of grain legumes, with the biolistic method being utilized in a limited number of instances. Of the various grain legumes, genetic modification has been successfully achieved in soybean, and the CRISPR/Cas9 system has also been extensively utilized [26]. Nevertheless, the utilization of CRISPR/Cas9 technology has the potential to overcome these limitations and obstacles [27]. The development of reliable and repeatable regeneration techniques results in the commercially successful production of several species of genetically engineered grain legumes.

4 Application of CRISPR/Cas9 in Grain Legumes

The CRISPR-mediated gene-editing technique has been successfully applied to various legumes, such as soybean, chickpea, lentil, and *Medicago truncatula*, among others. Leguminous plants are cultivated extensively across the globe. The implementation of CRISPR/Cas9 technology has demonstrated enhancements in crop productivity, quality, and resilience against both biotic and abiotic stressors.

Legume species domestication has been extensively researched, and currently, the CRISPR/Cas9 mechanism is being employed in the process of domestication to introduce and enhance diverse characteristics. It is anticipated that the forthcoming process of crop domestication shall witness a significant acceleration, with the aid of CRISPR/Cas9, which shall effectively enhance numerous crop traits of commercial significance [28]. In recent times, there have been major advances in improving the nutritional value of legumes through the application of CRISPR/Cas9 technology.

4.1 Medicago truncatula *(Alfalfa)*

A forage crop, *Medicago truncatula* is used as a model crop due to its self-fertility, short life cycle, relatively simple transformation, diploidy, and smaller genome,. It is an excellent model for studying the molecular and physiological bases of leguminous crops. The CRISPR/Cas9 system exhibits a sub-optimal level of efficacy when applied to the polyploid alfalfa genome such as the tetraploid *M. sativa and M. falcata*. For effective gene editing in alfalfa, an improved CRISPR/Cas9 gene-editing system will be required. The *MsSGR* gene of *M. truncatula* has been effectively edited using CRISPR/Cas9. The outcomes indicated substantial differences in colour among the mutants. The presence of colour variation plays a crucial role in the attraction of insects and birds, thereby facilitating successful pollination. The

experimental results indicated that mutations exhibited a greenish colour and suggested that the CRISPR/Cas9 method of knocking out alfalfa genes holds significant potential for future research [29]. Bottero and colleagues employed the CRISPR/Cas9 system to modify the NOD26 gene in alfalfa to enhance protein levels [30]. *Phytoene desaturase* (*PDS*) genes are a popular choice among researchers due to their easily observable phenotypic traits, which can be used to assess the efficacy of CRISPR/Cas9 gene-editing tools. Meng and Haji [31] devised a CRISPR/Cas9 mechanism to induce specific changes in the *MtPDS* gene of *M. truncatula*. Their findings revealed that out of 309 T_0 transgenic plants, 32 displayed the albino phenotype. Sixteen of these 32 transgenic plants were arbitrarily chosen for sequencing, and the results confirmed that all the tested albino plants had alterations in the intended region of the *MtPDS* gene. The genome-wide association studies (GWAS) employing CRISPR/Cas9 were carried out to study the function of potential genes for nodulation in *M. truncatula* [32]. Additionally, Trujillo et al. [33] and Wang et al. [34] have conducted mutational studies on five different nodule-specific PLAT domain (*NPD1–5*) and nitrate peptide family (*NPD*) genes.

4.2 Glycine max *(Soybean)*

Soybean (*G. max* (L.) Merr.) holds significant value as a crop for its oil and protein content, making it a prime candidate for genetic enhancement through the CRISPR/Cas9 technology [35]. The soybean plant is a diploid species that has undergone an evolution from a palaeotetraploid ancestor. The soybean genome exhibits significant duplication, thereby presenting a significant barrier to conventional genetic methodologies aimed at elucidating gene functionality. One of the difficulties encountered in soybean transformation is the limited efficacy of *Agrobacterium*-mediated techniques, which is influenced by the type of tissue or cultivar used. Consequently, *Agrobacterium rhizogenes*-mediated hairy root transformation has acquired significant attention since it can be used to efficiently assess the effectiveness of gRNAs before the whole-plant transformation. This is due to the prompt way of obtaining transgenic hairy roots, which can be obtained in a matter of weeks [36]. In 2015, there were multiple reports of the effective utilization of the CRISPR/Cas9 technology for gene-editing purposes in soybean [37–40]. After these preliminary accomplishments, soybean researchers have attempted to improve gene-editing technology. Di et al. [41], for instance, used eleven different GmU6 promoters to determine which would be the most effective for driving gRNA expression in soybean hairy roots and found that the GmU6–8 and GmU6–10 promoters were the most active in improving editing efficiency (20.3% and 20.6%, respectively) than the rest of the nine GmU6 promoters (ranging from 2.8% to 17%). Additionally, the CRISPR/Cas9 technique has been effective in targeting three *GmLox* genes (*GmLox1*, *GmLox2*, and *GmLox3*) that encode three lipoxygenases (LOX1, LOX2, and LOX3) that produce a beany flavour that limits human intake [42]. They observed that the associated lipoxygenase activities had been eliminated in 60

T_0-positive transgenic plants harboring various sgRNAs and mutations (two of them are triple mutants and one is a double mutant). Li et al. [36] employed the CRISPR/Cas9 methodology to modify the conglycinin (7S) and glycinin (11S) storage protein genes in soybean. The researchers observed gene-editing efficiencies of 5.8%, 3.8%, and 43.7% for *Glyma.20 g148400*, *Glyma.03 g163500*, and *Glyma.19 g164900* genes, respectively. Furthermore, the manipulation of soybean plant architecture has been observed through the utilization of the CRISPR/Cas9 system.

The study carried out by Bao et al. [35] involved the selective targeting of squamosal promoter-binding protein-like genes, namely *GmSPL9a*, *GmSPL9b*, *GmSPL9c*, and *GmSPL9*. The results of the study indicated that the T_2 double homozygous mutant spl9a/spl9b exhibited a reduced plastochron length. Also, it has been observed that T_4 mutant plants exhibit an increment in the number of nodes on the main stem as well as an increase in branch numbers. Zheng et al. [43] described simple binary vector systems using Cas9 and egg cell-specific promoters (ECp). The two genes, *GmAGO7a* and *GmAGO7b*, which encode ARGONAUTE7 (AGO7), were specifically targeted due to their significant role in regulating leaf patterns in soybean. Their findings indicate that the promoters can generate mutations and that it is possible to obtain several distinct mutations independently. Virdi et al. [44] conducted a study wherein they employed CRISPR/Cas9 mutagenesis to generate several knockout alleles including one in-frame allele for the *β-ketoacyl synthase 1* (*KASI*) gene, which is involved in the conversion of sucrose to oil. The findings of the study revealed that the function of the genes was lost.

Because of the importance of Soybean, a substantially higher number of CRISPR studies have been conducted on it compared to other legumes. These studies primarily focus on modifying its nutritional value and plant architecture, specifically leaf patterns and nodule numbers. Nonetheless, the establishment of stable soybean genetic transformation remains elusive due to the recalcitrant nature of this crop towards transformation. The enhancement of transformation efficiency has the potential to propel CRISPR research in soybean toward subsequent genetic research, owing to its efficiency, multiplex editing, and high-throughput mutagenesis capability [35].

4.3 Cicer arietinum *(Chickpea)*

Chickpea is a crop of significant commercial importance on a global scale. The application of genome-editing techniques presents a viable solution for addressing the challenges encountered during its cultivation. Badhan et al. [45] conducted a study with the objective of editing genes associated with drought tolerance, namely *4-coumarate ligase* (*4CL*) and *Reveille 7* (*RVE7*), using CRISPR/Cas9 technology in chickpea protoplasts. The results suggested that the knock-out of the RVE7 gene displayed a high level of editing efficiency in vivo. The findings of this study

indicate that the utilization of protoplasts enables the application of the CRISPR/Cas9 DNA-free gene editing technique for enhancing drought tolerance in chickpea genes. This study represents the initial and singular instance in which CRISPR/Cas9 gene editing has been employed in chickpea research.

4.4 Arachis hypogea *(Peanuts or Groundnut)*

Groundnut is an important leguminous crop that exhibits a notable concentration of oleic acid. One of the primary breeding goals for peanuts is to increase their oil content. The oil possesses significant industrial utility and benefits, such as prolonged shelf life and antioxidant properties. The CRISPR/Cas9 gene-editing system was leveraged in a study that edited the *ahFAD28* gene, which is responsible for the conversion of oleic acid to linoleic acid in fatty acids. The CRISPR/Cas9 system has effectively modified the gene to introduce desirable traits. The targeted mutation of this gene was achieved using groundnut protoplast and culture [46]. In a separate experiment, the utilization of the CRISPR/Cas9 tool was explored to edit the allergen gene (*Ara h 2*) in peanuts. The altered version of this gene resulted in enhanced nutritional qualities of peanuts for individuals with peanut allergies. Shu et al. [47] performed a study wherein CRISPR/Cas9 was employed to investigate the functions of Nod factor receptors (NFRs) in peanut nodule formation, particularly in the initiation of a symbiotic relationship with rhizobia. The mutants that underwent editing to contain two *AhNFR5* genes exhibited a Nod-phenotype. Conversely, the mutants that were picked for containing two *AhNFR1* genes were still capable of forming nodules following inoculation.

4.5 Vigna radiata *(Mungbean)*

Given the current accessibility of complete genome sequencing and the extensive collection of 1481 mung bean entries that have undergone comprehensive evaluation for different agronomical characteristics [48], there exists a significant opportunity for harnessing CRISPR/Cas9 gene editing in mung bean breeding initiatives. The successful implementation of CRISPR/Cas9-mediated gene editing in cowpea (*V. unguiculata*) has been reported, wherein a symbiosis receptor-like kinase gene was targeted to disrupt symbiotic nitrogen fixation [49]. The efficacy of CRISPR/Cas9 in a *Vigna* system implies the potential applicability of genome editing in additional species, such as mung bean. The initial objectives for gene editing in mung bean would encompass resistance to diseases and other desirable traits. The development of mung bean cultivars that exhibit resilience to fluctuating climate conditions would facilitate the global expansion of mung bean agriculture.

4.6 Vigna ungiculata *(Cowpea)*

Cowpea is a leguminous crop that is well-suited for cultivation in warm and arid regions [50]. It is considered as an orphan grain legume. Globally, the production of cowpeas increased from 9.7 MT in 2009 to 14.4 MT in 2019. Cowpeas exhibit a protein level of 25% by dry mass and exhibit a high-lysine content, thereby enabling them to serve as complementary dietary ingredients in cereal crop-based diets.

The recalcitrance of cowpea towards transformation has been observed to impede the regular application of CRISPR/Cas9 techniques. Che et al. [51] have reported enhanced transformation efficiencies ranging from 4.5% to 37% across nine cowpea varieties. Che and associates employed a CRISPR/Cas9 framework that drives the expression of Cas9 under the control of soybean elongation factor (GmEF1A2) promoter and a gRNA under the promoter of VuU6 to generate a total of 35 T_1 plants. Che and colleagues specifically targeted the *VuSPO11-1* gene to facilitate the production of asexual plants that are suitable for hybridization. Juranić et al. [52] designed a transient approach to evaluate CRISPR/Cas9 constructs within a 48-h timeframe using *Agrobacterium* infiltration of detached leaflets, as a means of surmounting the challenge of testing such constructs in stable cowpea transformants.

5 Conclusion

Legumes have been a staple in human and livestock diets since ancient times. The uniqueness of these plants lies in their possession of multiple nutritional benefits and their ability to withstand various diseases. Population growth has exerted a major supply burden for these crops on the food supply network. Researchers are introducing new variations for various crop traits [53, 54]. The application of the CRISPR/Cas9 tool has become increasingly significant in contemporary genome editing, with potential implications for achieving global food security. This widely used tool demonstrates crop modification without the use of transgenes. Legume crop genome editing using the CRISPR/Cas system has not yet reached its full potential [55] and it is anticipated that CRISPR/Cas9 in combination with other molecular approaches would significantly improve legumes and increase their yield.

CRISPR/Cas9 technology has undergone significant advancements, which have expanded the range of possibilities for accurately and effectively manipulating genes through genetic material addition or deletion. The utilization of CRISPR/Cas9 technology presents innovative opportunities for the exploration of functional genomics and the enhancement of diverse characteristics in grain legume crops. The efficacy of genome editing in enhancing legume quality depends on the presence of proficient procedures for plant transformation and complete plant regeneration, as well as a favorable regulatory framework and substantiation of societal endorsement of gene-edited crops.

References

1. Lewis, G.P., Schrire, B., Mackinder, B., Lock, M.: Legumes of the World, p. 577. Royal Botanic Gardens, Kew, EJB, Richmond (2005)
2. Bauchet, G.J., Bett, K.E., Cameron, C.T., Campbell, J.D., Cannon, E.K.S., Cannon, S.B., Carlson, J.W., Chan, A., Cleary, A., Close, T.J., Cook, D.R., Cooksey, A.M., Coyne, C., Dash, S., Dickstein, R., Farmer, A.D., Fernández-Baca, D., Hokin, S., Jones, E.S., Kang, Y., Monteros, M.J., Munoz-Amatriain, M., Mysore, K.S., Pislariu, C.I., Richards, C., Shi, A., Town, C.D., Udvardi, M., von Wettberg, E.B., Young, N.D., Zhao, P.X.: The future of legume genetic data resources: challenges, opportunities, and priorities. Legume Sci. **1**, e16 (2019)
3. Varshney, R.K., Pandey, M.K., Bohra, A., Singh, V.K., Thudi, M., Saxena, R.K.: Toward the sequence-based breeding in legumes in the post-genome sequencing era. Theor. Appl. Genet. **132**, 797–816 (2019)
4. Mousavi-Derazmahalleh, M., Bayer, P.E., Hane, J.K., Valliyodan, B., Nguyen, H.T., Nelson, M.N., Erskine, W., Varshney, R.K., Papa, R., Edwards, D.: Adapting legume crops to climate change using genomic approaches. Plant Cell Environ. **42**, 6–19 (2019)
5. Varshney, R.K., Kudapa, H., Pazhamala, L., Chitikineni, A., Thudi, M., Bohra, A., Gaur, P.M., Janila, P., Fikre, A., Kimurto, P., Ellis, N.: Translational genomics in agriculture: some examples in grain legumes. Crit. Rev. Plant Sci. **34**, 169–119 (2015)
6. Rouet, P., Smih, F., Jasin, M.: Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol. Cell. Biol. **14**, 8096–8106 (1994)
7. Bibikova, M., Golic, M., Golic, K.G., Carroll, D.: Targeted chromosomal cleavage and mutagenesis in drosophila using zinc-finger nucleases. Genet. **161**, 1169–1175 (2022)
8. Zhang, F., Cong, L., Lodato, S., Kosuri, S., Church, G.M., Arlotta, P.: Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat. Biotechnol. **29**, 149–153 (2011)
9. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F.: Multiplex genome engineering using CRISPR/Cas systems. Science. **339**, 819–823 (2013)
10. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M.: RNA-guided human genome engineering via Cas9. Science. **339**, 823–826 (2013)
11. Chen, K., Wang, Y., Zhang, R., Zhang, H., Gao, C.: CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu. Rev. Plant Biol. **70**, 667–697 (2019)
12. Gaj, T., Gersbach, C.A., Barbas, C.F.: ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. **31**, 397–405 (2013)
13. Puchta, H., Fauser, F.: Synthetic nucleases for genome engineering in plants: prospects for a bright future. Plant J. **78**, 727–741 (2014)
14. Kantor, A., Mcclements, M.E., Maclaren, R.E.: CRISPR-Cas9 DNA Base-editing and prime-editing. Int. J. Mol. Sci. **21**(17), 6240 (2020)
15. Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., van der Oost, J., Regev, A., Koonin, E.V., Zhang, F.: Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. **163**(3), 759–771 (2015)
16. Jiang, F., Doudna, J.A.: CRISPR-Cas9 structures and mechanisms. Annu. Rev. Biophys. **46**, 505–529 (2017)
17. Zhu, H., Li, C., Gao, C.: Applications of CRISPR-Cas in agriculture and plant biotechnology. Nat. Rev. Mol. Cell Biol. **21**, 661–677 (2020)
18. Jones, H.D.: Regulatory uncertainty over genome editing. Nat. Plants. **1**, 14011 (2015)
19. Kim, J., Kim, J.S.: Bypassing GMO regulations with CRISPR gene editing. Nat. Biotechnol. **34**, 1014–1015 (2016)
20. Turnbull, C., Lillemo, M., Hvoslef-Eide, T.A.K.: Global regulation of genetically modified crops amid the gene edited crop boom – a review. Front. Plant Sci. **12**, 630396 (2021)

21. Yadav, R., Mehrotra, M., Singh, A.K., Niranjan, A., Singh, R., Sanyal, I., Lehri, A., Pande, V., Amla, D.V.: Improvement in agrobacterium-mediated transformation of chickpea (Cicer arietinum L.) by the inhibition of polyphenolics released during wounding of cotyledonary node explants. Protoplasma. **254**, 253–269 (2017)
22. Dewir, Y.H., Murthy, H.N., Ammar, M.H., Alghamdi, S.S., Al-Suhaibani, N.A., Alsadon, A.A., Paek, K.Y.: In vitro rooting of leguminous plants: difficulties, alternatives, and strategies for improvement. Hortic. Environ. Biotechnol. **57**, 311–322 (2016)
23. Negi, J., Rathinam, M., Sreevathsa, R., Kumar, P.A.: Transgenic Pigeonpea [Cajanus cajan (L). Millsp.]. Genetically Modified Crops: Current Status, Prospects and Challenges, Volume 1, pp. 79–96. Springer, Singapore (2021)
24. Bhowmik, P., Konkin, D., Polowick, P., Hodgins, C.L., Subedi, M., Xiang, D., Yu, B., Patterson, N., Rajagopalan, N., Babic, V., Ro, D.K.: CRISPR/Cas9 gene editing in legume crops: opportunities and challenges. Legume Sci. **3**(3), e96 (2021)
25. Sehaole, E.K.M.: Genetic transformation in agro-economically important legumes. Legumes Res. **1** (2022)
26. Zhang, P., Du, H., Wang, J., Pu, Y., Yang, C., Yan, R., Yang, H., Cheng, H., Yu, D.: Multiplex CRISPR/Cas9-mediated metabolic engineering increases soya bean isoflavone content and resistance to soya bean mosaic virus. Plant Biotechnol. J. **18**(6), 1384–1395 (2020)
27. Deng, F., Zeng, F., Shen, Q., Abbas, A., Cheng, J., Jiang, W., Chen, G., Shah, A.N., Holford, P., Tanveer, M., Zhang, D.: Molecular evolution and functional modification of plant miRNAs with CRISPR. Trends Plant Sci. **27**(9), 890–907 (2022)
28. Rasheed, A., Wassan, G.M., Khanzada, H., Solangi, A.M., Han, R., Li, H., Bian, J., Wu, Z.: Identification of genomic regions at seedling related traits in response to aluminium toxicity using a new high-density genetic map in rice (Oryza sativa L.). Genet. Resour. Crop. Evol. **68**, 1889–1903 (2021)
29. Wolabu, T.W., Cong, L., Park, J.J., Bao, Q., Chen, M., Sun, J., Xu, B., Ge, Y., Chai, M., Liu, Z., Wang, Z.Y.: Development of a highly efficient multiplex genome editing system in outcrossing tetraploid alfalfa (Medicago sativa). Front. Plant Sci. **11**, 1063 (2020)
30. Bottero, E., Massa, G., González, M., Stritzler, M., Tajima, H., Gómez, C., Frare, R., Feingold, S., Blumwald, E., Ayub, N., Soto, G.: Efficient CRISPR/Cas9 genome editing in alfalfa using a public germplasm. Front. Agron. **3**, 661526 (2021)
31. Merga, B., Haji, J.: Economic importance of chickpea: production, value, and world trade. Cogent Food Agric. **5**(1), 1615718 (2019)
32. Curtin, S.J., Tiffin, P., Guhlin, J., Trujillo, D.I., Burghart, L.T., Atkins, P., Baltes, N.J., Denny, R., Voytas, D.F., Stupar, R.M., Young, N.D.: Validating genome-wide association candidates controlling quantitative variation in nodulation. Plant Physiol. **173**, 921–931 (2017)
33. Trujillo, D.I., Silverstein, K.A., Young, N.D.: Nodule-specific PLAT domain proteins are expanded in the Medicago lineage and required for nodulation. New Phytol. **222**(3), 1538–1550 (2019)
34. Wang, L., Sun, S., Wu, T., Liu, L., Sun, X., Cai, Y., Li, J., Jia, H., Yuan, S., Chen, L.: Natural variation and CRISPR/Cas9-mediated mutation in GmPRR37 affect photoperiodic flowering and contribute to regional adaptation of soybean. Plant Biotechnol. J. **18**, 1869–1881 (2020)
35. Bao, A., Zhang, C., Huang, Y., Chen, H., Zhou, X., Cao, D.: Genome editing technology and application in soybean improvement. Oil Crop Sci. **5**(1), 31–40 (2020)
36. Li, C., Nguyen, V., Liu, J., Fu, W., Chen, C., Yu, K., Cui, Y.: Mutagenesis of seed storage protein genes in soybean using CRISPR/Cas9. BMC. Res. Notes. **12**, 1–7 (2019)
37. Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., Han, T., Hou, W.: CRISPR/Cas9-mediated genome editing in soybean hairy roots. PLoS One. **10**(8), e0136064 (2015)
38. Jacobs, T.B., LaFayette, P.R., Schmitz, R.J., Parrott, W.A.: Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol. **15**, 1–10 (2015)
39. Li, Z., Liu, Z.B., Xing, A., Moon, B.P., Koellhoffer, J.P., Huang, L., Ward, R.T., Clifton, E., Falco, S.C., Cigan, A.M.: Cas9-guide RNA directed genome editing in soybean. Plant Physiol. **169**(2), 960–970 (2015)

40. Sun, X., Hu, Z., Chen, R., Jiang, Q., Song, G., Zhang, H., Xi, Y.: Targeted mutagenesis in soybean using the CRISPR-Cas9 system. Sci. Rep. **5**(1), 1–10 (2015)
41. Di, Y.H., Sun, X.J., Hu, Z., Jiang, Q.Y., Song, G.H., Zhang, B., Zhao, S.S., Zhang, H.: Enhancing the CRISPR/Cas9 system based on multiple GmU6 promoters in soybean. Biochem. Biophys. Res. Commun. **519**(4), 819–823 (2019)
42. Wang, J., Kuang, H., Zhang, Z., Yang, Y., Yan, L., Zhang, M., Song, S., Guan, Y.: Generation of seed lipoxygenase-free soybean using CRISPR-Cas9. Crop J. **8**(3), 432–439 (2020)
43. Zheng, N., Li, T., Dittman, J.D., Su, J., Li, R., Gassmann, W., Peng, D., Whitham, S.A., Liu, S., Yang, B.: CRISPR/Cas9-based gene editing using egg cell-specific promoters in Arabidopsis and soybean. Front. Plant Sci. **11**, 800 (2020)
44. Virdi, K.S., Spencer, M., Stec, A.O., Xiong, Y., Merry, R., Muehlbauer, G.J., Stupar, R.M.: Similar seed composition phenotypes are observed from CRISPR-generated in-frame and knockout alleles of a soybean KASI Ortholog. Front. Plant Sci. **11**, 1005 (2020)
45. Badhan, S., Ball, A.S., Mantri, N.: First report of CRISPR/Cas9 mediated DNA-free editing of 4CL and RVE7 genes in chickpea protoplasts. Int. J. Mol. Sci. **22**(1), 396 (2021)
46. Yuan, M., Zhu, J., Gong, L., He, L., Lee, C., Han, S., Chen, C., He, G.: Mutagenesis of FAD2 genes in peanut with CRISPR/Cas9 based gene editing. BMC Biotechnol. **19**(1), 1–7 (2019)
47. Shu, H., Luo, Z., Peng, Z., Wang, J.: The application of CRISPR/Cas9 in hairy roots to explore the functions of AhNFR1 and AhNFR5 genes during peanut nodulation. BMC Plant Biol. **20**(1), 1–15 (2020)
48. Schafleitner, R., Nair, R.M., Rathore, A., Wang, Y.W., Lin, C.Y., Chu, S.H., Lin, P.Y., Chang, J.C., Ebert, A.W.: The AVRDC–the world vegetable center mungbean (Vigna radiata) core and mini core collections. BMC Genomics. **16**(1), 1–11 (2015)
49. Ji, J., Zhang, C., Sun, Z., Wang, L., Duanmu, D., Fan, Q.: Genome editing in cowpea Vigna unguiculata using CRISPR-Cas9. Int. J. Mol. Sci. **20**(10), 2471 (2019)
50. Cullis, C., Kunert, K.J.: Unlocking the potential of orphan legumes. J. Exp. Bot. **68**(8), 1895–1903 (2017)
51. Che, P., Chang, S., Simon, M.K., Zhang, Z., Shaharyar, A., Ourada, J., O'Neill, D., Torres-Mendoza, M., Guo, Y., Marasigan, K.M., Vielle-Calzada, J.P.: Developing a rapid and highly efficient cowpea regeneration, transformation and genome editing system using embryonic axis explants. The Plant J. **106**(3), 817–830 (2021)
52. Juranić, M., Nagahatenna, D.S., Salinas-Gamboa, R., Hand, M.L., Sánchez-León, N., Leong, W.H., How, T., Bazanova, N., Spriggs, A., Vielle-Calzada, J.P., Koltunow, A.M.: A detached leaf assay for testing transient gene expression and gene editing in cowpea (*Vigna unguiculata* [L.] Walp.). Plant Methods. **16**(1), 1–17 (2020)
53. Rasheed, A., Fahad, S., Hassan, M.U., Tahir, M.M., Aamer, M., Wu, Z.: A review on aluminum toxicity and quantitative trait loci maping in rice (Oryza sativa L). Appl. Ecol. Environ. Res. **18**(3), 3951–3964 (2020a)
54. Rasheed, A., Fahad, S., Aamer, M., Hassan, M.U., Tahir, M.M., Wu, Z.: Role of genetic factors in regulating cadmium uptake, transport and accumulation mechanisms and quantitative trait loci mapping in rice. A review. Appl. Ecol. Environ. Res. **18**(3), 4005–4023 (2020b)
55. Kumar, A., Kumar, S., Komal, R., N. and Singh, P.: Role of traditional ethnobotanical knowledge and indigenous communities in achieving sustainable development goals. Sustainability. **13**(6), 3062 (2021)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 16
Genetic Improvement in Leguminous Crops Through Genome Editing

Aida Dervishi, Muhammad Waqas Choudry, Rabia Riaz, and Allah Bakhsh

Abstract Leguminous crops belong to the Fabaceae family, known for their high nutritional properties and use for human and livestock consumption and for their ability to symbiotically fixing nitrogen which plays an important role in soil enrichment and sustainable agriculture. Over the years the genetic improvement of legumes has been carried out using conventional techniques of breeding based on the QTL and MAS selection mainly to increase the yield. Genome editing techniques have been successfully used in different legume crops, mainly on model crops and grain legumes such as chickpea, soybean and cowpea. The recent advancement in gene editing tools such as CRISPR-Cas technology contributed to the improvement of important agronomic traits in legume species and provide a great potential for studies of the traits such as tolerance to biotic and abiotic stress, increased yield and improvement of seed content chave recently been introduced in legumes utilizing genome editing tools. This chapter provides an overview of the main developments in genetic improvement methods applied and the achievements so far achieved in leguminous crops. Further studies aiming at the genetic improvement of minor or underutilized legumes are pivotal and a challenge in the future.

A. Dervishi
Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Tiranë, Albania

M. W. Choudry · R. Riaz · A. Bakhsh (✉)
Centre of Excellence in Molecular Biology, University of Punjab, Lahore, Pakistan
e-mail: allahbakhsh@cemb.edu.pk

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_16

1 Introduction

1.1 Nutritional and Ecological Value of Leguminous Crops

Legume is any plant species of Fabaceae (*Leguminosae*) family which constitutes 5% of the total 400,000 plant species known so far on earth. The term 'legume' has derived from Latin word *Legūmen* that means "beans inside pods". The Fabaceae family constitute major "founder crops", the ones domesticated earliest in known human history [1]. Among the "Big Eight' founder crops' group that known to be cultivated as early as 10,000 BC, four are legumes including; Pea (*Pisum sativum*), chick pea (*Cicer arietinum*), lentils (*Lens culinaris*), and bitter vetch (*Vicia ervilia*) [2]. Legume cultivation holds key importance in terms of worldwide grain food and forage production demands. The edible seed fruits of any leguminous plant are generally termed as pulses. The commonly cultivated grain legumes among the 20,000 worldwide distributed species are; common bean, faba bean, pea, chickpea, cowpea, pigeon pea, lentils, peanut, grass pea and horse gram [3]. According to Food and Agriculture organization (FAO) 2018 stats, common bean (dry bean) was the most cultivated grain legume worldwide with 34.5 million hectares of total cultivation area followed by chickpea (17.8 million ha) and cowpea (12.5 million ha). The estimated grain legume production exceeded 92 million tons worldwide. The major portions of grain legumes production are coming from India, China, Canada, Australia, USA, Brazil, Argentina and Russia. India is the largest producer of grain legumes contributing 1/4th of the total global production [4]. In Europe, soybeans, faba beans and field peas are presently the most cultivated legumes. Particularly, soybean production has dramatically increased in the last decade because of its high demand in livestock feed. According to European Commission (EC) reports, 943,000 ha of land was under soya cultivation in 2019 and expected to increase by 44% by 2030. However, only 43% of the total legume consumption in Europe is indigenously produced [5].

Nutritional Impact The United Nations celebrated 2016 as "International year of Pulses" to highlight the importance of nutritious seeds produced by legumes. Grain legumes are also sometimes referred to as 'poor man's meat' because of the high protein content they possess. The storage proteins in pulses make them the richest plant-based source of proteins which vary from 16–50% of the total dry weight. Along with this high protein content, the presence of adequate amounts of dietary fibers, vitamins, complex carbohydrates, sugars, minerals, and fatty acids making legume seeds as one of the healthiest foods sources [6]. Beans also contain some of the non-nutritional phytochemicals such as phytosterols, polyphenols, trypsin inhibiters, phytate, lectin and saponins. Some of these non-nutritional components of legume grains have been termed as anti-nutrients which means making nutrients less available in the body. This may result from reduced digestion, absorption, and bioavailability of nutrients. Enzyme inhibitors like trypsin/a-amylase inhibitors block the active site of these enzymes and hence no digestibility. Further, lectins are found to interfere with nutrients absorption in the small intestine by attaching

specific epithelial cell receptors [7]. However, in recent research, these phytochemicals are found to play a critical role in body's normal homeostasis through their antioxidant and anti-inflammatory activities [8]. Also, the consumption of pulses in daily diet is found to prevent cardiovascular diseases, obesity and type 2 diabetes control, as well as reduce risk of certain cancers including prostate and breast cancer [9].

Ecological Impact The members of the *Fabaceae* family are well known for their agronomic and ecological role related to their ability to fixate nitrogen [1]. They possess specialized structures in their roots called 'nodules'. Root nodules are symbiotic association structures that develop in the root hairs of leguminous plants with diazotrophic rhizobia through a complex bilateral signaling pathway initiated in nitrogen deficient soils. Rhizobia are a group of gram-negative bacteria that colonize root hairs in a host specific manner and fix gaseous atmospheric nitrogen into a usable form (i.e., ammonia) for plants. Diazotroph is any bacteria or archaea that assimilates atmospheric nitrogen into usable form. This process of harvesting gaseous nitrogen by the rhizosphere rhizobium colonies is termed biological nitrogen fixation (BNF). The symbiotic association is a two-way relation as the bacteria living in root nodules feeds upon the carbon-rich metabolites produced by the host plants. Moreover, legumes cropping has also been found to improve the soil structure (i.e., water retention), nutrients bioavailability (i.e., phosphorous mobilization) and breaking disease cycles through the activities of these rhizobial communities in root nodules. These unique features of legume crops play a critical part in maintaining soil health and adequate soil-nitrogen levels thus reducing the needs of artificial nitrogen fertilizers and pesticides. Further, this capacity of legumes has been extensively utilized in crop rotation systems for sustainable use of agricultural land, mitigating the Greenhouse gas (GHG) emissions and enhanced carbon sequestration processes [4]. The term 'crop rotation' refers to the process of growing different crops on the same land area in a specific sequence cycle so to maintain soil fertility, rhizosphere microbial diversity, nutrients availability and resistance to plant pests. It has been found that legumes rotation with cereal crops reduces greenhouse gas emissions by 5–7 times compared to non-legumes cultivation [5].

Challenges in Production Despite all these advantages global legumes cultivation has declined over the last few decades. According to FAO stats of 2015, only in Europe, the legumes cultivation area was reduced to 1.8 M ha from 5.8 M ha that was recorded in 1961 [10]. This decline in production was mainly attributed to the socio-economic factors and to the limited yield of the legumes in the field. Inconsistent yields effect the profitability of the crop in comparison to other crops grown at the same season [11]. Legumes output value is highly variable per unit of area and relatively low as compared to major cereals like rice, wheat and corn [12]. Pea crop, for example, has observed variability in market outputs ranging between 25–78% in Europe [10]. This low profitability of legumes is highly associated with unstable production yields rather than market prices. The factors limiting the production yields include both; (i) abiotic stresses i.e., salinity, drought, low soil fertil-

ity, extreme temperatures, and (ii) biotic stresses i.e., bacterial/fungal/viral diseases, and other pests' infestation.

2 Improvement in Legumes

2.1 Breeding of Legumes

The economy-driven trends of extensive cereal breeding programs, after the green revolution, is one of the reasons for the lack of focus on legumes breeding and cultivation in the last few decades. Furthermore, conventional breeding of legumes is constrained by the limited genetic diversity of available germplasms, collection facilities, and reference genome databases [13]. The natural inclination of legumes towards self-pollination is the main factor of their germplasm relatively low diversity. Breeders, in general, screen and select the favorable combination of genes/mutations in plant genotypes to provide the desired set of characteristics. This is done by the genome-wide screening of all the available wild and adapted germplasm of that species for the resistance against biotic and abiotic stresses [14]. Rapid advancement in genome sequencing and analysis technologies has helped breeders to link a variable phenotype in a population with a particular locus, termed quantitative trait loci (QTL). In the last decade, genome of around 35 legume crops have been sequenced along with complete transcriptome profile analysis [15]. The availability of this huge genomic data has built into valuable tools for marker-assisted selection (MAS) for desired phenotypes in leguminous crops by employing various genetic markers including simple sequence repeats (SSR), single nucleotide polymorphism (SNP), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) etc. [16].

2.2 Modern Genome Editing Tools

After the discovery of restriction endonucleases and their successful use in making a recombinant DNA molecule, the term 'Genetic Engineering' came to limelight. Genetic engineering (GE) essentially means the introduction of a foreign genetic material, using restriction enzymes and ligases, in a suitable host to provide the desired trait [17]. Since its development, technology has been continuously utilized in the various fields of crop improvement, having a significant global impact. The transformation of CRY1 family genes from *Bacillus Thuringiensis* into crops for insect pest resistance is one of the mainstream success of GE technology so far [18]. The next development in applied genetics was the discovery of homing endonuclease (HEs) or meganuclease I-SceI encoded by mobile genetic elements (MGEs) of the DNA for recombination process [19]. These meganuclease generate double

stranded breaks at specific sites in the DNA and then breaks are repaired by natural DNA repair pathways such as Homology directed repair (HDR) or Non-homologous ends joining (NHEJ) repair depending upon the repair mechanism available in the given cell. This double stranded break repair is generally accompanied with insertions or deletions (Indels) of some base pairs leading to genome editing in a locus-specific manner [20]. The genome editing success by using meganucleases was limited because of low frequency of its recognition sites in the DNA [21]. To overcome this limitation synthetic genomic scissors, Zinc finger nucleases (ZFN) were developed in 2002. ZNF is a hybrid molecule containing DNA binding domains, zinc fingers (His_2Cys_2) and a nuclease domain of Fok1 endonuclease. These zinc fingers can be engineered to recognize specific sites in the genome and this capacity is being utilized for genome editing by engineering ZNFs for a specific DNA regions [22]. One of the limitations in using zinc finger nuclease (ZFN) technology was compromised specificity depending upon the flanking DNA sequence of the recognition sites, called context-dependent specificity. On the similar pattern, transcription activator like effector nucleases (TALENs) were developed by combing DNA-recognizing domains of transcription activator (TAL) like effectors with nuclease domain of restriction enzyme Fok1 for site specific mutagenesis [23]. Despite similarity in basic concept, the major difference in TALENs and ZFNs is the frequency and accuracy of cleavage sites. Zinc finger domains recognize 3–4 bases while TALENs has specificity to a single bases conferred by the repeat variable di residues (RVD) of TALE proteins' DNA-binding domain making it possible to join several modules without interference in recognition sequence. Moreover, the design of TALENs is relatively simple making the DNA recognition and binding process less complex compared with ZFNs [24]. Now, coming to the most advanced genome editing tool termed as Cluster regularly interspaced palindromic sequences (CRISPR) – Crispr associated system (CAS) system. CRISPR-Cas genome editing system is tailored from the natural adaptive defense mechanism in most of the bacterial and archaeal species against consecutive infections of bacteriophages. CRISPR are simply virus-specific sequences, termed 'spacers' and are placed between regularly clustered repeats present throughout the bacterial and archaeal genomes called CRISPR loci [25]. CRISPR Loci are associated with a set of specialized proteins called CAS-proteins. These CAS proteins have highly specific nuclease activity mediated by RNA-DNA complementarity. The spacer sequences upon infection by bacteriophage got transcribed into crispr RNA (crRNA) and act as a guide for CAS protein to cleave the viral DNA and hence infectivity stops [26]. The adoption of this technology by artificially designing guide RNA (gRNA) and CAS protein constructs leads to an era of third generation genome editing. The RNA-DNA base pair complementarity makes CRISPR-Cas system more precise genome editing tool compared with ZFN and TALENs where protein-DNA interaction was used for targeting [24].

ZFN technology was employed in soybean to generate heritable mutations on Dicer-like 1 (DCL1) loci that lead to a defective miRNA precursor transcript processing [27], on Dicer-like 4 (DCL4a and DCL4b) loci for hairy root transformation by increasing the growth of lateral root [28]. TALEN technology have been

effectively used to improve seed nutritional characteristics, increasing oleic acid content in soybean [29] and peanut [30] or reducing linoleic acid content in soybean [31] by targeting fatty acid desaturase 2-1A (FAD2-1A) 2-1B, (FAD2-1B), and FAD3A gene family. TALEN was successfully applied in soybean to produce mutants by targeting Dicer-like2 gene [32].

3 CRISPR-Mediated Genome Improvement in Legumes

CRISPR-Cas based genome editing is a relatively simple process requiring only a Cas9 endonuclease activity and a guide RNA (gRNA). The gRNA further consists of i) a crisper RNA (crRNA) that binds the target sequence and, ii) a transactivating RNA (tracer RNA) that mediates target recognition and cleavage. Some variations on the basic pattern may include the use of a different Cas-protein (i.e., Cas12a, Cas13 etc.) with different Protospacer adjacent motif (PAM) requirements. PAM is 2–5 bp sequence that flanks the target sequence to facilitates the Cas-protein bindings and is often a limitation to CRISPR-Cas system's applicability [33]. The critical steps in plant genomic improvement through CRISPR-Cas includes; (i) optimization of gRNA and Cas9 constructs, (ii) successful transformation/use of suitable delivery vehicle, (iii) detection of resulting mutations and, (iv) regeneration of mutated callus [16]. CRISPR-based genetic improvements have been extensively explored in various crops to generate mutations and then selection of high-quality cultivars. However, the lack of optimized protocols for the successful transformation and regeneration of legumes plantlets from callus is a major barrier in their genome editing [34]. Browning of tissues are recalcitrant in-vitro rooting behavior are the key barriers in regeneration of many pulses [35]. Agrobacterium-mediated transformation using seed-tissues have achieved some of success in recent years, particularly in soybean [36] and model legumes *Medicago truncatula* and *Lotus japonicus* [37]. Other legume plants with successful reports of CRISPR-based editing includes cowpea, pea, chickpea, alfalfa and peanut as mentioned in Table 16.1.

Use of Cas9 variants to achieve diverse targeted sites, optimized guide RNA vector constructs, multiple gRNA targeting the same gene or different genes at the same time [52], and development of regeneration protocols are key areas of research in legume genetic improvements. Further, ongoing regulatory debates are focusing on non-specific complementarity binding of gRNA and off-target cleavages which may result in unwanted effects in the host [50].

Table 16.1 Genome editing of various legume crops for improved varieties

CRISPR-based genome editing					
Sr no.	Legume plant	Desired trait	Targeted genes	Results/mutation efficiency	References
1	Soybean	Increased isoflavone content and resistance to Soybean mosaic virus (SMV)	*GmF3H1, GmF3H2, & GmFNSII-1*	44.44% mutation efficiency; stable inheritance; doubled isoflavone content in leaves; reduction (1/3) of SMV coat protein	[38]
2	Soybean	Understanding the flowering time & adaptation to diverse environments	*GmFT2a, & GmFT5a*	Both genes collectively regulate flowering time; *GmFT2a* is critical for short day conditions while *GmFT5a* for long day; *GmFT5a* is essential for adaptation in higher latitudes	[39]
3	Soybean	Improvement in seed-oil composition	*GmFAD2-1A, & GmFAD21-B*	40% of null mutation founds for both genes; 80% increase in oleic acid content; and 1.3–1.7% decreased in linoleic acid.	[40]
4	Soybean	Attempt to modify storage-protein composition of seeds	Nine of the soybean seed storage protein coding genes were targeted	3.8–43.7% mutation efficiency; three of the genes namely, *Glyma.20 g148400, Glyma.03 g163500, & Glyma.19 g164900* were successfully mutated	[41]
5	Soybean	Improvement in plant architecture	*GmSPL9, GmSPL9a, GmSPL9b, & GmSPL9c*	In T2, double homozygous mutant for SPL9a/b showed shorter plastochron length; in T4 various mutations resulted in increased node number and branch number	[42]
6	Soybean	Improved taste	*LOX1, LOX2, & LOX3*	Triple gene mutant was obtained; reduced lipoxygenase activity	[43]
7	Cowpea	To develop asexual plant lineage	Meiosis controlling gene *VuSPO11-1*	4.5–37% of mutation efficiency in nine different cowpea genotypes	[44]
8	Chickpea	Genome free deployment of CRISPR in chickpea editing and understanding of drought tolerance genes	*4CL, & RVE7*	RNP complex-based successful editing of chickpea protoplast; 76–79% mutation efficiency for RVE7; 2% mutation efficiency for 4CL gene	[45]

(continued)

Table 16.1 (continued)

CRISPR-based genome editing					
Sr no.	Legume plant	Desired trait	Targeted genes	Results/mutation efficiency	References
9	Chickpea	Optimization of chickpea genome editing through CRISPR-Cas	*PsPDS*	42% mutation efficiency was achieved indicated by albino phenotypes	[46]
10	Pea	Optimization of pea genome editing through CRISPR-Cas	*PsPDS*	16–45% mutation efficiency with different vector constructs	[47]
11	Peanut	Oleic acid content	*ahFAD2a, & ahFAD2b*	$G_{451}T$, a new mutation was generated *ahFAD2b* which may result in high oleic acid content	[48]
12	Alfalfa	Achieving genome editing through CRISPR	*uidA, & NOD26*	Successfully mutated GUS with no activity; 11% mutation efficiency was found in NOD26 gene	[49]
13	Alfalfa	Understanding genes associated with growth and biomass development	*MsSPL8*	Successful mutations in 3–4 *MsSPL8* alleles; reduced leaf size and early flowering; decrease in internodal length and plant height	[50]
14	Bridsfoot trefoil	Identification of genes involved in symbiotic association of N_2 fixing bacteria in legumes	*SYMRK*	35% mutation efficiency with 2 biallelic homozygous mutagenic events	[51]

References

1. Vasconcelos, M.W., Grusak, M.A., Pinto, E., et al.: The biology of legumes and their agronomic, economic, and social impact. In: The Plant Family Fabaceae, pp. 3–25. Springer, Singapore (2020)
2. Weiss, E., Zohary, D.: The neolithic southwest Asian founder crops their biology and archaeobotany. Curr. Anthropol. **52**, 237–254 (2011)
3. Singh, M., Upadhyaya, H.D., Bisht, I.S.: Introduction. Genetic and Genomic Resources of Grain Legume Improvement, pp. 1–10. Elsevier (2013)
4. Dutta, A., Trivedi, A., Nath, C.P., Gupta, D.S., Hazra, K.K.: A comprehensive review on grain legumes as climate-smart crops: challenges and prospects. Environ. Challenges. **7**, 100479 (2022)
5. Ferreira, H., Pinto, E., Vasconcelos, M.W.: Legumes as a cornerstone of the transition toward more sustainable Agri-food systems and diets in Europe. Front. Sustain. Food Syst. **5**, 1–9 (2021)
6. Hughes, J., Pearson, E., Grafenauer, S.: Legumes—A comprehensive exploration of global food-based dietary guidelines and consumption. Nutrients. **14**(15), 3080 (2022)

7. Kumar, Y., Basu, S., Goswami, D., Devi, M., Shivhare, U.S., Vishwakarma, R.K.: Anti-nutritional compounds in pulses: implications and alleviation methods. Legum. Sci. **4**, **e111** (2022)
8. Singh, J., Basu, P.S.: Non-nutritive bioactive compounds in pulses and their impact on human health: an overview. Food Nutr. Sci. **3**(12), 1664–1672 (2012)
9. Bouchenak, M., Lamri-Senhadji, M.: Nutritional quality of legumes, and their role in cardio-metabolic risk prevention: a review. J. Med. Food. **16**(3), 185–198 (2013)
10. Zander, P., Amjath-Babu, T.S., Preissel, S., Reckling, M., Bues, A., Schläfke, N., Kuhlman, T., Bachinger, J., Uthes, S., Stoddard, F., Murphy-Bokern, D., Watson, C.: Grain legume decline and potential recovery in European agriculture: a review. Agron. Sustain. Dev. **36**(26), 20 (2016)
11. Jensen, E.S., Peoples, M.B., Hauggaard-Nielsen, H.: Faba bean in cropping systems. F. Crop. Res. **115**(3), 203–216 (2010)
12. Joshi, P.K., Asokan, M., Datta, K.K., Kumar, P.: Socioeconomic Constraints to Legumes Production in rice-Wheat Cropping Systems of India. Data and Sample, pp. 176–184. Cornell University, Ithaca (1994)
13. Varshney, R.K., Kudapa, H., Pazhamala, L., et al.: Translational genomics in agriculture: some examples in grain legumes. CRC. Crit. Rev. Plant Sci. **34**, 169–194 (2015)
14. Punia, M., Rolaniya, K.L., Jat, R.L.: Recent advancement in genetic improvement of food legume crops. In: Plant breeding- New perspectives (2022)
15. Bauchet, G.J., Bett, K.E., Cameron, C.T., et al.: The future of legume genetic data resources: challenges, opportunities, and priorities. Legume Sci. **1**, **e16** (2019)
16. Bhowmik, P., Konkin, D., Polowick, P., Hodgins, C.L., et al.: CRISPR/Cas9 gene editing in legume crops: opportunities and challenges. Legume Sci. **3**, **e96** (2021)
17. GreenaWay, P.J.: Basic steps in genetic engineering. Int. J. Environ. Stud. **15**(1), 11–24 (1980)
18. Abbas, M.S.T.: Genetically engineered (modified) crops (Bacillus thuringiensis crops) and the world controversy on their safety. Egypt. J. Biol. Pest Control. **28**(52), 1–12 (2018)
19. Rouet, P., Smih, F., Jasin, M.: Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol. Cell. Biol. **14**(12), 8096–8106 (1994)
20. Liu, M., Zhang, W., Xin, C., Yin, J., Shang, Y., Ai, C., Li, J., Meng, F.L., Hu, J.: Global detection of DNA repair outcomes induced by CRISPR-Cas9. Nucleic Acids Res. **49**(15), 8732–8742 (2021)
21. De Souza, N.: Primer: genome editing with engineered nucleases. Nat. Methods. **9**, 27 (2012)
22. Bibikova, M., Golic, M., Golic, K.G., Carroll, D.: Targeted chromosomal cleavage and mutagenesis in drosophila using zing-finger nucleases. Genetics. **161**(3), 1169–1175 (2002)
23. Li, T., Huang, S., Zhao, X., Wright, D.A., Carpenter, S., Spalding, M.H., Weeks, D.P., Yang, B.: Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Res. **39**(14), 6315–6325 (2011)
24. Khalil, A.M.: The genome editing revolution: review. J. Genet. Eng. Biotechnol. **18**(1), 68 (2020)
25. Hille, F., Richter, H., Wong, S.P., Bratovič, M., Ressel, S., Charpentier, E.: The biology of CRISPR-Cas: backward and forward. Cell. **172**(6), 1239–1259 (2018)
26. Rath, D., Amlinger, L., Rath, A., Lundgren, M.: The CRISPR-Cas immune system: biology, mechanisms and applications. Biochimie. **117**, 119–128 (2015)
27. Curtin, S.J., Michno, J.M., Campbell, B.W.: MicroRNA maturation and microRNA target gene expression regulation are severely disrupted in soybean dicer-like1 double mutants G3. Genes, Genomes, Genet. **6**(2), 423–433 (2016)
28. Curtin, S.J., Zhang, F., Sander, J.D.: Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol. **156**, 466–473 (2011)
29. Haun, W., Coffman, A., Clasen, B.M., et al.: Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol. J. **12**(7), 934–940 (2014)
30. Wen, S., Liu, H., Li, X., Chen, X., Hong, Y., Li, H., Lu, Q., Liang, X.: TALEN-mediated targeted mutagenesis of fatty acid desaturase 2 (FAD2) in peanut (Arachis hypogaea L.) promotes the accumulation of oleic acid. Plant Mol. Biol. **97**(1–2), 177–185 (2018)

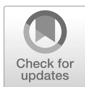
31. Demorest, Z.L., Coffman, A., Baltes, N.J., et al.: Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. BMC Plant Biol. **16**(225), 1–8 (2016)
32. Curtin, S.J., Xiong, Y., Michno, J.M., Campbell, B.W., Stec, A.O., Čermák, T., Starker, C., Voytas, D.F., Eamens, A.L., Stupar, R.M.: CRISPR/Cas9 and TALENs generate heritable mutations for genes involved in small RNA processing of Glycine max and Medicago truncatula. Plant Biotechnol. J. **16**(6), 1125–1137 (2018)
33. Gleditzsch, D., Pausch, P., Müller-Esparza, H., Özcan, A., Guo, X., Bange, G., Randau, L.: PAM identification by CRISPR-Cas effector complexes: diversified mechanisms and structures. RNA Biol. **16**(4), 504–517 (2019)
34. Ochatt, S., Conreux, C., Moussa, M., Colo, R., Despierre, G., Magnin-Robert, J.B., Raffiot, B.: Phytosulfokine-alpha, an enhancer of in vitro regeneration competence in recalcitrant legumes. Plant Cell Tissue Organ Cult. **135**, 189–201 (2018)
35. Yadav, R., Mehrotra, M., Singh, A.K., Niranjan, A., Singh, R., Sanyal, I., Lehri, A., Pande, V., Amla, D.V.: Improvement in agrobacterium-mediated transformation of chickpea (Cicer arietinum L.) by the inhibition of polyphenolics released during wounding of cotyledonary node explants. Protoplasma. **254**(1), 253–269 (2017)
36. Zheng, N., Li, T., Dittman, J.D., Su, J., Li, R., Gassmann, W., Peng, D., Whitham, S.A., Liu, S., Yang, B.: CRISPR/Cas9-based gene editing using egg cell-specific promoters in Arabidopsis and soybean. Front. Plant Sci. **11**, 1–15 (2020)
37. Lu, Q.S.M., Tian, L.: An efficient and specific CRISPR-Cas9 genome editing system targeting soybean phytoene desaturase genes. BMC Biotechnol. **22**(7), 1–16 (2022)
38. Zhang, P., Du, H., Wang, J., Pu, Y., Yang, C., Yan, R., Yang, H., Cheng, H., Yu, D.: Multiplex CRISPR/Cas9-mediated metabolic engineering increases soya bean isoflavone content and resistance to soya bean mosaic virus. Plant Biotechnol. J. **18**(6), 1384–1395 (2020)
39. Cai, Y., Wang, L., Chen, L., Wu, T., Liu, L., Sun, S., Wu, C., Yao, W., Jiang, B., Yuan, S., Han, T., Hou, W.: Mutagenesis of GmFT2a and GmFT5a mediated by CRISPR/Cas9 contributes for expanding the regional adaptability of soybean. Plant Biotechnol. J. **18**(1), 298–309 (2020)
40. Do, P.T., Nguyen, C.X., Bui, H.T., Tran, L.T.N., Stacey, G., Gillman, J.D., Zhang, Z.J., Stacey, M.G.: Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous GmFAD2-1A and GmFAD2-1B genes to yield a high oleic, low linoleic and α-linolenic acid phenotype in soybean. BMC Plant Biol. **19**(311), 1–14 (2019)
41. Li, C., Nguyen, V., Liu, J., Fu, W., Chen, C., Yu, K., Cui, Y.: Mutagenesis of seed storage protein genes in Soybean using CRISPR/Cas9. BMC Res. Notes. 12. **176**(1), 1–7 (2019)
42. Bao, A., Chen, H., Chen, L., et al.: CRISPR/Cas9-mediated targeted mutagenesis of GmSPL9 genes alters plant architecture in soybean. BMC Plant Biol. **19**(131), 1–12 (2019)
43. Wang, J., Kuang, H., Zhang, Z., Yang, Y., Yan, L., Zhang, M., Song, S., Guan, Y.: Generation of seed lipoxygenase-free soybean using CRISPR-Cas9. Crop J. **8**(3), 432–439 (2020)
44. Che, P., Chang, S., Simon, M.K., Zhang, Z., Ourada, J., O'Neill, D., Torres-Mendoza, M., Guo, Y., Marasigan, K.M., Vielle-Calzada, J.P., Ozias-Akins, P., Albertsen, M.C., Jones, T.J.: Developing a rapid and highly efficient cowpea regeneration and transformation system using embryonic axis explants. Plant J. **106**(3), 817–830 (2021)
45. Badhan, S., Ball, A.S., Mantri, N.: First report of CRISPR/Cas9 mediated DNA-free editing of 4CL and RVE7 genes in chickpea protoplasts. Int. J. Mol. Sci. **22**(1), 1–15 (2021)
46. Gupta, S.K., Vishwakarma, N.K., Malakar, P., Vanaspati, P.: Development of an agrobacterium -delivered codon-optimized CRISPR/Cas9 system for cheakpea genome editing. Protoplasma. **260**(5), 1437–1451 (2023)
47. Li, G., Liu, R., Xu, R.: Development of an agrobacterium-mediated CRISPR/Cas9 system in pea (Pisum sativum L.). Crop J. **11**(1), 132–139 (2023)
48. Yuan, M., Zhu, J., Gong, L., He, L., Lee, C., Han, S., Chen, C., He, G.: Mutagenesis of FAD2 genes in peanut with CRISPR/Cas9 based gene editing. BMC Biotechnol. **19**, 1–7 (2019)

49. Bottero, E., Massa, G., González, M., Stritzler, M., Tajima, H., Gómez, C., Frare, R., Feingold, S., Blumwald, E., Ayub, N., Soto, G.: Efficient CRISPR/Cas9 genome editing in alfalfa using a public germplasm. Front. Agron. **3**, 1–8 (2021)
50. Singer, S.D., Burton Hughes, K., Subedi, U., Dhariwal, G.K., Kader, K., Acharya, S., Chen, G., Hannoufa, A.: The CRISPR/Cas9-mediated modulation of squamosa promoter-binding protein-like 8 in Alfalfa leads to distinct phenotypic outcomes. Front. Plant Sci. **12**, 1–17 (2022)
51. Wang, L., Wang, L., Tan, Q., Fan, Q., Zhu, H., Hong, Z., Zhang, Z., Duanmu, D.: Efficient inactivation of symbiotic nitrogen fixation related genes in Lotus japonicus using CRISPR-Cas9 front. Plant Sci. **7**, 1–13 (2016)
52. Abdelrahman, M., Wei, Z., Rohila, J.S., Zhao, K.: Multiplex genome-editing Technologies for Revolutionizing Plant Biology and Crop Improvement. Front. Plant Sci. **12**, 1–15 (2021)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 17
Soybean Improvement and the Role of Gene Editing



Nihal Öztolan Erol

Abstract Soybean is a major agricultural crop that is used for food, feed, and industrial products. However, soybean production is facing several challenges, including pests, diseases, and environmental factors. In recent years, there has been a growing interest in using gene editing technologies to improve soybean traits. Gene editing technologies offer a promising new approach to improving soybean production and quality.

Gene editing technologies can be used to precisely alter the soybean genome. There are a number of different gene editing technologies that can be used to improve soybeans. One of the most commonly used technologies is CRISPR/Cas9, which uses a protein called Cas9 to cut DNA at a specific location. This can be used to insert, delete, or modify genes. Other gene editing technologies include zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs). Gene editing technologies have the potential to revolutionize soybean breeding. This can be used to introduce new traits, such as resistance to pests and diseases, or to improve existing traits, such as yield and oil content.

The use of gene editing technologies in soybean improvement is still in its early stages, but the potential benefits are significant. Gene editing technologies offer a more precise and efficient way to improve soybean production than traditional breeding methods. They also offer the potential to create new varieties of soybeans that are better able to meet the challenges of a changing world.

1 Soybean Production and Its Economic Value

Soybean is a very important crop that provides substantial oil and protein nutrition for the increasing human population. Soybean cultivation has been rooted back in ancient times c. 6000–9000 years ago, in East Asia [1]. Its massive production has reached its highest in the last century with the help of improving breeding

N. Ö. Erol (✉)
Sabancı University Nanotechnology Research and Application Center, İstanbul, Turkey
e-mail: nihal.oztolan@sabanciuniv.edu

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_17

techniques. Soybean production has increased since 1961 from 20–30 million tons to 350 million tons per year [2].

Soybean is very rich in oil and protein compared to other members of the legume family. Therefore, it meets a considerable demand for animal feed and oil production. Over three-fourths of soybean by weight is used for feeding livestock, poultry, and aquaculture production, so some countries increasingly export soybean products while others import to meet the demand for soybean-based animal feed. The rest is consumed by humans as an industrial oil, biofuel, food ingredients (lecithin, emulsifier, and proteins), and food (soy sauce, tempeh, soy milk, and tofu). Soybean is introduced as a rich protein source for plant-based diets as it consists of 40% of the dry matter including nine essential amino acids. Therefore, it is very important for the vegetarian and vegan diet, it provides high nutrition with its protein content [3]. Soybean seeds are the most important part of the plant, so throughout the domestication process, traits improving soybean seed quality and yield have been artificially selected for efficient utilization in the food industry and agriculture.

Soybean domestication has led to a significant reduction in genetic diversity due to selective sweeps, resulting in the fixation of beneficial traits. Studies have shown that nucleotide fixation during soybean domestication and improvement has resulted in a reduction of genetic diversity compared to wild soybean populations. Furthermore, the fixation of key genes involved in the regulation of traits such as seed size, pod dehiscence, and photoperiodic flowering has played a crucial role in shaping the morphology and adaptation of soybean to different environments. These genetic changes have contributed to increased yield and better adaptation to a range of environmental conditions, making soybean a globally important crop. However, the reduced genetic diversity resulting from selective sweeps also raises concerns regarding the resilience and adaptability of soybean crops in the face of new and changing environmental challenges.

2 Genetically Modified Soybean

Genetic modification of an organism traces back to the domestication of organisms. However, public perception misinterprets this term and people think that genetic modification of organisms came out with the developments in biotechnology in the late twentieth century. Breeding practices have long been used by humans and are striking evidence of genetic modification. With the discovery of recombination techniques in bacteria, genetic modification techniques have been gradually improved over the years and used first in producing medicines and then crops.

Recombinant DNA technologies are the fundamentals of genetic modifications in living organisms. Briefly, a target gene cassette-containing vector is transferred by a virus or a bacterium into living cells of an organism to insert a specific genetic sequence into that organism's genome. The first genetically modified soybean was produced in the 1990s. Glyphosate-resistant soybean cultivation together with the glyphosate herbicide dramatically decreased labour that occurred due to tillage of

the soil, at the same time this dual application increased genetically modified soybean production.

Despite its recalcitrant nature of regeneration under tissue culture techniques, several studies showed that soybean has been used for gene editing of flowering time, seed oil content, lateral root growth, and defence mechanism [4] (Table 17.1).

Curtin et al. (2011) first published the research about hairy-root and whole-plant transformation mediated by *Agrobacterium rhizogenes* using the zinc-finger nuclease (ZFN) method to target *DICER LIKE (DCL), RNA-DEPENDENT RNA POLYMERASE (RDR),* and *HUA ENHANCER1 (HEN1)* genes in the root cells [5].

Table 17.1 Soybean traits and associated genes that are modified by gene editing techniques CRISPR/Cas9, zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), *Agrobacterium tumafaciens,* and bean pod mottle virus mediated transformations

Trait	Gene	Gene editing technique	References
Pod shattering	*GmPdh1*	CRISPR/Cas9	[27]
Determinacy	*GmTfl1*		[33]
	GmSOC1		[35]
	GmLHY		[32]
Photoperiodicity	*GmFT2*		[39]
	GmE2		[42]
	GmPRR37		[15]
	GmFKF1		[37]
	GmCPDK38		[43]
Photomorphogenesis and flowering time	*GmPHYA/ GmPHYB*		[38]
Seed sugar transportation, seed oil and protein content	*GmSWEET10*		[15]
Seed sugar transportation, seed oil and protein content Stachyose content	*GmMFT* *GmSTS*		[48] [17]
Rafinose content	*GmRS*		[16]/ [17]
Lipoxigenase	*GmLox*		[15]
Salt stress tolerance	*GmAITR*		[66]
Seed size	*GmSSS1*		[54]
Seed thickness	*GmST1*		[74]
	GmDCL		[5]/ [6]
	GmRDR	ZFN	[5]
	GmHEN1	ZFN/TALEN	[5]
Fatty acid	*GmFAD2–1*		[7]/ [8]
Fatty acid Albino	*GmFAD3* *GmPDS*	TALEN TALEN	[9] [10]
Drought resistance	*GmNFYA13*	*Agrobacterium tumefaciens*	[64]
Seed hardness	*GmHs1*	*Agrobacterium tumefaciens*	[44]
Seed size, biotic and abiotic stress resistance	*GmFAD3*	Bean pod mottle virus	[53]

They continued using the ZFN method to create double mutants of *DCL1a* and *DCL1b* for *Agrobacterium rhizogenes*-mediated whole-plant transformation [6]. ZFNs were also used to deliver multiple different DNA donors in *the FAD2-1a* locus (Glyma.10 g278000) by using a biolistic bombardment technique on immature embryo explants [7]. This research successfully regenerated fertile plants and transmitted the insert to the next generation.

FAD2-1a and *FAD2-1b* loci were mutated by using transcription activator-like effector nucleases (TALEN), to convert oleic acid into linoleic acid to increase the polyunsaturated fatty acid. The study used the hairy-root transformation method mediated by *Agrobacterium rhizogenes* [8]. The same research group also targeted *FAD2-1a, FAD2-1b,* and *FAD3* to convert linoleic acid into oleic acid by using the TALEN technique and again successfully transformed soybean immature embryo explants [9].

Du et al. (2016) conducted a study to compare two gene editing techniques, TALEN and CRISPR-Cas9 in parallel with testing the transformation efficiency by using soybean-specific *U6-10* and *Arabidopsis*-specific *U6-26* promoters in soybean [10]. They targeted a gene encoding phytoene desaturase (PDS), a rate-limiting enzyme involved in the carotenoid biosynthesis pathway. Hairy root transformation mediated by *Agrobacterium rhizogenes* successfully resulted in mutated buds. The study suggested the usage of CRISPR/Cas9 with species-specific promoters to acquire a highly transformation-efficient, cost-efficient, and easy-to-construct transformation technique [10].

CRISPR/Cas9 technique outperforms other precise gene editing techniques by its cost-efficient and easily applicable features. This technique accelerates soybean breeding and supports soybean production. Cytoplasmic male sterility (*GmAMS1*) [11], flowering time (*LNK2*) [12], seed oil content (*GmFAD2*) [13, 14]), seed lipoxygenase, raffinose, and stachyose contents (*GmLox, GmRS, GmSTS*) [15–17], plant growth and structure (*GmLHY* and *GmSPL9*) [18, 19] are some of the traits, which have a significant role in soybean breeding, that were studied in the last decade.

Investigation of causal alleles of certain traits has provided the most crucial information for gene editing applications. QTL mapping, using GWAS and linkage mapping analysis, along with functional genetic mutations unravel the causative nucleotide changes. With the introduction of nucleic acid-cutting enzymes and nucleases, gene editing breathes new life into plant breeding by precise editing, and soybean breeding will definitely benefit from this transformative new breeding techniques.

3 Agronomically Important Soybean Traits and the Use of Gene Editing

Soybean breeding plays a crucial role in the production of soybeans around the world as it helps to develop soybean varieties that can adapt to different environmental conditions. By breeding soybean varieties that are good quality, and tolerant to various biotic and abiotic stresses, soybean production can be increased and stabilized. Additionally, breeding efforts have resulted in soybean varieties with

desirable traits such as high yield, improved nutritional quality, and enhanced oil and protein content, which are important for meeting the increasing demand for soybean products worldwide. Overall, soybean breeding has been instrumental in improving soybean production by developing varieties that are better adapted to the diverse environmental conditions in different regions of the world. A rapid and precise gene editing might help to improve elite soybean varieties. Soybean improvement might be accelerated by introducing a non-synonymous mutation with the help of gene editing.

Although trading and migrating routes had caused the dissemination of a certain type of cultivated soybean seeds towards Eastern Asia and North America, local landraces had provided efficient genetic resources for soybean breeding in adaptation to the environment. The idea of a single origin of soybean domestication does not completely explain the existence of allelic variation among cultivated varieties. Because local genetic diversity had provided location-specific causal alleles associated with the traits of interest. Therefore, to improve plants' yield capacity and seed quality in terms of oil and protein, there were several genes functionally identified to be responsible for plant architectural, physiological, and morphological changes in organs by using CRISPR/Cas9 method.

3.1 Pod Shattering Resistance

Pod shattering resistance, to prevent seed dispersal and yield loss, is an important agronomical trait that has come along with domestication [20]. Angiosperms develop their seeds within the fruit and disperse them when there is an abscission between pedicel and lemma. This decreases the harvest output and was taken under control by artificially selecting pod-shattering resistant plants. Four pod-shattering resistance-associated genes were identified in soybean: *GmSHAT1-5, Pdh1, NST1A,* and *Glyma09g06290.*

Dong et al., (2014) identified a causal polymorphism in *the GmSHAT1-5* gene and the pod-shattering resistant domesticated soybeans, which were diversified from wild soybeans, derived from this single haplotype [21]. *GmSHAT1-5* is responsible for the lignification of fiber cap cells in the pod ventral suture which causes thickening in domesticated soybeans. The sample collection included both *Glycine max* and *Glycine soja* varieties gathered from the seed bank of the Chinese Academy of Agricultural Sciences (Beijing). The pod indehiscent allele from *Glycine max* showed a 13-fold higher expression than *Glycine soja*. It seems like domestication significantly affects pod-shattering traits; however, this research did not reveal the origin of the indehiscent allele.

Zhang and Singh (2020) identified a locus called *NST1A,* which showed epistasis with *Pdh1*. *NST1A* was a NAC family gene, a paralog of *GmSHAT1-5* [22]. Likewise, in NAC family transcription factors in *Arabidopsis thaliana*, a premature stop codon was identified to be responsible for gain-of-function mutation, where it provided pod shattering-resistance despite the existence of *the Pdh1* allele [22, 23]. The indehiscent *NST1A* allele was predominantly found in Southern China and Japan, this

implies that local wild cultivars in those regions were selected for the indehiscent *NST1A* allele independent of low humid conditions.

A genome-wide association study genotyped 211 soybean accessions including modern and wild cultivars collected from the National Center for Soybean Improvement of China by using NJAU 355 K SoySNP array containing 282,469 SNPs. A quantitative trait locus was identified on chromosome nine and within that locus, a candidate gene *Glyma09g06290* was found homologous to *Arabidopsis thaliana* basic helix-loop-helix, a gene responsible for silique dehiscence. Quantitative polymerase chain reaction analysis also indicated that *the Glyma09g06290* gene was highly expressed in pod indehiscent varieties [24].

Another gene regulating pod shattering in the domesticated soybean is *Pdh1*. *Pdh1* showed high homology to dirigent family genes, which were initially known as a stereoselective bimolecular phenoxy radical coupling of (E)-coniferyl alcohol, for producing lignan [25]. The functional *Pdh1* was found to be highly expressed in the lignified inner-sclerenchyma cells of the seed pod [26]. The inner sclerenchyma physical properties changed when *Pdh1* expression increased, and pod shattering started. As the relation between *Pdh1* and lignin was not clear yet, the gene might be responsible for lignin deposition in the seed pod. A non-synonymous nucleotide substitution that produces a stop codon results in pod-shattering-resistant varieties. Under low humidity *pdh1* allele containing soybeans showed significantly lower shattering scores than those with the *Pdh1* allele. This pod shattering-resistance associated allele was seen in more than 50% of Chinese and a considerable proportion of South Asian and North American landraces. However, Japanese and Korean landraces showed a very low frequency of this allele. The origin of domestication by selecting the indehiscent *Pdh1* allele might be originated from Huang-Huai-Hai Valley [22]. This infers that low humidity conditions provided selective pressure on *the pdh1* allele to protect seeds from dispersion. Zhang et al. (2022) provided a CRISPR/Cas9 gene editing solution for pod shuttering-susceptibility in a summer adapted soybean cultivar HC6 found in Huang-Huai-Hai [27]. They performed QTL mapping by using a recombinant inbred line population of HC6 and a pod shattering-resistant variety JD12 and they found a reproducible major allele at the *Pdh1* locus, SNP A/T that causes a nonsense variant (HC6/JD12). The resistant allele T was associated with low humidity regions in China, whereas the susceptible one A with high humidity regions in China, Japan, and Korea. Having known the contrasting effect, causal allele in different haplogroups facilitated the application of CRISPR/Cas9, the precise gene editing. This finally provided a gene therapy for pod shattering in soybean cultivars.

3.2 Shoot Growth Habit

Planting and harvesting time remarkably affect soybean yield, therefore, farmers must choose the appropriate maturity type regarding the environmental conditions. Soybean determinacy is an important agronomic trait that identifies the maturity type. Determinacy is governed by genes and environmental signals, which control

the generation of shoot apical meristem and transition to floral meristem. Soybean can be classified into three groups of determinacies: determinate, semi-determinate, and indeterminate. Indeterminate varieties, which are late maturing, show a prolonged vegetative phase with active stem and branch apices producing new nodes with leaves. Whereas determinate varieties, which are early maturing, cease stem and branch apical growth with photo-periodical floral induction.

Phenotypic variation amongst soybean landraces provided a good genetic resource for soybean breeding. Soybean planting management aims to maximize yield capacity and quality. It was found that, when indeterminate varieties are early-planted, they maintain an active vegetative growth for a long time and adequately accumulate amino acids and nutrients to allocate them towards seeds to increase yield quality and capacity. On the other hand, when determinate varieties are late-planted, yield capacity and moisture decrease. However, early planting of indeterminate soybean varieties can carry some risks. For example, late frost or extended pathogen infection might cause to decrease in yield capacity and even delay harvesting. To avoid the risks, determinate and indeterminate varieties are planted accordingly to maximize soybean production in the field [28–30].

In the cultivated soybean varieties, two genetic loci were identified to be associated with the determinacy trait: *Dt1* and *Dt2*. The *Dt1* allele is dominant or incompletely dominant on the *dt1* allele; the *Dt2* allele is dominant on the *dt2* allele. Soybean plants with *Dt1/Dt1* genotype are identified as indeterminate with *dt2/dt2* and semi-determinate with *Dt2/Dt2*. However, the *dt1/dt1* genotype shows a determinate phenotype when the *Dt2* locus is either recessive or dominant homozygous or heterozygous. Therefore, the *Dt1* locus has an epistatic effect on the *Dt2* locus [31, 32]. Their antagonistic behavior regulates flowering time and plant stem growth.

Dt1 is induced by *E3* and *E4* under long day conditions, interacts with bZIP family transcription factor FDc1, and binds to the promoter of *APETALA1* for delaying flowering. On the other way, when *APETALA1* binds to the promoter of *Dt1*, it inhibits its expression, thus promotes flowering [33]. *Dt1* locus encodes a phosphatidylethanolamine-binding protein (PEBP) family protein called GmTfl1 (or GmTfl1b) which is an ortholog of Arabidopsis TERMINAL FLOWER1 that controls plant height and internode length. *GmTfl1b* is expressed in the shoot apical meristem until flowering initiation [34]. Four independent single nucleotide polymorphisms on *GmTfl1* were identified in *Glycine max*, which makes non-synonymous amino acid changes, whereas these nucleotide changes were not observed in *Glycine soja.* This infers that the determinacy trait was introduced by the domestication process of soybean.

Four homologous *Tfl1* genes have been found in soybean. *Tfl1b* has been already known but the functions of other homologous genes are still not clear. Wang et al. (2023) identified the function of *Tfl1c* and *Tfl1d* by using CRISPR/Cas9 knock out double mutation [33]. Results showed that *tfl1c/tfl1d* double mutant soybeans flowered earlier than the wild type. The interaction of these homologous genes with *APETALA1* was also confirmed. Likewise*, Tfl1c* and *Tfl1d* interacted with FDc1 and inhibited four homologous genes of *APETALA1* and thus delaying flowering.

Dt2 encodes the MADS-domain factor that binds to the promoter of *Dt1* and inhibits the plant stem growth to start the transition from the vegetative phase to the

reproductive phase in soybean. There are two homologs of *the SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* gene, known as the positive regulator of flowering which is activated downstream of *FLOWERING LOCUS T* (*FT*) in *Arabidopsis thaliana* [35], in soybean: *GmSOC1a* and *GmSOC1b.* These homologous genes interact in parallel with the *Dt2* locus and initiate flowering and inhibit stem elongation and node generation. A CRISPR/Cas9 gene knock out application mediated by *Agrobacterium tumefaciens* produced homozygous *soc1a, soc1b,* and *soc1a/soc1b* null mutants. Due to the lack of interaction between *Dt2* and *SOC1* in these mutant soybeans, *Dt1* expression was increased that caused delayed flowering.

Gibberellic acid biosynthesis is also very important for prolonged shoot growth. Plant height regulator genes in soybean, such as the dwarf gene *GmDW1, LATE ELONGATED HYPOCOTYL* (*GmLHY*), and an R2R3 MYB transcription factor *GmGAMYB* positively regulate gibberellic acid pathway [32]. A CRISPR/Cas9 knock out mutation application mediated by *Agrobacterium tumefaciens* produced a quadruple *LHY* mutant soybean (*Gmlhy1a,1b,2a,2b*), where it showed decreased plant height and gibberellic acid (GA3) level [19].

These functionally identified genes have provided insights into the molecular mechanisms underlying determinacy in soybean. Further studies utilizing techniques like CRISPR/Cas9 gene editing have deepened our understanding of these genes' functions and their interactions. Overall, unravelling the genetic and environmental factors controlling determinacy in soybean will contribute to the development of improved varieties with enhanced yield capacity and quality.

3.3 Photo-Periodicity

Soybean is a photo-period sensitive short-day plant, so it does not flower under long-day conditions. However, its adaptation to large latitudes requires a range of genetic variations of short-day activated genes. A group of genes has been identified to be involved in soybean latitudinal adaptation by regulating floral initiation.

Cryptochromes are generally known as blue-light receptor proteins involved in plant development and circadian clock. In *Arabidopsis thaliana* CRY1 was found to be responsible for mediation of blue-light induced de-etiolation, and CRY2 for photo-periodic flowering. *GmCRY1a* promotes blue-light-induced cell-wall elongation inhibition and, in contrast to *Arabidopsis thaliana,* regulates photo-periodic flowering by increasing the expression of *FT* mRNA [36].

The maturity loci in soybean, *E1, E2, E3,* and *E4* have a predominant effect on mediating photo-periodic flowering and maturity. The dominant *E1, E2, E3,* and *E4* allele delay flowering and the recessive allele facilitates adaptation to high latitudes by promoting early maturing, whereas *J, FT2a, FT5a, Time of flowering (Tof)16,* and *Tof18* facilitate adaptation to low latitudes [1, 37]. *J* locus, encoding a homolog of *EARLY FLOWERING 3 (ELF3)* in *Arabidopsis thaliana,* functions as a suppressor of the *E1* allele and promotes early flowering. On the other hand, *E1* suppresses *FT2a* and *FT5a,* and also, two homologs of the red-light photoreceptor phytochrome A (phyA) *E3* and *E4* interact with plant circadian clock evening complex (LUX)

and *E1* to delay flowering [37, 38]. Therefore, the phyA-LUX-E1-FT pathway regulates photo-periodic floral initiation in soybean. Cai et al. (2018) produced *GmFT2a* frameshift mutant soybeans having a 1 base-pair insertion or short deletion by applying sgRNAs CRISPR/Cas9 vectors through *Agrobacterium tumefaciens* mediated transformation [39]. They showed that the *ft2a* soybeans were flowering later than the wild types under short and long day photoperiodic conditions. Another study that targets the photoperiodicity of a short-day soybean variety showed that a frameshift mutation of *E1* caused truncated protein production. This unfunctional proteins thus disinhibited *GmFT2a/5a* and therefore, initiated flowering under long day conditions [40]. Another flowering repressor gene *GmPRR37* was identified by Wang et al. (2020) [41]. A CRISPR/Cas9 knock-out mutant *gmprr37* showed early flowering under long day conditions. Wang et al. (2023) clarified the function of *E2* and its homologous genes *E2-Like a* and *E2-Like b* [42]. They designed a single and double mutants of *E2* and its homologous genes through CRISPR/Cas9 knock out method to investigate their function in flowering and grain yield and their interaction with *E1*. It was found that *E2* is the major regulator of flowering but *E2-Like a* and *E2-Like b* were redundant. Only double mutants *e2/e2-like a* or *e2/e2-like b* initiated flowering earlier than *e2* types. Additionally, Li et al. (2022) discovered the interaction between photoperiodicity and plant protection by identifying the function of *GmCDPK38*, a calcium-dependent protein kinase encoding gene [43]. A cutworm susceptible soybean was mutated through CRISPR/Cas9 and *GmCDPK38* sgRNA vectors delivered by *Agrobacterium tumefaciens. gmcdpk38* soybeans delayed flowering and produced more defence-related metabolites under long-day conditions. This might suggest a beneficial soybean improvement strategy for growing resistant soybean at low latitudes. Recently, a new locus identified from GWAS, named *Tof8,* encodes a homolog of *the Arabidopsis thaliana FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1)* gene. There are two orthologous *FKF1* genes were found in soybean: *FKF1a* and *FKF1b.* These orthologous genes were found to be involved in flowering time and maturity by activating *E1* transcription through CRISPR/Cas9 knock-out mutation and two haplotypes of *FKF1b* were identified to be a part of regulating the latitudinal effect on flowering which might be a cause of natural selection during environmental adaptation [37].

Soybean flowering is dependent on a short photoperiod, so it usually flowers in spring or fall. Unravelling the mechanism underlying the latitudinal effect on flowering time will support soybean breeding across a wide range of latitudes. Moreover, it will be possible to produce long-day soybean varieties through genetic manipulation to speed up the breeding process.

3.4 Seed Quality

Seed traits determine the quality of the product. The seed coat, seed oil, oligosaccharides, lipoxigenases and protein contents are the important seed traits, which were improved during the domestication process. Breeding practices has shaped the modern soybean seed traits and improved the plant for a better human digestibility,

consumption, harvestability and endurance. Gene editing accelerates this process by functionally identifying the genes of interest associated with the desired seed traits.

Improved seed coat water impermeability protects embryos for sustainable seed storage and provides resistance to environmental deterioration in the field, such as pathogens, and mechanical and imbibition damages. Seed coat impermeability correlates with seed viability and longevity in soybean breeding programs. Additionally, hard seeds contain high calcium levels and increase the nutritional value of soyfoods [44]. As an adverse effect, hard seededness is unfavourable during post-harvest processing for vegetable oil and soyfood [45, 46]. Indeed, high seed water permeability facilitates water absorption and makes the seed easy to germinate. Jang et al. (2015) identified a seed hardness locus q*HS1*, which encodes an endo-1,4-β-glucanase [45]. A single nucleotide substitution from A to G in this gene dysfunctions the substrate-enzyme cleft domain and causes permeable seed coat in soybean. Likewise, a single nucleotide substitution from C to T by using *Agrobacterium tumefaciens* mediated transformations in *the GmHs1–1* gene, which encodes a calcineurin-like metallophosphoesterase transmembrane protein, showed increased permeability in the soybean seed coat [44]. Chandra et al. (2020) made inter-specific crosses between *Glycine max* and *Glycine soja* to understand the genetic inheritance of seed coat impermeability by using 217 recombinant inbred lines [46]. They phenotyped seed coat impermeability by looking into slow and rapid imbibition rates of the offspring. They identified three linked markers on chromosome 2, this locus was previously identified by Sun et al. (2015) and Jang et al. (2015) [44, 45]. Additionally, the phenotyping results revealed semi-permeable genotypes that might cause by minor alleles, and one of them was found to be associated with leaflet width, phytophthora resistance, and seed tocopherol. This implies that seed coat-identifying genes diversify in nature, and they maintain seed protection and coat-related alleles. The process of soybean breeding to improve seed coat impermeability should consider the involvement of the minor alleles as a potential genetic gain.

Another seed trait that was subjected to artificial selection during the domestication process is seed oil content. Cultivated soybean seeds contain more oil than wild seeds, which shows the effect of domestication on selecting high oil capacity in the seeds [47]. Soybean oil has a great economical value in the market, after palm oil, it is the second most-produced vegetable oil in the world between 2018 and 2023. China is the world leader in the production and consumption of soybean oil (USDA, 2023). It is used for human consumption and shows tremendous health benefits. Soybean oil consists of 15% saturated and 85% unsaturated fatty acids, which is responsible for lowered blood cholesterol levels, and decreased coronary heart disease [48]. On the other hand, soybean seeds became one of the most consumed vegan proteins for vegetarian and vegan diets. Seed oil increases with sugar mobilization towards the embryo. The underlying reason was investigated and found that sugar flux-controlling genes are upregulated and provide precursors for fatty acid biosynthesis. However, increasing seed oil content adversely affects seed protein content, which eventually influences the consumption of soybean as a source of protein.

Sugars Will Eventually be Exported Transporters (SWEET) gene family is responsible for the sugar transportation across cellular membranes having a role in the mobilization of carbohydrates from source to sink organs to sustain healthy growth and development of a plant [49, 50]. Bi-parental QTL mappings and genome-wide association studies dissect the genetic mechanisms by revealing QTLs on chromosomes 5, 7, 8, 10, 15, and 20, which are associated with seed oil, sugar, and protein contents [15, 51]. A major QTL has been identified on chromosome 15, *GmSWEET10a* (Glyma.15G049200), that affects seed oil, size, and protein content [15, 47]. This locus encodes a member of the SWEET gene family, a sugar transporter gene, ensuring sucrose efflux and allocating sugar from the mother seed coat to the filial embryo. Wang et al. (2020) showed that the frequency of the allele, which is significantly associated with seed oil content increase, is higher among landraces and cultivated soybeans than wild varieties [52]. Therefore, there was a strong artificial selection during the domestication of soybean. Zhang et al. (2020) identified a two-base-pair CC deletion in exon 6 of the cultivated and high-seed oil-containing soybean varieties [47]. They also unravelled that this gene shows a pleiotropic effect on seed oil and protein contents since the varieties having CC alleles available are significantly rich in protein. Additionally, Wang et al. (2020) identified a homologous locus of *GmSWEET10a*, named *GmSWEET10b*, by conducting a knock-out mutation through CRISPR/Cas9 showing a similar effect on seed oil and protein content while changing seed size; however, they could not find a significant artificial selection for this locus [52]. Cai et al. (2023) showed a similar antagonistic effect between seed oil and protein contents by indicating contrasting synthesis of oil and protein under changing expression of *the GmMFT* gene through CRISPR/Cas9 mediated knock-out mutants [48]. The reason underlying this antagonistic behavior might be that during the domestication process, carbohydrate transportation to seeds enhances fatty acid biosynthesis rather than protein synthesis. Wang et al. (2019) showed that the metabolic composition of soybean seeds unravelled how protein-rich seeds produced nitrogen-assimilating amino acids -free asparagine, free 3-cyanoalanine, free glutamic acid, L-malic acid, free glutamine, and free aspartic acid- and at the same time, they expressed the negative correlation to seed oil content [15]. Therefore, for a rich protein seed content synthesis of these amino acids are crucial.

Research on soybean seed oil-rich varieties always shows big seed in size. This might explain why seed size-regulating genes co-segregate with seed oil-regulating genes. However, seed size-regulating genes do not always correlate with seed oil or protein contents. Silencing of the *GmFAD3* gene, encoding omega-3 fatty acid desaturase, showed increased seed size without changing seed protein and oil content [53]. This implies that the seed size trait controlling *GmFAD3* is not linked with seed sugar efflux-controlling genes. Likewise, *the soybean seed size 1* (*GmSSS1*) gene, a homolog of *the Arabidopsis thaliana SPY* gene encodes an O-GlcNAc transferase protein and controls seed and pod size by showing pleiotropy, that was identified through CRISPR/Cas9 mediated knock-out mutants, and it might be achieved through cell expansion and division [54]. The independence of seed size-regulating

genes implies the epistatic effect of the size-regulating genes on *GmSWEET10a, GmSWEET10b,* and *GmMFT*.

Seed protein content is not the sole trait correlating with seed oil content, seed shape and coat color regulating genes are also co-segregating with the oil content. Soybean seed shape associated seed thickness (*ST1*) locus, encoding a UDP-D-glucuronate 4-epimerase, shows a pleiotropic effect on seed color and oil content through CRISPR/Cas9 mediated knock-out mutants. It acts upon seed shape by turning a flat seed into a round and catalyses the biosynthesis of UDP-galacturonic acid and participates in the glycolytic pathway. The conversion of UDP-glucuronic acid to UDP-galacturonic acid, which is a pectin precursor, promotes cell wall protein production. Simultaneously, glycerol-3-phosphate esterification produces triacylglycerol, which is a lipid biosynthesis precursor, and seeds show high oil content. The population analysis of 1209 soybean accessions, including 122 wild accessions, 542 landraces, and 545 cultivated soybeans, one haplotype, which shows a C to T polymorphism at nucleotide 203 of the *ST1* locus, was found the most frequent within the population and was significantly associated with high seed oil content and round shape. Another very interesting finding was that seed coat color determining locus *l*, a highly unstable transposon-induced locus, when reversed, co-segregates with *ST1*. This might explain the high frequency of yellow, round, and high oil involving cultivated soybean seeds [74].

The anthropogenic impact of oil-rich soybean domestication cannot be overseen. One reason could be that seeds rich in oil might be a good source of energy storage in the human body, so a simultaneous increase in seed oil and size might drive people to select oil-rich varieties. Another reason might be that carbon and nitrogen are primarily required for high seed protein content, so under limited nitrogen resources, oil accumulating large seeds might have been selected by humans. However, this hypothesis does not explain why seed oil accumulation still competes with protein accumulation under nitrogen-sufficient conditions. Soybean protein is a crucial supplement for vegetarian diet. Therefore, upregulating the production of nitrogen assimilating amino acids will enhance the soybean protein level. Gene editing will provide confirmation of the gene functions that are involved in soybean protein production.

3.5 Abiotic and Biotic Stress Resistance

Changing climatic conditions and pollution of air, soil, and water create devastating effects on soybean production. Abiotic and biotic stress conditions cause vulnerable soybean plants; indeed, their negative effects can be inherited and result in yield loss. Major abiotic stress conditions around the world for soybean are drought, salinity, cold, and flooding stresses [55]. For example, soybean production has been declining in Argentina, one of the major soybean producers, due to drought stress, which decreases the production, and so does the export and crushing [56]. QTL studies identified causal loci, which can promote marker-assisted breeding for

abiotic and biotic stress resistance. However, only a few of them have been validated through gene editing; otherwise, most of them are still in the candidate status.

The improved root system, enhanced water uptake, effective stomatal conductance, and slow wilting are some of the avoidance strategies in soybean from drought stress. Soybean breeding in light of genetic mechanisms can provide drought-resistant soybean crops and save soybean production under drought conditions. Throughout the investigations, several drought resistance-conferring genes have been functionally identified. A crosstalk between plant hormones and transcription factors regulates plant response to stress conditions. NAC (NAM, ATAF, and CUC) [57, 58], MYB [59], WRKY [59], AREB [60], DREB [61, 62], AP2/ERF [63] transcription factors were found to be involved in this collaboration. Overexpression of soybean *GmNFYA13,* a nuclear localization protein, was found to be responsible for gaining resilience to salt and drought stress in transgenic soybean plants. Abscisic acid (ABA) is one of the plant hormones which control the physiological adaptations of a plant under stress conditions. For example, stomatal closure is induced by increasing ABA to prevent water loss. When ABA is artificially induced in soybean, *GmNFYA13* expression was increased. This infers that *the GmNFYA13* gene is involved in abscisic acid-mediated stress response in soybean plants [64].

Soybean is a salt-sensitive plant, increased Na^+ ions change cellular ion balance and damage cells. *Cation Diffusion Facilitator 1, Arabidopsis K^+ Transporter 1,* and also some transcription factors that are generally involved in abiotic and biotic stress conditions, such as MYB, WRKY, AP2/ERF, and NAC are associated with salt stress resistance in soybean [65]. Wang et al. (2021) identified an ABA and salt induced transcription repressor *GmAITR* in soybean to reduce the salinity stress related phenotypes without losing its fitness [66]. A CRISPR/Cas9 knock out mutant technique, *gmaitr* inhibited the expression of ABA and showed tolerance to salt stress.

Moreover, flooding stress is another abiotic stress that affects soybean production under ill-drained soils. Soybean roots are primary organs that are affected by flooding stress, limited oxygen uptake causes hypoxia and reduced energy production. To overcome this stress, plants undergo alternative energy-producing metabolic activities. Transcriptomic and proteomic studies unravel a group of proteins that are involved in cell wall modification, methylglyoxal detoxification, hypoxia reduction, pathogen defence, reactive oxygen species scavengers and chaperons, and energy production through glycolysis induction and alcohol fermentation [67–69].

Soybean production around the world is challenged by increasing negative impacts of fungus, bacterium, phytoplasma, nematode, and virus infections. Natural and artificial selection strategies improved soybean resistance over the years and sustain its development and reproduction despite dynamic spatial and temporal conditions. Soybean breeding for biotic stress resistance is a very active process. Due to changing climate conditions, pathogen populations shift, and new races are introduced to host plants. This activates new protection mechanisms and beneficial mutations in resistance-conferring genes provide endurance to plants. The selection pressure on beneficial mutations can occur both naturally and artificially. Zhao et al.

(2015) investigated nucleotide fixation of pathogen resistance in wild and cultivated varieties, and their study revealed that *Glyma20g08290* (homolog of *Arabidopsis thaliana RPM1* gene) is a naturally selected locus, which is associated with *Pseudomonas syringae* in soybean (Ashfield et al., 1995) and found in wild soybean varieties [70, 71].

Marker-assisted selection provides causal QTLs for vertical and horizontal resistance. It unravels major R genes, which maintain vertical resistance in soybean to soybean cyst nematode (*Rhg*), Phytophthora root and stem rot (*Rps*), soybean rust (*Rpp*), frog eye leaf spot (*Rcs*), bacterial blight (*Rpg*), and soybean mosaic virus (*Rsv* and *Rsc*) [65, 70, 72, 73]. R genes provide full protection in a race-specific manner. Horizontal resistance is controlled by multiple minor effect genes and confers resistance against many soybean diseases such as sudden death syndrome, Sclerotinia stems rot, root-knot nematode, and most Pythium species [73]. However, this type of protection is not pathotype specific so it is more long-lasting than vertical resistance. In vertical resistance, environmental conditions might cause genetic changes in avirulent proteins, which are recognized by pathotype-specific R genes, or shift in pathogen populations. On the other hand, utilization of molecular markers associated with R genes is more feasible than pursuing a soybean breeding strategy for minor allelic resistance.

4 Conclusion

QTLs have been identified for a number of traits in soybean. These QTLs can be used to develop marker-assisted breeding programs to improve soybean cultivars for resistance to these stresses. Gene editing is a newer technology that can be used to rapidly and efficiently introduce and edit specific genes. Gene editing is a complementary approach to marker-assisted breeding, and the two technologies can be used together to accelerate the development of improved soybean cultivars. The advantages of using gene editing for soybean improvement:

- Gene editing is a precise technology that can be used to target specific genes.
- Gene editing is a rapid technology that can be used to develop new cultivars in a shorter time frame than conventional breeding methods.

The challenges of using gene editing for soybean improvement:

- Gene editing is a regulated technology, and there are a number of regulatory hurdles that must be overcome before gene-edited soybeans can be commercialized.
- There is some public opposition to the use of gene editing in food crops.

Despite the challenges, gene editing is a promising technology that has the potential to revolutionize soybean improvement. By combining gene editing with marker-assisted breeding, we can develop soybean cultivars that are more resistant to

abiotic and biotic stresses, have improved yield and nutritional quality, and are better suited to the changing climate.

References

1. Sedivy, E.J., Wu, F., Hanzawa, Y.: Soybean domestication: the origin, genetic architecture and molecular bases. New Phytol. **214**, 539–553 (2017)
2. Ritchie, H., Roser, M.: Forests and deforestation. Our World in Data. Retrieved from https://ourworldindata.org/forests-and-deforestation (2021)
3. Fraanje, W., Garnett, T.: Soy: Food, Feed, and Land Use Change. (Foodsource: Building Blocks). Food Climate Research Network. University of Oxford (2020)
4. Baloglu, M.C., Celik Altunoglu, Y., Baloglu, P., Yildiz, A.B., Türkölmez, N., Özden Çiftçi, Y.: Gene-editing technologies and applications in legumes: Progress, evolution, and future prospects. Front. Genet. **13**, 859437 (2022)
5. Curtin, S.J., Zhang, F., Sander, J.D., Haun, W.J., Starker, C., Baltes, N.J., Reyon, D., Dahlborg, E.J., Goodwin, M.J., Coffman, A.P., Dobbs, D., Joung, J.K., Voytas, D.F., Stupar, R.M.: Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol. **156**, 466–473 (2011)
6. Curtin, S.J., Michno, J.M., Campbell, B.W., Gil-Humanes, J., Mathioni, S.M., Hammond, R., Gutierrez-Gonzalez, J.J., Donohue, R.C., Kantar, M.B., Eamens, A.L., Meyers, B.C., Voytas, D.F., Stupar, R.M.: MicroRNA maturation and MicroRNA target gene expression regulation are severely disrupted in soybean dicer-like1 double mutants. G3 (Bethesda, Md.). **6**, 423–433 (2016)
7. Bonawitz, N.D., Ainley, W.M., Itaya, A., Chennareddy, S.R., Cicak, T., Effinger, K., Jiang, K., Mall, T.K., Marri, P.R., Samuel, J.P., Sardesai, N., Simpson, M., Folkerts, O., Sarria, R., Webb, S.R., Gonzalez, D.O., Simmonds, D.H., Pareddy, D.R.: Zinc finger nuclease-mediated targeting of multiple transgenes to an endogenous soybean genomic locus via non-homologous end joining. Plant Biotechnol. J. **17**, 750–761 (2019)
8. Haun, W., Coffman, A., Clasen, B.M., Demorest, Z.L., Lowy, A., Ray, E., Retterath, A., Stoddard, T., Juillerat, A., Cedrone, F., Mathis, L., Voytas, D.F., Zhang, F.: Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol. J. **12**, 934–940 (2014)
9. Demorest, Z.L., Coffman, A., Baltes, N.J., Stoddard, T.J., Clasen, B.M., Luo, S., Retterath, A., Yabandith, A., Gamo, M.E., Bissen, J., Mathis, L., Voytas, D.F., Zhang, F.: Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. BMC Plant Biol. **16**, 225 (2016)
10. Du, H., Zeng, X., Zhao, M., Cui, X., Wang, Q., Yang, H., Cheng, H., Yu, D.: Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. J. Biotechnol. **217**, 90–97 (2016)
11. Chen, X., Yang, S., Zhang, Y., Zhu, X., Yang, X., Zhang, C., Li, H., Feng, X.: Generation of male-sterile soybean lines with the CRISPR/Cas9 system. Crop J. **9**, 1270–1277 (2021)
12. Li, Z., Cheng, Q., Gan, Z., Hou, Z., Zhang, Y., Li, Y., Li, H., Nan, H., Yang, C., Chen, L., Lu, S., Shi, W., Chen, L., Wang, Y., Fang, C., Kong, L., Su, T., Li, S., Kou, K., Wang, L., Kong, F., Liu, B., Dong, L.: Multiplex CRISPR/Cas9-mediated knockout of soybean LNK2 advances flowering time. Crop J. **9**, 767–776 (2021)
13. Xiao, Z., Jin, Y., Zhang, Q., Lamboro, A., Dong, B., Yang, Z., Wang, P.: Construction and functional analysis of CRISPR/Cas9 vector of FAD2 gene family in soybean. Phyton-Int. J. Exp. Bot. **91**, 349–361 (2022)
14. Zhou, J., Li, Z., Li, Y., Zhao, Q., Luan, X., Wang, L., Liu, Y., Liu, H., Zhang, J., Yao, D.: Effects of different gene editing modes of CRISPR/Cas9 on soybean fatty acid anabolic metabolism based on GmFAD2 family. Int. J. Mol. Sci. **24**, 4769 (2023)

15. Wang, J., Zhou, P., Shi, X., Yang, N., Yan, L., Zhao, Q., Yang, C., Guan, Y.: Primary metabolite contents are correlated with seed protein and oil traits in near-isogenic lines of soybean. Crop J. **7**, 651–659 (2019)
16. Cao, L., Wang, Z., Ma, H., Liu, T., Ji, J., Duan, K.: Multiplex CRISPR/Cas9-mediated raffinose synthase gene editing reduces raffinose family oligosaccharides in soybean. Front. Plant Sci. **13**, 1048967 (2022)
17. Lin, W., Kuang, H., Bai, M., Jiang, X., Zhou, P., Li, Y., Chen, B., Li, H., Guan, Y.: Multiplex genome editing targeting soybean with ultra-low anti-nutritive oligosaccharides. Crop J.
18. Bao, A., Chen, H., Chen, L., Chen, S., Hao, Q., Guo, W., Qiu, D., Shan, Z., Yang, Z., Yuan, S., Zhang, C., Zhang, X., Liu, B., Kong, F., Li, X., Zhou, X., Tran, L.P., Cao, D.: CRISPR/Cas9-mediated targeted mutagenesis of GmSPL9 genes alters plant architecture in soybean. BMC Plant Biol. **19**, 131 (2019)
19. Cheng, Q., Dong, L., Su, T., Li, T., Gan, Z., Nan, H., Lu, S., Fang, C., Kong, L., Li, H., Hou, Z., Kou, K., Tang, Y., Lin, X., Zhao, X., Chen, L., Liu, B., Kong, F.: CRISPR/Cas9-mediated targeted mutagenesis of GmLHY genes alters plant height and internode length in soybean. BMC Plant Biol. **19**, 562 (2019)
20. Spengler, R.N.: Anthropogenic seed dispersal: rethinking the origins of plant domestication. Trends Plant Sci. **25**, 340–348 (2020)
21. Dong, Y., Yang, X., Liu, J., et al.: Pod shattering resistance associated with domestication is mediated by a NAC gene in soybean. Nat. Commun. **5** (2014)
22. Zhang, J., Singh, A.K.: Genetic control and geo-climate adaptation of pod dehiscence provide novel insights into soybean domestication. G3 (Bethesda, Md.). **10**, 545–554 (2020)
23. Parker, T.A., Lo, S., Gepts, P.: Pod shattering in grain legumes: emerging genetic and environment-related patterns. Plant Cell. **33**, 179–199 (2021)
24. Hu, D., Kan, G., Hu, W., Li, Y., Hao, D., Li, X., Yang, H., Yang, Z., He, X., Huang, F., et al.: Identification of loci and candidate genes responsible for pod dehiscence in soybean via genome-wide association analysis across multiple environments. Front. Plant Sci. **10**, 811 (2019)
25. Davin, L.B., Wang, H.-B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G.: Stereoselective bimolecular Phenoxy radical coupling by an auxiliary (Dirigent) protein without an active center. Science. **275**, 362–366 (1997)
26. Funatsuki, H., Suzuki, M., Hirose, A., Inaba, H., Yamada, T., Hajika, M., Komatsu, K., Katayama, T., Sayama, T., Ishimoto, M., et al.: Molecular basis of a shattering resistance boosting global dissemination of soybean. Proc. Natl Acad. Sci. USA. **111**, 17797–17802 (2014)
27. Zhang, Z., Wang, J., Kuang, H., et al.: Elimination of an unfavorable allele conferring pod shattering in an elite soybean cultivar by CRISPR/Cas9. aBIOTECH. **3**, 110–114 (2022)
28. Singh, M., Siler, T. Soybean planting considerations for maximum profits. MSU Extension Soybeans. https://www.canr.msu.edu/news/soybean-planting-and-time-management-considerations (2022)
29. Staton, M. Reducing soybean harvest losses in 2017. MSU Extension Soybeans. https://www.canr.msu.edu/news/reducing_soybean_harvest_losses_in_2017 (2017)
30. Staton, M. Overview of 2020 soybean on-farm research projects. MSU Extension Soybeans. https://www.canr.msu.edu/news/soybean_on_farm_research_trials (2020)
31. Tian, X., Wang, X., Lee, R., Li, Y., Specht, J.E., Nelson, R.L., McClean, P.E., Qiu, L., Ma, J.: Artificial selection for determinate growth habit in soybean. Proc. Natl. Acad. Sci. **107**, 8563–8568 (2010)
32. Zhou, X., Wang, D., Mao, Y., Zhou, Y., Zhao, L., Zhang, C., Liu, Y., Chen, J.: The organ size and morphological change during the domestication process of soybean. Front. Plant Sci. **13**, 913238 (2022)
33. Wang, L., Lin, C., Li, B., Su, T., Li, S., Li, H., He, F., Gou, C., Chen, Z., Wang, Y., Qin, J., Liu, B., Kong, F., Yue, L., Lu, S., Fang, C.: Two soybean homologues of TERMINAL FLOWER 1 control flowering time under long day conditions. Crop J. **11**(3), 704–712 (2023)

34. Liu, B., Watanabe, S., Uchiyama, T., Kong, F., Kanazawa, A., Xia, Z., Nagamatsu, A., Arai, M., Yamada, T., Kitamura, K., Masuta, C., Harada, K., Abe, J.: The soybean stem growth habit gene Dt1 is an Ortholog of Arabidopsis TERMINAL FLOWER1. Plant Physiol. **153**, 198–210 (2010)
35. Yoo, S.K., Chung, K.S., Kim, J., Lee, J.H., Hong, S.M., Yoo, S.J., Yoo, S.Y., Lee, J.S., Ahn, J.H.: CONSTANS activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to promote flowering in Arabidopsis. Plant Physiol. **139**, 770–778 (2005)
36. Zhang, Q., Li, H., Li, R., Hu, R., Fan, C., Chen, F., Wang, Z., Liu, X., Fu, Y., Lin, C.: Association of the circadian rhythmic expression of GmCRY1a with a latitudinal cline in photoperiodic flowering of soybean. Proc. Natl. Acad. Sci. U. S. A. **105**, 21028–21033 (2008)
37. Li, H., Du, H., He, M., Wang, J., Wang, F., Yuan, W., Huang, Z., Cheng, Q., Gou, C., Chen, Z., Liu, B., Kong, F., Fang, C., Zhao, X., & Yu, D.: Natural variation of FKF1 controls flowering and adaptation during soybean domestication and improvement. New Phytol. Advance online publication. (2023)
38. Zhao, F., Lyu, X., Lyu, X., Ji, R., Ji, R., Liu, J., Liu, J., Zhao, T., Zhao, T., Li, H., Li, H., Liu, B., Liu, B., Pei, Y., Pei, Y.: CRISPR/Cas9-engineered mutation to identify the roles of phytochromes in regulating photomorphogenesis and flowering time in soybean. Crop J. **10**, 1654–1664 (2022)
39. Cai, Y., Chen, L., Liu, X., Guo, C., Sun, S., Wu, C., Jiang, B., Han, T., Hou, W.: CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. Plant Biotechnol. J. **16**, 176–185 (2018)
40. Han, J., Guo, B., Guo, Y., Zhang, B., Wang, X., Qiu, L.J.: Creation of early flowering germplasm of soybean by CRISPR/Cas9 technology. Front. Plant Sci. **10**, 1446 (2019)
41. Wang, L., Sun, S., Wu, T., Liu, L., Sun, X., Cai, Y., Li, J., Jia, H., Yuan, S., Chen, L., Jiang, B., Wu, C., Hou, W., Han, T.: Natural variation and CRISPR/Cas9-mediated mutation in GmPRR37 affect photoperiodic flowering and contribute to regional adaptation of soybean. Plant Biotechnol. J. **18**, 1869–1881 (2020)
42. Wang, L., Li, H., He, M., Dong, L., Huang, Z., Chen, L., Nan, H., Kong, F., Liu, B., Zhao, X.: GIGANTEA orthologs, E2 members, redundantly determine photoperiodic flowering and yield in soybean. J. Integr. Plant Biol. **65**, 188–202 (2023)
43. Li, X., Hu, D., Cai, L., Wang, H., Liu, X., Du, H., Yang, Z., Zhang, H., Hu, Z., Huang, F., Kan, G., Kong, F., Liu, B., Yu, D., Wang, H.: CALCIUM-DEPENDENT PROTEIN KINASE38 regulates flowering time and common cutworm resistance in soybean. Plant Physiol. **190**, 480–499 (2022)
44. Sun, L., Miao, Z., Cai, C., Zhang, D., Zhao, M., Wu, Y., Zhang, X., Swarm, S.A., Zhou, L., Zhang, Z.J., Nelson, R.L., Ma, J.: GmHs1-1, encoding a calcineurin-like protein, controls hard-seededness in soybean. Nat. Genet. **47**, 939–943 (2015)
45. Jang, S.J., Sato, M., Sato, K., Jitsuyama, Y., Fujino, K., et al.: A single-nucleotide polymorphism in an Endo-1,4-β-Glucanase gene controls seed coat permeability in soybean. PLoS One. **10**, e0128527 (2015)
46. Chandra, S., Taak, Y., Rathod, D.R., Yadav, R.R., Poonia, S., Sreenivasa, V., Talukdar, A.: Genetics and mapping of seed coat impermeability in soybean using interspecific populations. Physiol. Mol. Biol. Plants. **26**, 2291–2299 (2020)
47. Zhang, H., Goettel, W., Song, Q., Jiang, H., Hu, Z., Wang, M.L., An, Y.C.: Selection of GmSWEET39 for oil and protein improvement in soybean. PLoS Genet. **16**(11), e1009114 (2020). https://doi.org/10.1371/journal.pgen.1009114
48. Cai, Z., Xian, P., Cheng, Y., Zhong, Y., Yang, Y., Zhou, Q., Lian, T., Ma, Q., Nian, H., Ge, L.: MOTHER-OF-FT-AND-TFL1 regulates the seed oil and protein content in soybean. New Phytol. Advance online publication. (2023)
49. Chen, L.-Q., Hou, B.-H., Lalonde, S., Frommer, W.B.: Sugar transporters for intercellular exchange and nutrition of pathogens. Nature. **468**, 527–532 (2010)

50. Patil, G., Valliyodan, B., Deshmukh, R., Prince, S., Nicander, B., Zhao, M., Sonah, H., Song, L., Lin, L., Chaudhary, J., Liu, Y., Joshi, T., Xu, D., Nguyen, H.T., Mutava, R.N.: Soybean (Glycine max) SWEET gene family: insights through comparative genomics, transcriptome profiling and whole genome re-sequence analysis. BMC Genomics. **16**, 520 (2015)
51. Huang, J., Ma, Q., Cai, Z., Xia, Q., Li, S., Jia, J., Chu, L., Lian, T., Nian, H., Cheng, Y.: Identification and mapping of stable QTLs for seed oil and protein content in soybean [Glycine max (L.) Merr.]. J. Agric. Food Chem. **68**, 6448–6460 (2020)
52. Wang, S., Liu, S., Wang, J., Yokosho, K., Zhou, B., Yu, Y.C., Liu, Z., Frommer, W.B., Ma, J.F., Chen, L.Q., Guan, Y., Shou, H., Tian, Z.: Simultaneous changes in seed size, oil content and protein content driven by selection of SWEET homologues during soybean domestication. Natl. Sci. Rev. **7**, 1776–1786 (2020)
53. Singh, A.K., Fu, D.Q., El-Habbak, M., Navarre, D., Ghabrial, S., Kachroo, A.: Silencing genes encoding omega-3 fatty acid desaturase alters seed size and accumulation of bean pod mottle virus in soybean. Mol. Plant-Microbe Interact. **24**, 506–515 (2011)
54. Zhu, W., Yang, C., Yong, B., Wang, Y., Li, B., Gu, Y., Wei, S., An, Z., Sun, W., Qiu, L., He, C.: An enhancing effect attributed to a nonsynonymous mutation in SOYBEAN SEED SIZE 1, a SPINDLY-like gene, is exploited in soybean domestication and improvement. New Phytol. **236**, 1375–1392 (2022)
55. Chugh, V., Kaur, D., Purwar, S., Kaushik, P., Sharma, V., Kumar, H., Rai, A., Singh, C.M., Kamaluddin, Dubey, R.B.: Applications of molecular markers for developing abiotic-stress-resilient oilseed crops. Life (Basel, Switzerland). **13**, 88 (2022)
56. Boroughs, B.: Oilseeds and products annual (Report no. AR2023–0004). US department of agriculture: foreign agricultural service. https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Oilseeds%20and%20Products%20Annual_Buenos%20Aires_Argentina_AR2023-0004.pdf (2023)
57. Lu, P.L., Chen, N.Z., An, R., Su, Z., Qi, B.S., Ren, F., Chen, J., Wang, X.C., Tao, J.J.: A novel drought-inducible gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. Plant Mol. Biol. **63**, 289–305 (2007)
58. Yang, C., Huang, Y., Lv, P., Antwi-Boasiako, A., Begum, N., Zhao, T., Zhao, J.: NAC transcription factor GmNAC12 improved drought stress tolerance in soybean. Int. J. Mol. Sci. **23**, 12029 (2022)
59. Arya, H., Singh, M.B., Bhalla, P.L.: Towards developing drought-smart soybeans. Front. Plant Sci. **12**, 750664 (2021)
60. Fuganti-Pagliarini, R., Ferreira, L.C., Rodrigues, F.A., Molinari, H.B.C., Marin, S.R.R., Molinari, M.D.C., Marcolino-Gomes, J., Mertz-Henning, L.M., Farias, J.R.B., de Oliveira, M.C.N., Neumaier, N., Kanamori, N., Fujita, Y., Mizoi, J., Nakashima, K., Yamaguchi-Shinozaki, K., Nepomuceno, A.L.: Characterization of soybean genetically modified for drought tolerance in field conditions. Front. Plant Sci. **8**, 448 (2017)
61. Hou, Z., Li, Y., Cheng, Y., Li, W., Li, T., Du, H., Kong, F., Dong, L., Zheng, D., Feng, N., Liu, B., Cheng, Q.: Genome-wide analysis of DREB genes identifies a novel salt tolerance gene in wild soybean (Glycine soja). Front. Plant Sci. **13**, 821647 (2022)
62. Kidokoro, S., Watanabe, K., Ohori, T., Moriwaki, T., Maruyama, K., Mizoi, J., Myint Phyu Sin Htwe, N., Fujita, Y., Sekita, S., Shinozaki, K., Yamaguchi-Shinozaki, K.: Soybean DREB1/CBF-type transcription factors function in heat and drought as well as cold stress-responsive gene expression. Plant J. **81**, 505–518 (2015)
63. Zhang, G., Chen, M., Li, L., Xu, Z., Chen, X., Guo, J., Ma, Y.: Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. J. Exp. Bot. **60**, 3781–3796 (2009)
64. Ma, X.J., Fu, J.D., Tang, Y.M., Yu, T.F., Yin, Z.G., Chen, J., Zhou, Y.B., Chen, M., Xu, Z.S., Ma, Y.Z.: GmNFYA13 improves salt and drought tolerance in transgenic soybean plants. Front. Plant Sci. **11**, 587244 (2020)
65. Du, H., Fang, C., Li, Y., Kong, F., Liu, B.: Understandings and future challenges in soybean functional genomics and molecular breeding. J. Integr. Plant Biol. **65**, 468–495 (2023)

66. Wang, T., Xun, H., Wang, W., Ding, X., Tian, H., Hussain, S., Dong, Q., Li, Y., Cheng, Y., Wang, C., Lin, R., Li, G., Qian, X., Pang, J., Feng, X., Dong, Y., Liu, B., Wang, S.: Mutation of GmAITR genes by CRISPR/Cas9 genome editing results in enhanced salinity stress tolerance in soybean. Front. Plant Sci. **12**, 779598 (2021)
67. Komatsu, S., Yamamoto, R., Nanjo, Y., Mikami, Y., Yunokawa, H., Sakata, K.: A comprehensive analysis of the soybean genes and proteins expressed under flooding stress using transcriptome and proteome techniques. J. Proteome Res. **8**, 4766–4778 (2009)
68. Yin, X., Komatsu, S.: Comprehensive analysis of response and tolerant mechanisms in early-stage soybean at initial-flooding stress. J. Proteome. **169**, 225–232 (2017)
69. Khan, M.N., Ahmed, I., Ud Din, I., Noureldeen, A., Darwish, H., Khan, M.: Proteomic insight into soybean response to flooding stress reveals changes in energy metabolism and cell wall modifications. PLoS One. **17**, e0264453 (2022)
70. Zhao, S., Zheng, F., He, W., Wu, H., Pan, S., Lam, H.M.: Impacts of nucleotide fixation during soybean domestication and improvement. BMC Plant Biol. **15**, 81 (2015)
71. Ashfield, T., Keen, N.T., Buzzell, R.I., Innes, R.W.: Soybean resistance genes specific for different pseudomonas syringae avirulence genes are allelic, or closely linked, at the RPG1 locus. Genetics. **141**, 1597–1604 (1995)
72. Cook, D.E., Lee, T.G., Guo, X., Melito, S., Wang, K., Bayless, A.M., Wang, J., Hughes, T.J., Willis, D.K., Clemente, T.E., Diers, B.W., Jiang, J., Hudson, M.E., Bent, A.F.: Copy number variation of multiple genes at Rhg1 mediates nematode resistance in soybean. Science (New York, N.Y.). **338**, 1206–1209 (2012)
73. Lin, F., Chhapekar, S.S., Vieira, C.C., Da Silva, M.P., Rojas, A., Lee, D., Liu, N., Pardo, E.M., Lee, Y.C., Dong, Z., Pinheiro, J.B., Ploper, L.D., Rupe, J., Chen, P., Wang, D., Nguyen, H.T.: Breeding for disease resistance in soybean: a global perspective. TAG. Theor. Appl. Genet. Theoretische und angewandte Genetik. **135**, 3773–3872 (2022)
74. Li, J., Zhang, Y., Ma, R., Huang, W., Hou, J., Fang, C., Wang, L., Yuan, Z., Sun, Q., Dong, X., Hou, Y., Wang, Y., Kong, F., Sun, L.: Identification of ST1 reveals a selection involving hitchhiking of seed morphology and oil content during soybean domestication. Plant Biotechnol. J. **20**, 1110–1121 (2022)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 18
CRISPR/Cas-Based Precision Breeding of Oilseed Rape (*Brassica napus* L.) – Recent Improvements

Justyna Boniecka

Abstract The seeds of oilseed rape (*Brassica napus* L.) are used in large-scale production of one of the most health-promoting plant oils in the food industry, as well as for animal feed and biofuel production. Thus, increasing the yield of this crop is of crucial economic and ecological importance. However, conventional breeding programs are slow, laborious and time-consuming. Hence, along with the discovery of the possibility to apply CRISPR/Cas technology to edit plant genomes and to accelerate the breeding process, much effort has been put into applying this technology to study specific genes and biosynthetic pathways, especially in species with many gene copies such as *B. napus*. Here, recent improvements in generating CRISPR/Cas-induced mutations in the *B. napus* genome, delivering CRISPR/Cas reagents into oilseed rape plant cells, fast-checking the efficiency of targeted mutagenesis of CRISPR/Cas reagents, and oilseed rape transformation and regeneration procedures are described. Finally, new applications of CRISPR/Cas tools in oilseed rape precision breeding are discussed, focusing mainly on applications verified in field.

Oilseed rape (canola; *Brassica napus* L.) belongs to the genus *Brassica*. It is a domesticated allotetraploid, which originated from spontaneous hybridization between two diploid species – turnip rape (*B. rapa*) and cabbage (*B. oleraceae*) – about 7500 years ago [1]. Today, oilseed rape is one of the most important oil crops worldwide, being cultivated in Europe and Asia predominantly as winter forms and in Australia, Canada and northern Europe as spring ones, providing food, feed and biofuel. This success is attributable to intensive breeding for seed quality traits in the last century. The first milestone in oilseed rape quality breeding was achieved by the introduction of the first low-erucic acid (EA – has a bitter taste and in high doses

J. Boniecka (✉)
Department of Genetics, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń, Toruń, Poland

Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University in Toruń, Toruń, Poland
e-mail: jboniecka@umk.pl

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_18

has been implicated in cardiac health problems) varieties (0-varieties), at the beginning of the 1970s, resulting in high-quality oil with high levels of desirable unsaturated fatty acids. The second milestone was the establishment (in the mid-1980s) of cultivars with low seed glucosinolates (GSL – in monogastric animals their digestion results in the release of toxic by-products that can cause liver and kidney damage along with lymph dysfunction) content (low EA and GSL together – 00-varieties), making possible the use of oilseed rape press residues as protein-rich fodder for animals [2].

However, these breeding programs have significantly reduced genetic diversity in modern breeding pools [3–5]. Both of the mentioned seed quality traits – low EA and low GSL content – originated from single genetic resources. Low EA derived from the German spring cultivar (cv.) "Liho", which carried spontaneous mutations in two *B. napus FATTY ACID ELONGASE 1* (*FAE1*) homologs, while low GSL content was first identified in the Polish spring-type cv. "Bronowski", which was found to possess at least three recessive genes for low GSL content. Large-scale crossing programs facilitated the introgression of these traits into all the different ecogeographical forms of oilseed *B. napus*. The result was the release in 1974 of the first 00-quality spring variety, "Tower", with zero EA and low GSL content (in 1978, the term "canola", derived from "Canadian oil", was adopted to identify these varieties). The first 00 winter oilseed rape cultivar, "Librador", was released in Germany in 1981. Consequently, modern oilseed rape breeding material has a relatively narrow genetic diversity [2].

This narrow genetic diversity markedly restricts breeding progress, which relies on the availability of genetic variation to introduce new desirable traits into crops. Initially, breeders sought genetic variation from landraces and heirloom varieties. The emergence of mutation breeding in the 1940s allowed for the artificial induction of new genetic variation into plant genomes. This approach is extremely crude, as it introduces thousands of random mutations, both wanted and unwanted, with the latter requiring several rounds of backcrossing to remove. Moreover, in polyploid species, where most genes are multiple-copy genes with redundant functions, it is very inefficient to change traits through random mutations. However, all in all, this technology greatly increased the amount of genetic variation available to breeders, and, interestingly, falls within the definition of "conventional and traditional" breeding and thus is lightly regulated [6]. The first commercial varieties developed through mutation breeding were registered in the 1950s, and now over 3400 varieties are listed on the FAO/IAEA Mutant Variety Database, including 21 of *B. napus* obtained mainly by applying gamma rays [7].

New breeding technologies, such as genome editing, are providing plant breeders with access to a far broader range of genetic variation. Moreover, compared with the traditional random mutagenesis, genome editing not only reaches the same end point as conventional breeding but gets there with a greater: i) degree of precision – no/limited number of unexpected mutations (hence it being termed "precision breeding"), and ii) speed, which is very important as, with conventional plant breeding, it may take 10–15 years or more to get new crops to the market [6]. The current legislation in the EU and many other countries puts plants improved by means of

genome editing “in the same box” as typical Genetically Modified Organisms (GMOs). However, very favorable legislature for the production and consumption of such plants in the USA, Japan, Australia, some countries from South and Central America, and others such as Canada [8] is stimulating changes in the legislation in the EU. It is important to understand that simple genome edited crops, i.e., where no foreign genetic sequence is introduced, are indistinguishable and equivalent to conventionally bred crops and therefore could be regulated in a similar way [6].

Genome editing technologies encompass sequence changes within an organism or incorporation of valuable sequences into a germplasm, both resulting in an alteration of genotype without extensive backcross. These technologies are based on sequence-specific nucleases (SSNs) such as zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs), and CRISPR (Clustered, regularly interspaced, short, palindromic repeats)/Cas (CRISPR associated nuclease; with *Streptococcus pyogenes* Cas9 being the most commonly applied). The first two are relatively time-consuming to construct, as protein engineering of their DNA binding domains is required to achieve the requisite target specificity. The latter is the only RNA-guided endonuclease that targets DNA sites through nucleotide base pairing and, thus, has become the preferred SSN for genome editing in plants [9, 10]. SSNs create DNA double strand breaks (DSBs) at predefined genomic loci, which are then repaired through intrinsic erroneous, non-homologous end joining (NHEJ) or a donor DNA introducing homology-directed repair (HDR) pathways [11].

The CRISPR/Cas9 application uses a ~100-nt single guide RNA (gRNA or sgRNA), which is a combination of the crRNA and trans-activating crRNA (tracrRNA) originally encoded by the CRISPR loci [12], to direct Cas9 nuclease to a specific DNA site. The complex *Sp*Cas9-sgRNA targets with its 20 nt guide sequence, being part of the crRNA (the rest of the crRNA sequence and the tracrRNA is a scaffold), genomic sequence of 20 nt – protospacer – upstream to a 5’-NGG-3′ protospacer adjacent motif (PAM), and introduces a DSB near the PAM sequence and originates blunt ends [13] that, in plants, will be repaired preferentially by NHEJ, often introducing indel mutations.

Due to the possibility of introducing mutations at multiple sites concurrently, genome editing is an excellent option to significantly accelerate the breeding process of, especially, polyploid species such as *B. napus,* with many gene targets (at least one homolog from each of the A and C genomes) [1]. CRISPR/Cas technology offers great potential when working on complex traits such as crop yield and disease tolerance, which requires simultaneously targeting multiple loci, related or unrelated, within a single cell. The technology has already been successfully applied to generate mutations in multiple locations in oilseed rape genomes by co-expressing multiple guide RNAs [14–17].

Oilseed rape is a high-value commodity crop, which has already been modified and produced as traditional GMOs, despite the costs associated with regulatory compliance for such plants [6, 18]. Hence, it is not surprising that there is interest in investing in its precision breeding with the use of genome editing technologies. Here, recent works attempting to improve various steps on the way towards *B. napus* precision breeding are reviewed, as well as propositions/solutions that could

potentially improve the system even further. Since reviews, some of them very comprehensive, on the application of CRISPR/Cas in oilseed rape for gene function research and genetic improvements have been published only recently [16, 17, 19–21], here, only some work that included field trials with *Brassica* CRISPR/Cas modified species will be described.

1 Optimization of CRISPR/Cas Reagents and Ways of Their Expression

Mutating homeologs from both subgenomes of *B. napus* is necessary due to their functional genetic redundancy. An optimization of CRISPR/Cas constructs/reagents is essential, as the gene editing efficiency depends to a large extent on the selection of a Cas protein and/or guide RNAs. Constructs encoding *Sp*Cas9 or *Lachnospiraceae bacterium* Cas12a (Cpf1; *Lb*Cas12a) endonucleases, with one or more guide RNA expression cassettes enabling production of either a single one-guide transcript, multiple one-guide transcripts or a single multi-guide transcript are the most widely used for CRISPR/Cas-mediated genome editing in most plant species. An example of the single, multi-guide transcript production is the CRISPR/Cas9 system based on endogenous tRNA processing that was used, for example, to introduce mutations in the genome of *Brassica oleracea* – a construct with tandemly arrayed tRNA-sgRNA architecture to express multiple sgRNAs [22]. Another example of the single, multi-guide transcript production is the CRISPR/Cas system, where the multi-guide transcript produced by a Pol III from only one promoter is processed by the same nuclease involved in the generation of DSBs – Cpf1 [23]. The variety of CRISPR/Cas based nucleases and their corresponding guide RNA backbones [24] can be explored applying expanded cloning toolkits that contain not only modules encoding some of the mentioned elements but a number of promoters that allow expression of CRISPR/Cas nucleases and their guide RNAs in monocots and dicots, e.g. [25].

In plants, like in other organisms, not all transformants containing Cas-guide RNA encoding sequences display high levels of mutations in the target gene, with efficiencies reported to vary from a few percent to close to 100% [26, 27]. The efficiency with which mutations are generated in target genes depends on multiple factors, including the choice of target sites in selected genes, as well as the nature of the coding and regulatory sequences of the Cas gene and guide RNA construct. In recent studies, several architectural parameters of Cas constructs designed for plants have been investigated, including the codon usage of the Cas gene, the number of nuclear localization signals (NLSs) in the Cas enzyme, the nature of the promoters and terminators of the Cas gene, the length and sequence of the conserved region of the guide RNA and the terminator sequence of the guide RNA, and the relative orientations of the various expression cassettes in the final transfer DNA (T-DNA) [24, 28].

Marked improvements in the efficiency of targeted mutagenesis (even up to 80% more mutated plants) of *Brassica* species – *B. oleracea* and *B. napus* – were observed when using a modified version of Cas9. The application of a plant-optimized Cas9 CDS with one intron coupled with a tRNA guide architecture [22, 29] and with two guides per gene targeting two unrelated *B. oleracea* genes helped to outperform the system targeting these genes with two constructs each carrying information on two guides, each under its own Pol III promoter, and the human-codon-optimized *Hs*Cas9. The potato intron IV was originally added to avoid expression in bacteria during cloning and, as a side effect, can also increase expression *in planta* [24]. The application of the improved construct resulted in 100% of plants mutated in two of the four loci [29]. The same improved system but with one guide to edit two copies of a gene was very successful in *B. napus* and outperformed the system targeting these copies with the same guide but not coupled with the tRNA guide architecture and instead using the *Hs*Cas9 [29]. It is worth noting that, in the improved system, the transcription of sgRNA and Cas9 was in head-to-head divergent orientation, which has been shown previously to often result in a highly active CRISPR/Cas system. It was proposed that a weak terminator after Cas9 enables Pol II read through that could interfere with Pol III transcription of sgRNAs in some T-DNA construct architectures, and that this limiting factor can be corrected by divergent transcription of Cas9 and sgRNAs [24]. Another version of Cas9 with 13 introns – ZCas9 + 13int – this time prepared by introducing 13 *Arabidopsis thaliana* introns into the *Zea mays* codon-optimized version [30] (again coupled with tRNA guide architecture, with a single guide targeting a *B. oleracea* gene *GA4* or two copies of the gene in *B. napus*, with divergent transcription of Cas9 and sgRNAs) improved markedly the number of edited plants in comparison to the system not coupled with the tRNA guide architecture, head-to-tail orientation, and using the *Hs*Cas9 [29].

However, no matter what kind of modifications of the Cas9 are proposed, the CRISPR/Cas9 system still has some limitations. One of these is the strict PAM dependence, which constrains the availability of target sites, especially to coding regions, as noncoding regions are relatively poor in 5′-NGG-3′ sites. Other limitations of *Sp*Cas9 include its large size for viral delivery and the low efficiency in gene targeting caused by blunt DSBs. A solution comes with the CRISPR/Cas12a (Cpf1) system with i) a single crRNA (~42 nt, which is less than half that of Cas9, making it more suitable for multiplexed genome editing and packaging into viral vectors) and ii) Cas12a, an endonuclease smaller than *Sp*Cas9, which might facilitate viral delivery. This system requires a 5′-TTTN-3′ PAM sequence and introduces 5′ staggered ends, with 4–5 nt overhangs, at sites distal from 5′ T-rich PAM, at the end of the protospacer sequence, which has been proposed to favor gene insertions [31, 32].

Recently, *Lachnospiraceae bacterium* Cas12a (*Lb*Cas12a) was shown to generate edits in the genome of *Brassica oleracea* [29], which is a good indication that this endonuclease may function in *B. napus* as well. Working with this nuclease, four different constructs were analyzed for their efficiency in inducing mutations in a *B. oleracea* gene. In the case of all constructs, the nuclease encoding sequence was under the UBI10 promoter and was followed by the sequence of the *Pisum*

sativum rbcS E9 terminator. These sequences were in a head-to-head orientation with the RNA guide expressing cassette under the *At*U6–26 promoter.

In the case of the first construct, an *Arabidopsis*-optimized *Lb*Cas12a CDS carrying a "temperature tolerant" D156R mutation (ttAtCas12a) was encoded. The codon-optimized *Lb*Cas12a CDS carrying the mentioned mutation was shown to have increased activity in comparison to a non-mutated variant of the protein when *Arabidopsis* plants were grown for 2 weeks at 22°C or, especially, at 28°C. It is noteworthy that even the non-mutated variant, when put at 28°C, performed better at 4 of 5 loci tested [33]. Similarly, repeated heat stress treatments of *Arabidopsis* or *Citrus* plants had a major effect on the rate of mutagenesis by CRISPR/*Sp*Cas9 – in *Arabidopsis* it increased approximately five-fold in somatic tissues and up to 100-fold in the germline. It was also proposed that *Sp*Cas9 is more active in creating double-stranded DNA breaks at 37°C than at 22°C [34]. The first construct encoding the temperature-tolerant enzyme had a four guide (targeting one gene) expressing cassette, serving to produce transcript to be processed by the ttAtCas12a nuclease. The second construct encoded ttAtCas12a nuclease and a pre-gRNA transcript carrying information on one guide RNA (4 separate constructs each containing one of the four guides used in the first construct scenario). The pre-gRNA is a self-processing ribozyme-flanked guide expression cassette named Ribozyme-gRNA-Ribozyme (RGR). The RGR molecule contains ribozymes (possess nuclease activity) – Hammerhead (HH) type ribozyme at the 5′-end, and the terminal hepatitis delta virus (HDV) ribozyme at the 3′-end of the RGR, and thus undergoes self-catalyzed cleavage to generate the desired gRNA. However, the introduction of a self-cleavable ribozyme in the 5′ end of the transcript is unnecessary when a 5′-G is added to the Cas12a DR (direct repeat – region of the crRNA whose proper folding is important for nuclease activity), which is compatible with the Pol III promoter AtU6–26 transcription start site. The HDV ribozyme removes the poly-A tail from the transcript, leaving no additional nucleotides at the 3′ position of the crRNA with a classical construct carrying unprocessed U6 termination signal, which conserves a spurious tail of adenines at the 3′ position of the Cas12 crRNA [29, 32, 35–37]. The third construct contained the human-codon-optimized CDS, with modified CDS by inserting the D156R mutation to give ttHsCas12a, and the singular guide RGR expression cassette (one construct to target only one of the four target sites). The fourth construct contained ttAtCas12a, with an addition of 8 *Arabidopsis* introns generating ttAtCas12a + int, and the singular guide RGR expression cassette (one construct to target only one of the four target sites). The application of the singular guide architecture markedly increased the number of mutant plants, when applying constructs with ttAtCas12a, in two target regions from 0 to 10% and from 3 to 50% of the screened plants. The other two systems were applied only to compare the efficiency of the system in one target region, where the application of the second construct increased the number of mutant plants from 3 to 50%. By using the third constructs, where instead of ttAtCas12a, ttHsCas12a CDS was applied, researchers showed that both nucleases performed equally well. Next, the application of ttAtCas12a + int further increased the number of plants with edits in the target region from 50 to 68% [29].

Although it is clear that the temperature-tolerant version of the Cas nuclease works well in *B. oleracea,* it would be interesting to compare its activity with the *Arabidopsis*-codon-optimized version. Based on the results presented, it is hard to conclude whether the heat tolerant enzyme works better than the "wild type" version and it is not clear whether heat stress increases the efficiency of the CRISPR/Cas system in *B. oleracea*, and thus potentially in *B. napus*, as was shown for *Arabidopsis* [33, 34].

2 Delivery of CRISPR/Cas Reagents and Elements Carrying Information on CRISPR/Cas Reagents into Oilseed Rape Plant Cells

Oilseed rape plants bearing edited alleles have been predominantly obtained using *Agrobacterium tumefaciens*-mediated transformation. In one study, several essential factors that affect the transformation efficiency, such as *Agrobacterium* strains, selection marker genes and genotypes of oilseed rape were analyzed. Comparison of different *Agrobacterium* strains showed that the GV3101 had higher transformation efficiency than C58C1 and EHA105. The transformation efficiency was 3.7–4.8%, 2.2–22.5%, and 1.6–5.9% when the hypocotyl of Westar was infected by GV3101 and screened under hygromycin, kanamycin and basta, respectively. The transformation efficiency of Westar was highest and ZS11 was lowest when five different genotypes of oilseed rape (Westar, ZS9, ZS11, GY284 and WH3417) were infected by GV3101 [38].

The auxotrophic *A. tumefaciens* strain LBA4404 Thy-, which exhibits the inability to survive, proliferate or grow in the absence of thymidine, provides a method for the transformation and regeneration of plant cells that does not need an *Agrobacterium* counter-selective agent to cure plant tissue of *Agrobacterium* [39], and recently this strain has been used in a genotype-independent method of oilseed rape plant transformation and regeneration [40]. Another important element of this genotype-independent method is the application of the ternary pVir system with the T-DNA binary vector (with sequences encoding, e.g. CRISPR/Cas reagents) and an improved accessory plasmid [40] that is characterized by small size, enhanced vector stability, an improved bacterial selectable marker and amended *vir* genes. Application of the pVir system resulted in more efficient T-DNA delivery and stable plant transformation in difficult-to-transform maize elite inbreds [40, 41].

However, some drawbacks remain in these strategies, as they involve delivery of DNA-based CRISPR/Cas reagents that are first integrated into the genome, and expressed as a transgenic construct, and then segregated away by breeding as null segregants to leave only the desired edited allele/s [16]. T-DNA removal is crucial because regulatory constraints for gene-edited crops are likely to be less for those that do not contain foreign DNA [6]. Moreover, it can be important when a loss-of-function phenotype must be confirmed by complementation of the CRISPR/

Cas-induced mutation. A CRISPR/Cas construct still present in the mutant can target the complementation transgene and interfere with the resulting phenotypes [24]. Furthermore, as long as the T-DNA encoding the reagents of the CRISPR/Cas system is present in a genome, a progressive gene-editing process can take place, both during selection on appropriate media with antibiotics (which may lead to chimeras) and during the growth of the transgenic plants, and even in next generations [16, 42]. Therefore, DNA-free gene editing has received extensive attention in recent years.

One of the commonly applied T-DNA free oilseed rape genome editing methods is protoplast transfection, which is a transient (no T-DNA integration) alternative for delivery of CRISPR/Cas vectors [43, 44]. CRISPR/Cas reagents can be delivered into plant cells as ribonucleoproteins (RNPs) composed of purified recombinant enzyme Cas and *in vitro*-transcribed or synthesized gRNA as well. Particle bombardment can be used to deliver RNPs into explants, whereas polyethylene glycol (PEG)-mediated transfection and lipofection can be used to deliver RNPs into protoplasts. Although CRISPR/Cas RNPs has become an attractive approach for genetic engineering, its editing efficiency remains modest [45]. As an example, although Murovec et al. [46] were able to obtain mutation frequencies of 0.09 to 2.25% and 1.15 to 24.51% in *B. oleracea* and *B. rapa*, respectively, no mutations were detected after PEG-mediated transfection of oilseed rape (cv. "Topaz") protoplasts [46].

Although researchers have used protoplast systems to show the potential of CRISPR/Cas reagents to edit a locus or loci of interest, lack of protoplast regeneration protocols to obtain edited plants has been a major bottleneck in this system [16, 43]. Although *B. napus* protoplast regeneration was shown to be possible before the era of genome editing [47], only recently was a protocol for regeneration of protoplasts of oilseed rape in combination with genome editing proposed. Targeted genes – *BnGTR* – controlling glucosinolate transport from the vegetative tissues to seeds were mutated with high frequency [48]. According to this protocol, relatively high concentrations of auxins are essential for protoplasts to form cell walls and maintain cell division, and thereafter auxin should be reduced for callus formation and shoot induction. For shoot regeneration, relatively high concentrations of cytokinin are required, with the best combinations resulting in up to 45% shoot regeneration [48].

3 Fast-Checking the Efficiency of Targeted Mutagenesis of CRISPR/Cas Reagents

The process of plant genome editing is usually time-consuming as, in most instances, there is a need for regeneration of plants from tissue culture. Therefore, to have confidence that specific genome editing components will work, it is important to test the CRISPR/Cas system of choice and, especially, the gRNAs, before plant genome editing.

To assess the efficiency of CRISPR/Cas mutagenesis, the plant protoplast system is commonly applied. This system was used to analyze the efficiency of CRISPR/Cas vectors designed and built to target oilseed rape loci encoding proteins involved in the metabolism of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) – RelA/SpoT Homeologs (RSH]), which are likely to be involved in seed development, maturation and longevity [44, 49, 50]. Importantly, before checking the efficiency of editing, the transfection efficiency of the protoplast system can be determined with a GFP expressing vector, with the help of a microscope. Similarly, one can actually check whether CRISPR/Cas constructs were delivered into protoplasts and with what frequency. It has been shown that the efficiency of PEG-mediated transfection depends on cultivar and concentration of PEG used for the transfection. Using this approach, followed with multiplex amplicon sequencing, it was determined that the rate of particular CRISPR/Cas-mediated edits at one of the *RSH* loci was in the range of 1–3% [44]. Genome editing events can be detected in many different ways, many of which were mentioned in Shillito et al. [51].

Very recently, another interesting system was proposed to avoid the traditional lengthy explant transformation and regeneration process to study the gene-editing efficiency of various CRISPR/Cas constructs in oilseed rape – hairy-root cultures [52]. In this system, plant infection with *Agrobacterium rhizogenes* strains harboring a hairy-root-inducing (Ri) plasmid causes an abnormal rooting on hosts' tissues. After an agrobacterial infection at wounded sites, a T-DNA from the Ri plasmid is transferred to the host cells, where it is stably integrated into the plant genome. Subsequently, the expression of T-DNA genes leads to the induction of hairy roots. Agrobacterial strains carrying both Ri plasmid and artificial binary vector have been widely used for delivering foreign DNA into plant cells. Recently, using this system, researchers examined the mutation efficiency of nine different CRISPR/Cas9 constructs to edit the auxin biosynthetic gene *TRYPTOPHAN AMINOTRANSFERASE* (*BnTAA1*; two paralogs). They showed that the plant-codon-optimized *Sp*Cas9 with the potato IV2 intron (pcoCas9) is more efficient in mutating the targeted loci than *Staphylococcus aureus* Cas9 (*Sa*Cas9; PAM – 5′-NNGRRT-3′) encoded by a gene that is about 1 kb shorter than *pcoCas9* – 83.05% of mutated loci versus 47.98%. Moreover, they observed a slight increase in efficiency when using a longer version of 35S promoter (1.3 kb) compared to the shorter one (0.4 kb) – 67.29% mutated loci vs 58.7%, respectively. The efficiency of mutagenesis was also increased in the presence of the SV40 nuclear localization signal (NLS) – by 25% (75.82% with NLS-Cas9 versus 50.53% with Cas9). With the most efficient construct – NLSpcoCas9 – 96.95% loci were mutated, with less influence on the promoter choice. Next, among pcoCas9 constructs, NLSpcoCas9 induced homozygous mutations with the highest efficiency (33% of the mutated loci) [52].

The hairy-root system is a fast and straightforward system – it makes it possible to evaluate the most effective gene-editing construct within approximately 2 months after transformation. According to the protocol of Jedličková et al. [52], hypocotyls of 18-day-old seedlings are used for *Agrobacterium* injection, and the first calli and hairy roots are detected after 2 weeks. Another advantage of this system is the

relatively high transformation efficiency (number of seedlings with emerging hairy roots over the number of injected seedlings). Three cultivars were tested (DH12075, Westar and Topas), and it was observed that the transformation efficiency was 97%, 84% and 42%. It is worth noting that Topas is a cultivar recalcitrant to petiole-based transformation. Hence, this system seems to work in a genotype-independent manner. Furthermore, hairy roots with homozygous/biallelic mutations can be used to some degree for functional gene studies, e.g. when the analyzed genes encode proteins involved in the production of metabolites, as the hairy roots have been used for the production of secondary metabolites or to investigate phytoremediation processes. Next, as it has been shown in the case of *B. napus*, using the edited hairy roots, one can regenerate plants applying appropriate protocols [52].

4 Oilseed Rape Plant Regeneration

One of the milestones of oilseed rape molecular breeding has been an efficient *in vitro* regeneration, though regeneration rates are genotype-dependent. Several elite *B. napus* varieties have not been easy to transform. At present, the reported varieties for *A. tumefaciens*-mediated genetic transformation include spring oilseed rape varieties (e.g., cv. "Westar", "862" and "Haydn") and semi-winter varieties (e.g., cv. "J9707", "J9712" and "ZS6") [17]. For many years, researchers aiming to improve *B. napus* plant regeneration protocols have focused on applying explant/chemical approaches, which are based on testing explants and different ratios of auxin and cytokinin to induce cells to differentiate into whole plants [53]. So far, the most widely used method for the development of genetically modified *B. napus* plants has been the *Agrobacterium*-mediated hypocotyl transformation [e.g., 16, 17, 38, 54–57]. However, this method, even after modifications and other methods applied so far are genotype-dependent [40, 58].

Recently, an alternative, genotype-independent *Agrobacterium*-mediated *B. napus* transformation method was developed that is rapid and amenable for high-throughput transformation and CRISPR/Cas-mediated genome editing; the method is based on epicotyl and higher stem (internodal) segments (3–4 mm), and it has been successfully implemented in multiple oilseed rape genotypes, though with varying transformation efficiencies. Epicotyl segments produced significantly higher rates of shoot formation compared to hypocotyl segments across all genotypes tested [40].

An efficient and likely genotype-independent system to obtain edited plants is plant regeneration from hairy roots. Recently, an optimized regeneration protocol for *B. napus* cultivar DH12075 was proposed [52]. Using this system, in combination with embryo rescue (21–28 days after pollination) from seeds containing torpedo-stage embryos or older, it is possible to obtain transgene-free T1 plants with desired mutations roughly 1 year after agrobacterial transformation. Moreover, the protocol was used for regenerating plants from hairy-root cultures of Topas, a variety referred to as being in many instances recalcitrant to transformation and plant

regeneration. The system nonetheless has some disadvantages. The *rol* genes, which are crucial for hairy root formation, encoded on the T-DNA of the Ri plasmid, are integrated into the plant genome, and their presence in regenerated plants is responsible for altered growth characteristics called "Ri phenotype". Thus, to obtain transgene free and Ri phenotype free plants, one has to carry out segregation analyses to obtain plants with neither the Ri T-DNA nor the CRISPR/Cas T-DNA [52].

Recently, to improve plant regeneration, molecular genetic-based methods have been studied [53]. These methods require transfer of sequences encoding morphogenic factors/developmental regulators, including *WUSCHEL* (*WUS2*) or *BABY BOOM* (*BBM*), that significantly improve the efficiency of plant regeneration and allow the regeneration of thus-far recalcitrant genotypes [53, 59]. However, in order to avoid genotype-specific pleiotropic effects, including abnormal plant growth and infertility, the expression of these genes must be regulated. To solve this problem, one can use the system based on overexpressing of a fusion protein combining transcription factor GROWTH REGULATING FACTOR 4 (GRF4) and its co-factor GRF-INTERACTING FACTOR 1 (GIF1) or of *Arabidopsis GRF5* and/or its homologs, which helps to enhance plant regeneration and transformation without affecting plant growth and fertility [60, 61]. In oilseed rape (cv. "BNS3"), overexpression of *AtGRF5*, *AtGRF6*, *AtGRF9* or *BnGRF5-LIKE* was shown to significantly increase transgenic callus production of hypocotyl explants; however, it had no significant impact of shoot formation [61].

Tissue culture procedures are often technically demanding, time-consuming and laborious. Hence, no-tissue-culture-required delivery methods, which are genotype-independent, such as nanoparticles [62] or virus delivery [63, 64], could be very helpful to further extend the application of CRISPR/Cas in oilseed rape genome editing. The floral-dip method, commonly used for *Agrobacterium*-mediated transformation of *A. thaliana* plants [65], is another such method. This approach was already applied to transform *B. napus* plant but not in combination with genome editing [66, 67]. The drawback of this method is that, to transform oilseed rape plants in this way, one must wait a relatively long time until the plants reach inflorescence, which is much longer for *B. napus* than for *A. thaliana*. To overcome this problem, it is possible to generate and use rapid flowering lines. One such example is fast-flowering mini maize, amenable to transformation and editing, with a seed-to-T1-seed time of 5.5 months compared to over 9 months for other genotypes [68].

5 New Applications of CRISPR/Cas Technology in Oilseed Rape Precision Breeding

CRISPR/Cas9 system was first applied in oilseed rape to target two *ALCATRAZ* (*ALC*) homologous genes for site-directed mutagenesis to avoid seed loss during mechanical harvest by increasing shatter resistance [69]. Since that time, this system has been applied for oilseed rape gene function research and genetic improvement relating to weed control, flowering, self-incompatibility, plant hormone biology,

abiotic and biotic stress resistance, grain composition and pod shatter reduction. Since most of these achievements were well described in recent review reports [16, 17, 19–21], here, only the most recent applications of the CRISPR/Cas technology in *B. napus* (and closely related species) precision breeding that have been accompanied by field experiments will be summarized.

CRISPR/Cas9 technology was used to create targeted mutations on two homologous copies of the *FAE1* gene (on the A08 and C03 chromosomes), which plays a decisive role in the synthesis of erucic acid, in three *B. napus* germplasms with high EA (>30%) and high oil (>50%). The EA content was significantly reduced by more than 10 percentage points in the mutant of *BnC03.FAE1*, while the double mutation of *BnA08.FAE1* and *BnC03.FAE1* resulted in nearly zero EA in three *BnFAE1*-edited germplasms, and the oleic acid content was increased in different degrees. The confirmed homozygous T2 mutant lines without Cas9 were grown in an experimental farm in China, and the field management was performed in line with standard breeding practice. The agronomic yield-related traits, including plant height, branch height, branch number, silique length, number of siliques per plant, 1000-seed weight and yield per plant, were measured. It was concluded that growth and yield of the mutant plants were not significantly different in comparison to wild type plants. These results provide a way for future low-EA breeding, broadening the resources of *B. napus* with low EA [57].

CRISPR/Cas9 *MYB28*-edited *Brassica oleracea* plants were the subject of the first CRISPR/Cas field trial in the United Kingdom approved and regulated by the UK Department for Environment, Food & Rural Affairs after the reclassification of gene-edited crops as genetically modified organisms by the European Court of Justice on July 25, 2018. The *MYB28* gene encodes a transcription factor characterized as a key regulator of aliphatic glucosinolate (A-GSL) biosynthesis in *Brassica* genus. A-GSL derivatives may contribute to the putative health-promoting effects of cruciferous plant vegetables. Knocking out of *MYB28* resulted in downregulation of A-GSL biosynthesis genes and reduction in accumulation of the methionine-derived glucosinolate – glucoraphanin, the precursor for isothiocyanate sulforaphane, which is believed to have health-promoting effects – in leaves and florets of field-grown broccoli plants [70]. These results demonstrate the potential for the gene-edited plants to express the improved traits when grown in field conditions.

6 Conclusions

CRISPR/Cas-mediated genome-editing offers great potential for both genetic improvement and biological research. Hence, this technology is being constantly developed, especially to make it more widely applicable and efficient for economically important crop species, such as oilseed rape. One of the improvements in the CRISPR/Cas technology is the application of new or improved CRISPR/Cas reagents (e.g., Cas12a, with its differing PAM requirement comparing to Cas9) and/or ways of their expression, which have increased the scope and efficiency of

targeted mutagenesis. An important step was developing and improving methods of delivering CRISPR/Cas reagents, or of sequences encoding them, which has a tremendous impact on the final time that it takes to obtain transgene-free CRISPR/Cas edited plants and/or the efficiency of transformation and regeneration. Next, important protocols have been developed for analyses of different CRISPR/Cas variants/gRNAs to assess their efficiencies before applying to obtain edited plants. Lastly, setting up genotype-independent regeneration protocols to obtain mutations in a desired elite germplasm has been a tremendous achievement.

However, although the presented tools and methods serve to make a major contribution to more efficient and rapid gene discovery and functional characterization, some CRISPR/Cas variants and methods applied in the frame of genome editing in plants await implementation in *B. napus* precision breeding. These include the application of editing technologies such as homology-directed gene editing or prime-editing to enable the most precise and defined edits in this crop. Furthermore, increasing the overall efficiency of regeneration protocols remains an active area of research.

The CRISPR/Cas system has even further scalability; one can use it to either regulate gene expression or introduce a single base change or introduce/remove epigenetic marks, by using a system where, respectively, a transcriptional activator/repressor or a base editor or a protein introducing/removing epigenetic marks is linked to a Cas endonuclease that functions as a nickase or a "dead" Cas [71, 72]. While some of these applications have already been applied in *B. napus* precision breeding [16, 20], some still await implementation.

The next reasonable step is to introduce the gene-edited traits through introgression breeding into elite varieties or, when genotype dependency does not play a role, apply the CRISPR/Cas technique to directly modify these elite varieties.

Acknowledgments The research experiments conducted by JB, financed from the funds of the National Science Centre in Poland (2017/01/X/NZ1/01981) and the Ministry of Science and Higher Education in Poland (1189-B research grant from the Faculty of Biological and Veterinary Sciences at the Nicolaus Copernicus University in Toruń), were the inspiration to write this publication. The Author acknowledges the European COST Action CA18111 – Genome Editing in Plants for providing an excellent platform to discuss genome editing-related topics.

References

1. Chalhoub, B., Denoeud, F., Liu, S., et al.: Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science. **345**, 950–953 (2014)
2. Friedt, W., Snowdon, R.: Oilseed rape. In: Vollmann, J., Rajcan, I. (eds.) Handbook of plant breeding, oil crops, pp. 91–126. Springer, New York (2009)
3. Hasan, M., Seyis, F., Badani, A.G., Pons-Kühnemann, J., Friedt, W., Lühs, W., Snowdon, R.J.: Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. Genet. Resour. Crop Ev. **53**, 793–802 (2006)
4. Bus, A., Körber, N., Snowdon, R.J., Stich, B.: Patterns of molecular variation in a species-wide germplasm set of *Brassica napus*. Theor. Appl. Genet. **123**(8), 1413–1423 (2011)

5. Qian, L., Qian, W., Snowdon, R.J.: Sub-genomic selection patterns as a signature of breeding in the allopolyploid *Brassica napus* genome. BMC Genomics. **15**, 1170 (2014)
6. Hundleby, P., Harwood, W.: Regulatory constraints and differences of genome-edited crops around the globe. In: Wani, S.H., Hensel, G. (eds.) Genome editing, pp. 319–341. Springer, Cham (2022)
7. FAO/IAEA: Mutant variety database, Vienna. https://mvd.iaea.org. Accessed 05 May 2023 (2022)
8. Halford, N.G.: Legislation governing genetically modified and genome-edited crops in Europe: the need for change. J. Sci. Food Agr. **99**(1), 8–12 (2019)
9. Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D.L., Wei, P., Cao, F., Zhu, S., Zhang, F., Mao, Y., Zhu, J.K.: Efficient genome editing in plants using a CRISPR/Cas system. Cell Res. **23**(10), 1229–1232 (2013)
10. Nekrasov, V., Staskawicz, B.J., Weigel, D., Jones, J.D.G., Kamoun, S.: Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. Nat. Biotechnol. **31**, 691–693 (2013)
11. Zhang, H., Zhang, J., Lang, Z., Botella, J.R., Zhu, J.-K.: Genome editing-principles and applications for functional genomics research and crop improvement. Crit. Rev. Plant Sci. **36**, 291–309 (2017)
12. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E.: A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. **337**(6096), 816–821 (2012)
13. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., Zhang, F.: Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. **8**(11), 2281–2308 (2013)
14. Zaman, Q.U., Chu, W., Hao, M., Shi, Y., Sun, M., Sang, S.F., Mei, D., Cheng, H., Liu, J., Li, C., Hu, Q.: CRISPR/Cas9-mediated multiplex genome editing of JAGGED gene in *Brassica napus* L. Biomolecules. **9**(11), 725 (2019)
15. Zhang, K., Nie, L., Cheng, Q., Yin, Y., Chen, K., Qi, F., Zou, D., Liu, H., Zhao, W., Wang, B., Li, M.: Effective editing for lysophosphatidic acid acyltransferase 2/5 in allotetraploid rapeseed (*Brassica napus* L.) using CRISPR-Cas9 system. Biotechnol. Biofuels. **12**(225), 1–18 (2019)
16. Gocal, G.F.W.: Gene editing in *Brassica napus* for basic research and trait development. In Vitro Cell Dev.-Pl. **57**, 731–748 (2021)
17. Tian, Q., Li, B., Feng, Y., Zhao, W., Huang, J., Chao, H.: Application of CRISPR/Cas9 in rapeseed for gene function research and genetic improvement. Agronomy. **12**(4), 824 (2022)
18. Jorasch, P.: The global need for plant breeding innovation. Transgenic Res. **28**, 81–86 (2019)
19. Chang, T., Guan, M., Zhou, B., Peng, Z., Xing, M., Wang, X., Guan, C.: Progress of CRISPR/Cas9 technology in breeding of *Brassica napus*. Oil Crop Sci. **6**(2), 53–57 (2021)
20. Li, J., Yu, X., Zhang, C., Li, N., Zhao, J.: The application of CRISPR/Cas technologies to *Brassica* crops: current progress and future perspectives. aBIOTECH. **3**, 146–161 (2022)
21. Ali, E., Zhang, K.: CRISPR-mediated technology for seed oil improvement in rapeseed: challenges and future perspectives. Front. Plant Sci. **14**, 1086847 (2023)
22. Ma, C., Zhu, C., Zheng, M., Liu, M., Zhang, D., Liu, B., Li, Q., Si, J., Ren, X., Song, H.: CRISPR/Cas9-mediated multiple gene editing in *Brassica oleracea* var. *capitata* using the endogenous tRNA-processing system. Hortic. Res. **6**, 20 (2019)
23. Zetsche, B., Heidenreich, M., Mohanraju, P., Fedorova, I., Kneppers, J., DeGennaro, E.M., Winblad, N., Choudhury, S.R., Abudayyeh, O.O., Gootenberg, J.S., Wu, W.Y., Scott, D.A., Severinov, K., van der Oost, J., Zhang, F.: Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. Nat. Biotechnol. **35**, 31–34 (2017)
24. Castel, B., Tomlinson, L., Locci, F., Yang, Y., Jones, J.D.G.: Optimization of T-DNA architecture for Cas9-mediated mutagenesis in *Arabidopsis*. PLoS One. **14**(1), e0204778 (2019)
25. Hahn, F., Korolev, A., Sanjurjo Loures, L., Nekrasov, V.: A modular cloning toolkit for genome editing in plants. BMC Plant Biol. **20**, 179 (2020)

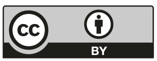
26. Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Nekrasov, V.: Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods. **9**, 39 (2013)
27. Ahmad, N., Rahman, M.U., Mukhtar, Z., Zafar, Y., Zhang, B.: A critical look on CRISPR-based genome editing in plants. J. Cell. Physiol. **235**, 666–682 (2020)
28. Ordon, J., Bressan, M., Kretschmer, C., Dall'Osto, L., Marillonnet, S., Bassi, R., Stuttmann, J.: Optimized Cas9 expression systems for highly efficient *Arabidopsis* genome editing facilitate isolation of complex alleles in a single generation. Funct. Integr. Genomic. **20**, 151–162 (2019)
29. Lawrenson, T., Chhetry, M., Clarke, M., Hundleby, P., Harwood, W.: Improved *Sp*Cas9 and *Lb*Cas12a genome editing systems in *Brassica oleracea* and *Brassica napus*. BioRxiv. (2022). https://doi.org/10.1101/2022.05.16.492057
30. Grützner, R., Martin, P., Horn, C., Mortensen, S., Cram, E.J., Lee-Parsons, C.W.T., Stuttmann, J., Marillonnet, S.: High-efficiency genome editing in plants mediated by a Cas9 gene containing multiple introns. Plant Commun. **2**(2), 100135 (2020)
31. Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., van der Oost, J., Regev, A., Koonin, E.V., Zhang, F.: Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. **163**(3), 759–771 (2015)
32. Tang, X., Lowder, L.G., Zhang, T., Malzahn, A.A., Zheng, X., Voytas, D.F., Zhong, Z., Chen, Y., Ren, Q., Li, Q., Kirkland, E.R., Zhang, Y., Qi, Y.: A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat. Plants. **3**, 17018 (2017)
33. Schindele, P., Puchta, H.: Engineering CRISPR/LbCas12a for highly efficient, temperature-tolerant plant gene editing. Plant Biotechnol. J. **18**(5), 1118–1120 (2020)
34. Le Blanc, C., Zhang, F., Mendez, J., Lozano, Y., Chatpar, K., Irish, V.F., Yannick, J.: Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. Plant J. **93**(2), 377–386 (2017)
35. Gao, Y., Zhao, Y.: Self-processing of ribozyme-flanked RNAs into guide RNAs *in vitro* and *in vivo* for CRISPR-mediated genome editing. J. Integr. Plant Biol. **56**(4), 343–349 (2014)
36. Bernabé-Orts, J.M., Casas-Rodrigo, I., Minguet, E.G., Landolfi, V., Garcia-Carpintero, V., Gianoglio, S., Vázquez-Vilar, M., Granell, A., Orzaez, D.: Assessment of Cas12a-mediated gene editing efficiency in plants. Plant Biotechnol. J. **17**(10), 1971–1984 (2019)
37. Wolter, F., Puchta, H.: *In planta* gene targeting can be enhanced by the use of CRISPR/Cas12a. Plant J. **100**(5), 1083–1094 (2019)
38. Dai, C., Li, Y., Li, L., Du, Z., Lin, S., Tian, X., Li, S., Yang, B., Yao, W., Wang, J., Guo, L., Lu, S.: An efficient *Agrobacterium*-mediated transformation method using hypocotyl as explants for *Brassica napus*. Mol. Breed. **40**, 96 (2020)
39. Ranch, J.P., Liebergesell, M., Garnaat, C.W., Huffman, G.A.: Auxotrophic *Agrobacterium* for plant transformation and methods thereof. US Patent. 8334429 (2012)
40. Cao Chu, U., Kumar, S., Sigmund, A., Johnson, K., Li, Y., Vongdeuane, P., Jones, T.J.: Genotype-independent transformation and genome editing of *Brassica napus* using a novel explant material. Front. Plant Sci. **11**, 579524 (2020)
41. Anand, A., Bass, S.H., Wu, E., Wang, N., McBride, K.E., Annaluru, N., Miller, M., Hua, M., Jones, T.J.: An improved ternary vector system for *Agrobacterium*-mediated rapid maize transformation. Plant Mol. Biol. **97**, 187–200 (2018)
42. Yang, Y., Zhu, K., Li, H., Han, S., Meng, Q., Khan, S.U., Fan, C., Xie, K., Zhou, Y.: Precise editing of *CLAVATA* genes in *Brassica napus* L. regulates multilocular silique development. Plant Biotechnol. J. **16**(7), 1322–1335 (2018)
43. Lin, C.S., Hsu, C.T., Yang, L.H., Lee, L.Y., Fu, J.Y., Cheng, Q.W., Wu, F.H., Hsiao, H.C., Zhang, Y., Zhang, R., Chang, W.J., Yu, C.T., Wang, W., Liao, L.J., Gelvin, S.B., Shih, M.C.: Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. Plant Biotechnol. J. **16**(7), 1295–1310 (2018)

44. Boniecka, J., Turkan, S., Nekrasov, V., Ruttink, T., Eeckhaut, T., Van Laere, K.: Targeted mutagenesis in oilseed rape (*Brassica napus* L.) protoplasts using CRISPR/Cas. Presented at the 2nd PlantEd conference plant genome editing, the wide range of applications, Lecce, 20–22 September 2021
45. Zhang, Y., Iaffaldano, B., Qi, Y.: CRISPR ribonucleoprotein-mediated genetic engineering in plants. Plant Commun. **2**(2), 100168 (2021)
46. Murovec, J., Guček, K., Bohanec, B., Avbelj, M., Jerala, R.: DNA-free genome editing of *Brassica oleracea* and *B. rapa* protoplasts using CRISPR-Cas9 ribonucleoprotein complexes. Front. Plant Sci. **9**, 1594 (2018)
47. Hu, Q., Andersen, S.B., Hansen, L.N.: Plant regeneration capacity of mesophyll protoplasts from *Brassica napus* and related species. Plant Cell Tiss. Org. **59**, 189–196 (1999)
48. Li, X., Sandgrind, S., Moss, O., Guan, R., Ivarson, E., Wang, E.S., Kanagarajan, S., Zhu, L.H.: Efficient protoplast regeneration protocol and CRISPR/Cas9-mediated editing of glucosinolate transporter (*GTR*) genes in rapeseed (*Brassica napus* L.). Front. Plant Sci. **12**, 680859 (2021)
49. Boniecka, J., Prusińska, J., Dąbrowska, G.B., Goc, A.: Within and beyond the stringent response-RSH and (p)ppGpp in plants. Planta. **246**(5), 817–842 (2017)
50. Boniecka, J., Kotowicz, K., Skrzypek, E., Dziurka, K., Rewers, M., Jędrzejczyk, I., Wilmowicz, E., Berdychowska, J., Dąbrowska, G.B.: Potential biochemical, genetic and molecular markers of deterioration advancement in seeds of oilseed rape (*Brassica napus* L.). Ind. Crop. Prod. **130**, 478–490 (2019)
51. Shillito, R.D., Whitt, S., Ross, M., Ghavami, F., Vleesschauwer, D.D., D'Halluin, K., Hoecke, A.V., Meulewaeter, F.: Detection of genome edits in plants—from editing to seed. In Vitro Cell Dev.–Pl. **57**, 595–608 (2021)
52. Jedličková, V., Mácová, K., Štefková, M., Butula, J., Staveníková, J., Sedláček, M., Robert, H.S.: Hairy root transformation system as a tool for CRISPR/Cas9-directed genome editing in oilseed rape (*Brassica napus*). Front. Plant Sci. **13**, 919290 (2022)
53. Gordon-Kamm, W., Barone, P., Svitashev, S., Sander, J.D., Kumar, S., Jones, T.: Strategies for CRISPR/Cas9-mediated genome editing: from delivery to production of modified lants. Burleigh Dodds Science Publishing Limited, Cambridge (2021)
54. De Block, M., De Brouwer, D., Tenning, P.: Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. **91**(2), 694–701 (1989)
55. Jonoubi, P., Mousavi, A., Majd, A., Salmanian, A.H., Jalali Javaran, M., Daneshian, J.: Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by *Agrobacterium tumefaciens*. Biol. Plantarum. **49**(2), 175–180 (2005)
56. Boniecka, J., Rybicka, A., Schweighofer, A., Trejgell, A.: Rape (*Brassica napus* L.) transformation and shoot organogenesis: important steps towards successful genome editing. Presented at the 1st PlantEd Conference Plant Genome Editing, State of the Art, Novi Sad, 6–7 November 2019
57. Liu, Y., Du, Z., Lin, S., Li, H., Lu, S., Guo, L., Tang, S.: CRISPR/Cas9-targeted mutagenesis of *BnaFAE1* genes confers low-erucic acid in *Brassica napus*. Front. Plant Sci. **13**, 848723 (2022)
58. Rani, T., Yadav, R.C., Yadav, N.R., Rani, A., Singh, D.: Genetic transformation in oilseed brassicas – a review. Indian J. Agr. Sci. **83**(4), 367–373 (2013)
59. Gordon-Kamm, B., Sardesai, N., Arling, M., Lowe, K., Hoerster, G., Betts, S., Jones, T.: Using morphogenic genes to improve recovery and regeneration of transgenic plants. Plants. **8**(2), 38 (2019)
60. Debernardi, J.M., Tricoli, D.M., Ercoli, M.F., Hayta, S., Ronald, P., Palatnik, J.F., Dubcovsky, J.: A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat. Biotechnol. **38**(11), 1274–1279 (2020)
61. Kong, J., Martin-Ortigosa, S., Finer, J., Orchard, N., Gunadi, A., Batts, L.A., Thakare, D., Rush, B., Schmitz, O., Stuiver, M., Olhoft, P., Pacheco-Villalobos, D.: Overexpression of the transcription factor *GROWTH-REGULATING FACTOR5* improves transformation of dicot and monocot species. Front. Plant Sci. **11**, 572319 (2020)

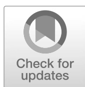
62. Demirer, G.S., Silva, T.N., Jackson, C.T., Thomas, J.B., Ehrhardt, D.W., Rhee, S.Y., Mortimer, J.C., Landry, M.P.: Nanotechnology to advance CRISPR-Cas genetic engineering of plants. Nat. Nanotechnol. **16**(3), 243–250 (2021)
63. Dinesh-Kumar, S.P., Voytas, D.F.: Editing through infection. Nat. Plants. **6**(7), 738–739 (2020)
64. Ellison, E.E., Nagalakshmi, U., Gamo, M.E., Huang, P.J., Dinesh-Kumar, S., Voytas, D.F.: Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. Nat. Plants. **6**(6), 620–624 (2020)
65. Clough, S.J., Bent, A.F.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**(6), 735–743 (1998)
66. Verma, S.S., Chinnusamy, V., Bansa, K.C.: A simplified floral dip method for transformation of *Brassica napus* and *B. carinata*. J. Plant Biochem. Biot. **17**(2), 197–200 (2008)
67. Li, J., Tan, X., Zhu, F., Guo, J.: A rapid and simple method for *Brassica napus* floral-dip transformation and selection of transgenic plantlets. Int. J. Biol. **10**(1), 127–131 (2010)
68. McCaw, M.E., Lee, K., Kang, M., Zobrist, J.D., Azanu, M.K., Birchler, J.A., Wang, K.: Development of a transformable fast-flowering mini-maize as a tool for maize gene editing. Front. Genome Ed. **2**, 622227 (2021)
69. Braatz, J., Harloff, H.J., Mascher, M., Stein, N., Himmelbach, A., Jung, C.: CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). Plant Physiol. **174**(2), 935–942 (2017)
70. Neequaye, M., Stavnstrup, S., Harwood, W., Lawrenson, T., Hundleby, P., Irwin, J., Troncoso-Rey, P., Saha, S., Traka, M.H., Mithen, R., Østergaard, L.: CRISPR-Cas9-mediated gene editing of *MYB28* genes impair glucoraphanin accumulation of *Brassica oleracea* in the field. CRISPR J. **4**(3), 416–426 (2021)
71. Razzaq, A., Saleem, F., Kanwal, M., Mustafa, G., Yousaf, S., Arshad, H.M.I., Hameed, M.K., Khan, M.S., Joyia, F.A.: Modern trends in plant genome editing: an inclusive review of the CRISPR/Cas9 toolbox. Int. J. Mol. Sci. **20**(16), 4045 (2019)
72. Van Eck, J.: Applying gene editing to tailor precise genetic modifications in plants. J. Biol. Chem. **295**(38), 13267–13276 (2020)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.



Chapter 19
Targeted Gene Editing in Pome Fruit Genetics and Breeding: State-of-the-Art, Application Potential and Perspectives



Hanne Claessen, Pollien Aert, and Nico De Storme

Abstract Even though traditional breeding of perennial fruit trees such as apple and pear has resulted in high performing cultivars in the past, it is a very lengthy and costly process that is unable to keep up with the increasing demands for improved yield, resistance and fruit quality posed by the growing world population and the rapidly changing climate. In the last decade, significant research advances have been made that can revolutionize pome fruit breeding to meet current needs, including the sequencing of apple and pear genomes, the increased understanding of associations between gene(s) and traits of interest, and the advancement in genetic engineering tools. In particular the emergence of genome-editing tools such as the CRISPR/Cas9 technology can significantly improve the speed and accuracy of pome fruit breeding programs. This chapter reviews the progress, opportunities and challenges of genome editing tools in apple and pear, and discusses the genetic basis of several important breeding goals to find possible targets for new gene-editing applications.

1 Introduction

The term "pome" refers to an accessory fruit produced by temperate tree species belonging to the maleae tribe of the Rosaceae family. The best-known and economically most important pome fruits are apple and pear. In 2020, apples were among the five most produced fruit crops worldwide together with watermelons, bananas,

H. Claessen · P. Aert
Laboratory for Plant Genetics and Crop Improvement (PGCI), Division of Crop Biotechnics, Department of Biosystems, University of Leuven, Leuven, Belgium

N. De Storme (✉)
Laboratory for Plant Genetics and Crop Improvement (PGCI), Division of Crop Biotechnics, Department of Biosystems, University of Leuven, Leuven, Belgium

Leuven Plant Institute (LPI), KU Leuven, Leuven, Belgium
e-mail: nico.destormde@kuleuven.be

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_19

oranges and grapes with 88.3 million metric tons [1]. Both apples and pears are commercially grown in over 50 countries with China being the top producer for both fruit crops [2]. Due to their economic importance and the wide geographic distribution of their production, ongoing breeding programs are present all over the world aiming to improve various traits including higher yield, increased disease resistance, and improved fruit quality [3–5].

Pome fruit breeding is an expensive and lengthy process due to biological characteristics typical of woody tree species, namely a long juvenile period (5–7 years), self-incompatibility, high heterozygosity, a limited available gene pool for new traits, and a large genome size and chromosome number [6, 7]. In a classical breeding scheme, selected parents are intercrossed to create hybrid seedling populations, consisting of a pool of unique genotypes, followed by a strict selection of the best performing progeny clone. Due to the limited genetic variation present in advanced breeding material and established cultivars, it is often necessary to use wild or semi-wild gene pools as sources for the introduction of new traits. Pre-breeding to obtain suitable parents from these (semi-)wild gene pools that can be crossed with elite germplasm takes decades due to the long life cycle and the need for repeated cycles. More specifically, the introgression of desired alleles originating from donor varieties with a minimum of linkage drag and concomitant selection for various other traits, requires multiple generations of hybridizations followed by selection, and thus significantly adds to the breeding time [7]. Alternatively, non-GMO breeding techniques that are used include interspecific hybridization, induced mutagenesis and polyploidization. Also, a wide range of genetic and molecular tools have been used to improve the efficiency and selection accuracy of pome fruit breeding programs such as genetic linkage maps, molecular markers and whole genome sequencing [8–11]. Each of these techniques have their own advantages and drawbacks, but many of the limitations remain similar to traditional breeding.

Recently, the introduction of genome editing techniques such as CRISPR/CAS has provided efficient ways to introduce precise mutations in plants. These techniques are especially beneficial in clonally propagated fruit tree species since they can generate improved breeding outcomes compared to conventional techniques without the extensive backcrossing and associated linkage drag that is necessary when introgressing new traits in established cultivars [12]. These genome editing techniques have the potential to greatly accelerate and improve the breeding process of woody fruit tree species, even though important challenges and limitations still need to be overcome to allow their broad scale application.

This chapter reviews the recent progress in the application of genome editing techniques in apple and pear, as well as the specific opportunities and challenges. We also outline and discuss important genes underlying economically interesting traits which could be used as valuable targets for gene-editing.

2 Genome Editing Technologies in Pome Fruit Trees

Genome editing refers to the use of genetic transformation techniques that can be used to precisely edit the plant genome [13]. There are three main genome editing approaches that are all based on the use of engineered nucleases, namely zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN) and clustered regularly interspaced short palindromic repeats associated nucleases (CRIPSR/cas) [3]. These nucleases can be designed to bind a specific target DNA sequence in the genome of the plant where they induce a double-strand break (DSB) which is subsequently repaired by one of the two following processes, homology directed DNA break repair (HDR) or non-homologous end joining (NHEJ) [14, 15]. Plant transformation is generally performed using *Agrobacterium tumefaciens* followed by regeneration of transgenic tissue *in vitro*. This paragraph describes the current use, adaptations and limitations of these three genome editing techniques in pome fruit breeding.

2.1 Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases were one of the first editing technologies [16]. They are artificial enzymes generated by fusing a zinc-finger DNA-binding domain to a nonspecific DNA-cleavage domain of the Fok I endonuclease enzyme [13, 17]. A pair of custom designed ZFNs bind to the DNA at the target location and together form an active dimer nuclease complex [18]. ZFNs have been successfully used for targeted mutagenesis in many species including *Arabidopsis* [19, 20], soybean [21], rice [22], and populus [23]. To our knowledge, only a single successful instance of ZFN application in pome fruit has been published [13]. The authors validated the use of ZFNs in apple and fig using a visual transgenic repair assay based on activation of a mutated uidA gene, which encodes the GUS reporter protein. The overall efficiency in apple was around 10% and the authors concluded that the genome editing approach was suitable for application in fruit tree species. However, although the technique has been around for almost three decades, the adoption of ZFNs in plant breeding is limited mainly due to their low efficiency, the complex construction of the zinc finger region that interacts with DNA and severe off-target effects [24–27].

2.2 Transcription Activator-Like Effector Nucleases (TALENs)

Another genome editing approach is based on the family of proteins known as transcription activator-like effectors (TALEs) which are produced in bacteria including plant pathogenic Xanthomonas species. These effector proteins have a DNA binding domain which is linked with a catalytic domain of an endonuclease like Fok I

[18, 25]. TALE nucleases (TALENs) with desired specificities can be created through modification of the DNA binding domain to target specific locations in the genome. The nucleases there act like molecular scissors that produce DSBs which are repaired with the cellular repair mechanisms leading to deletions, insertions, replacements or rearrangements [18, 25].

TALEN-mediated genome editing has been successfully applied in rice [28, 29], wheat [30], maize [31], and sugarcane [32, 33] among others. However, to our knowledge, no application of TALEN-based transformation has been applied in apple or pear or even any other woody fruit tree species such as citrus or prunus. Although TALENs have some advantages over ZFNs, such as lower toxicity and somewhat simplified construction, the CRISPR/Cas approach has quickly surpassed both ZFNs and TALENs as method of choice in plant genome editing [26, 27].

2.3 CRISPR/Cas Systems

Currently, clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing has become the most reliable and cost-effective approach in plant research. Unlike ZFN and TALEN, the DNA-recognition, based on RNA-DNA interactions, is faster, cheaper and generally more efficient [17, 34]. In plants, CRISPR/Cas 9 is the most used system and consists of a Cas 9 nuclease and a single guide RNA which replaces the original CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracr RNA). The gRNA contains a unique sequence of 20 bp which preceeds a protospacer adjacent motif (PAM). This gRNA binds the Cas 9 nuclease and directs it to a complementary target sequence on the genomic DNA. The two nuclease domains of the Cas 9 protein (RuvC and HNH) will then cleave the target sequence at three nucleotides upstream of the PAM site, leaving predominantly blunt ends [26, 35].

The CRISPR/Cas9 method has been widely adopted in plant research and has been used in many crops including pome fruit species. It was first used in apple to modify an apple phytoene desaturase (*PDS*) gene which encodes an essential plant carotenoid biosynthetic enzyme required for chlorophyll biosynthesis. Knock-out of this gene leads to an albino phenotype making it an easy visual marker [34, 36]. CRISPR/Cas9 was also used to reduce susceptibility to fire blight in apple [37], and induce early flowering in apple and pear [38]. It has also been applied in wild apple to target the *MsPDS* gene [39]. To date, nearly all applications in pome fruit use the CRISPR/Cas9 system, however alternative CRISPR systems can be applied as well [40]. One example is the CRISPR/Cas12 system, previously known as Cpf1, which also belongs to the class 2 CRISPR systems, but lacks the HNH domain and generates a staggered cut with a 5 nt 5′ overhang [41]. CRISPR/Cas12 has been successfully applied in other woody, perennial tree species such as poplar [42] and citrus [43]. Alternative CRISPR/Cas systems may be more suitable in specific situations, for example the CRISPR/Cas12 can be used to target T-rich regions of the genome which is difficult with CRISPR/Cas9 [44].

Base-editors are another type of genome-editing tools. They are derived from the CRISPR/Cas9 approach and allow precise nucleotide substitutions without double stranded breaks [45]. In plants, DSBs are generally repaired using the NHEJ mechanism which creates small insertions or deletions in the target sequence and usually results in gene knock-out. The alternative HDR mechanism can precisely introduce point mutations using template DNA, however it is very inefficient, especially in plants [45]. Base editors don't use DSBs, but create precise base substitutions of A-to-G or C-to-T using a nickase Cas9 (nCas) coupled to either an adenine deaminase (ABE) or a cytidine deaminase (CBE), respectively [45, 46]. The CBE-nCa9s fusion converts cytosine to uracil without cutting the DNA and this uracil is later converted to thymine through DNA replication or repair. Similarly, the ABE-nCa9s fusion converts adenine to inosine which is later converted to guanine. A uracil DNA glycosylase inhibitor protein (UGI) can be added to the construct to prevent uracil excision which lowers the efficiency of the transformation [46]. Malabarba et al. [45] applied a CBE base editing system including a UGI sequence and the nCas9-PmCDA1 fusion for the first time in apple and pear on two targets, *acetolactate synthase* (*ALS*) and *phytoene desaturase* (*PDS*). The authors induced a stop-codon in *PDS* and an amino-acid substitution in *ALS* in apple and pear resulting in chlorsulfuron herbicide resistant, dwarfed, albino plants. The study proved the feasibility of targeting multiple genes with base editing in apple and pear but also revealed important challenges that still need to be addressed.

2.4 Limitations of Genome Editing

Despite the advantages and many potential applications of these new genome-editing tools, important challenges still remain. While most studies currently use CRISPR-based methods for genome editing, many of the limitations listed here also apply to ZFN and TALEN methods.

A first major drawback of these genome-editing tools, including CRISPR-based methods, is insufficient target specificity and accuracy. Off-target cleavage can occur when the first 17–20 nucleotides of the sgRNA match with other regions in the genome instead of solely with the target and also in apple, these off-target mutations have been reported [45].

Another problem is the production of chimeras. Several of the studies on the genome editing of pear and apple discussed in this chapter report the production of chimeras during regeneration of transformed apple or pear plants [45]. Chimeras consist of genotypically distinct cells or tissues which may refer to transformed vs non-transformed cells, but also to transformed cells with different mutant alleles where the genome editing machinery has introduced distinct mutations in the same gene of different cells [45]. For example, Charrier et al. [38] reported a high rate of phenotypic chimeras (64% of regenerated transgenic plants) and editing chimeras (88% of pure albino phenotype transgenic plants) after CRISPR/Cas9 KO of the *PDS* gene in apple. The number of editing chimeras also seemed to increase during

the regeneration period, possibly due to the continued expression of the CRISPR/Cas9 cassette. Elimination of chimeras is necessary to establish stable mutants that can reliably pass on the desired trait to progeny. However, exclusion of chimerism through sexual reproduction of the transgenic plants is difficult in the highly heterozygous and self-incompatible woody fruit tree species. Alternatively, adventitious shoot regeneration may be used, but this process takes several rounds of regeneration [45]. Another possible method is the direct delivery of CRISPR/Cas9 ribonucleoprotein (RNPs) into protoplasts which could decrease the number of produced chimera [47]. Direct transfection of apple and grapevine protoplasts has already been performed by delivery of cas9 and gRNA using PEG solution [48, 49].

Transformation efficiency in fruit trees is generally still very low compared to herbaceous plants even using CRISPR/Cas9. One major obstacle contributing to this low efficiency is the highly recalcitrant nature of these species to both genetic transformation and *in vitro* regeneration [50]. In addition, this transformation and regeneration efficiency seems to vary significantly based on the target, transformed cell type, delivery method, regeneration method, species and even genotype. For example, pear transformation seems to happen at lower efficiency compared to apple: the use of the CRISPR-Cas9 system to knock out Terminal Flower 1 (TFL1) genes in apple and pear to obtain the early-flowering genotype, resulted in 93% of apple transgenic lines compared to 9% of pear transgenic lines [38]. A recent study attempted to increase transformation efficiency in apple through ectopic expression of *MdBBM1* which promotes plant regeneration [50]. However, it seems that extensive protocol optimization remains necessary for transformation and regeneration of different species and even different cultivars. This also makes obtaining a transformed plant, albeit less laborious than conventional transgenesis, still very cumbersome, especially since regenerated plants still require screening and quality control.

Finally, there is considerable public concern regarding transgenic plants. After the custom-designed nucleases are transformed into transgenic plants and have induced the desired DSB and mutation, they have to be removed to obtain transgene-free plants. This can happen via genetic segregation and back-crossing, but this method is less suitable for fruit trees [13]. Better approaches include transient expression of CRISPR/Cas9 or the use of preassembled CRISPR/Cas9 ribonucleoproteins (RNPs) [37, 51].

3 Application Potential of Genome-Editing for the Advancement of Important Pome Fruit Breeding Goals

3.1 Yield Improvement

Higher yield is one of the most important traits to achieve in plant breeding, albeit also one of the most difficult since it is a quantitative trait that is determined by many underlying small-effect genes and is highly influenced by environmental

conditions and management practices [52]. The complex background of yield makes this trait in itself very difficult to use as a specific breeding target, especially for targeted gene-editing. Instead, it can be considered as a combination of two traits, fruit number (crop load) and fruit size, which need to be carefully balanced and which are both still the result of a complex, multigenic regulation with significant environmental impact.

No known pome fruit breeding programs have increased crop load as a breeding target. Instead, a lot of research is focused on crop load management to minimize biennial bearing and low fruit size due to overcropping. Good management practices to optimize fruit load include cultivar-adapted pruning to optimize the light penetration into the canopy and the distribution of spurs versus extension shoots, thinning of flower buds, flowers and fruitlets, and well-considered root-stock choices to control vegetative growth [53, 54]. High and stable crop load in apple and pear are therefore mainly indirectly targeted in breeding programs through more specific objectives such as root stock breeding, optimized tree architecture and decreased biennial bearing tendency. Tree architecture and biennial bearing are discussed more elaborately later in this chapter.

Similarly, fruit size is also largely determined by environmental factors including orchard management, flower fertilization success, seed number and current crop load [55]. However, more information is available on the genetic basis of fruit size potential compared to crop load since studies often use single fruit weight as an indicative measurement. Fruit size is a complex quantitative trait that is controlled by multiple genes [56]. It depends on the number of cells, the size of the cells and the size of the intracellular spaces [57]. Many QTLs and several major-effect genes have been identified in different studies [56]. Some interesting major-effect genes in apple were homologous to *Arabidopsis* cell expansion gene *AtSAUR19* and tomato fruit size/shape determining genes *SlOVATE* and *SUN* [58]. Also, several miRNAs have been associated with fruit weight. For example, overexpression of miR172p in transgenic "Royal Gala" apple significantly reduced fruit size [56]. These miRNAs are part of a large superfamily of transcription factors that play important roles in growth, development and stress response in higher plants [59]. However, the exact functions of most of these genes and miRNAs remain elusive and more research is necessary before they can be reliably used to improve fruit size using genome-editing.

Still, yield as such is often not a primary breeding objective since many other traits are considered at least as important as high yield for the acceptance of a cultivar for commercial production. These traits include many practical aspects of fruit production such as efficient orchard management (trees must be easy to harvest and cheap to maintain), disease resistance, production stability, storability, and transportability. Also many fruit quality characteristics such as flavor, texture, firmness, fruit size and novelty are important for marketability. These traits are breeding objectives in their own right and the most important ones are further discussed in this chapter.

3.2 *Fruit Quality Attributes*

3.2.1 Sensorial Fruit Quality

The sensory or organoleptic quality of apple and other fresh fruits is determined by three main parameters, namely taste, texture and aroma. These three major sensory fruit quality attributes each independently impact the sensorial evaluation of the fruit, but also exhibit an intricate interplay that largely determines the overall taste and appreciation of fresh fruit. Some organoleptic quality attributes, like soluble sugar content, titratable acid (TA) and fruit firmness can be quantified using biochemical assays. However, these singular quality attributes only provide partial insights of the fruit quality and the evaluation of organoleptic fruit quality is therefore still mainly performed via sensory panels. Research on existing cultivars suggests that, in general, consumers prefer apples with firm, crisp texture, that are moderately juicy and that have a balance of sweet to acid taste. These sensory fruit quality attributes, together with a specific aroma profile, present one of the main targets for consumer-focused plant breeding in apples.

Genetic mapping and association studies in apple and other fruit species have revealed that many of these sensory fruit quality attributes including juiciness, crispness, mealiness, skin color, russet frequency, titratable acidity and soluble solids content have a complex regulation, and are quantitatively determined by a broad range of genomic regions with significant environmental impact [60–64]. This complex regulation with involvement of many genes as well as significant impact from the environment makes it more difficult to improve these fruit quality attributes through targeted gene editing, since (i) key regulatory genes have to be known and sequence-identified, (ii) desired alleles have to be characterized (additivity) together as well as the most optimal combination of alleles at different loci (dominant and epistatic effects), and (iii) all desired alleles have to be genetically engineered within the same generation.

Despite this complex regulation of various aspects of sensorial fruit quality, some major causative genes have been found to regulate specific aspects of fruit taste, texture or aroma and often the most desired allele, i.e. conferring an optimal level of a specific fruit quality parameter, is already known (as well as the genetic source). However, for most of these genes, a specific allelic DNA sequence variant conferring fine-tuned activity of the encoded protein, is desired, rather than a functional knock-out or null mutant, implying that desired traits can only be genetically introduced by advanced editing methods that either introduce nucleotide base pair switches (base editing) or enable allele replacement (HDR – homology directed recombination). These advanced gene editing techniques are only recently available for apple and other perennial fruit trees, and their use and applicability to modulate organoleptic fruit quality has, up till now, not yet been demonstrated. However, studies in other fruit species have shown that targeted editing of specific genes can be used to improve the sensorial quality of fresh fruit. In the following sections, key genes that determine the expression of different fruit quality attributes are outlined

together with their desired genetic configuration (allelic variants), with a predominant focus on genes that can be functionally depleted to obtain the desired phenotype.

Fruit taste refers to the basic sensory evaluation of the fruit commodity (sweet, sour, bitter, etc.) and for apple and other pome fruit species mainly depends on the content and composition of soluble sugars and organic acids [65]. Although the overall content of both these types of macromolecules is important, it is mainly the relative sugar/acidity ratio (TSS/acidity balance) of the fruit that determines the fruit's characteristic taste, implying that both aspects are linked and always need to be improved in parallel. The sweetness of the apple fruit is predominantly determined by sorbitol and the total content of soluble sugars (SSC), with no direct effect of single sugars (like sucrose, glucose, fructose, xylose), though with significant contribution from several volatile compounds, like esters and farnesene [66]. In line with the complex metabolic pathways of each of these sweetness-contributing chemicals, genetic assays have revealed that the sweetness of pome fruit is determined by multiple genetic loci, with each loci only having minor effects [56, 67–70]. For the fraction of fructose and sucrose in the total sugar pool, however, a major locus was identified on linkage group 1 that respectively explains 47% and 27% of the total variance [67]. This locus harbors the VIN1 vacuolar invertase (MDP0000149570), which enzymatically confers hydrolysis of sucrose into glucose and fructose to regulate the entry of sugars into different metabolic pathways [71]. Similarly, a pedigree-based QTL mapping approach in a "Honeycrisp"-derived germplasm identified and validated three large effect QTLs, i.e. on LG1, 13 and 16, that were consistent across multiple years for the total SSC content in apple fruit [70]. However, the causative genes have not yet been identified. Targeted gene expression studies throughout apple fruit development have shown that the accumulation of fructose in later stages coincides with an enhanced expression of the tonoplast monosaccharide transporters (TMTs) *MdTMT1* and *MdTMT2* which convert the excess amount of imported sugars into starch. At final fruit maturation, the accumulation of sucrose overlaps with an elevated expression and enhanced activity of the sucrose-phosphate synthases (SPS) *MdSPS5* and *MdSPS6*, which catalyze the transfer of a hexosyl group from UDP-glucose to D-fructose 6-phosphate to form UDP and D-sucrose-6-phosphate [72]. A combined QTL and transcriptomics study in Asian pear (*Pyrus pyrifolia*) identified two sucrose transport genes (*PpSUT*, LOC103964096, and LOC103940043) that are negatively correlated with the sugar content in ripening fruit [73]. In addition, two sorbitol dehydrogenase genes (*PpSDH genes*, LOC103960512 and LOC103960513), were also found to be negatively co-expressed with total sugar content in the fruit, indicating that these act as antagonists of fruit sweetness [73]. Plants harbor different types of sugar transporters, and the SWEET-class of sugar transporters (i.e., Sugar Will Eventually Be Exported Transporters) have been found to play a major role in sugar accumulation in the fruit of apple as well as various other fruit tree crops. Marker-based association studies thereby revealed that in particular three SWEET genes, i.e. namely *MdSWEET2e, MdSWEET9b, MdSWEET15a*, are significantly associated with total sugar content in the fruit, with MdSWEET15a and MdSWEET9b accounting for a relatively large

portion of the phenotypic variation (and located on a region harboring a QTL for sugar content) [74, 75].

Fruit acidity is a major determinant of the overall apple flavor and strongly influences the perception of other flavor traits such as sweetness and aroma. The pH of fresh apples generally ranges from 3.3 and 4.0, making them mildly acidic, and this is almost exclusively attributed to the accumulation of organic acids, with malic acid forming the major fraction (±90% of all organic acids) followed by quinic acid (±5%), citric acid (±1,5%) and small amounts of ascorbic, shikimic, maleic and tartaric acid [76, 77]. While in wild apple varieties fruit acidity is determined by the content of both malic and citric acid, in cultivated apple varieties citric acid is almost completely absent and fruit acidity is almost exclusively determined by malic acid [76, 78]. The concentration of malic acid can be measured sensorially through panel tasting, or more quantitatively by pH measurement, analytical methods (HPLC) or via titration of fruit juice, with the latter actually indicating the total content of organic acids as expressed in malic acid equivalents per fruit mass (TA: titratable acidity) [79]. Although acidity of freshly harvested apples is a complex trait, genetic studies have revealed that it is predominantly determined by two large effect QTLs together with various minor QTLs that mainly determine the variance in high acidity apple varieties (e.g. Ma4, Ma6, M2, and M3) [80–83]. More specifically, apple fruit acidity is genetically determined by one QTL on LG16, referred to as the *Ma* locus [84], and one on LG8, called the *Ma3* locus [85], with both QTLs jointly explaining 66% ± 5% of the phenotypic variation through an additive allele dosage model with incomplete dominance [86–88]. For the Ma3 locus on LG8, up till now, no candidate genes have been identified that control apple fruit acidity. In contrast, high resolution mapping and expression assays provided strong evidence that the aluminum-activated malate transporter gene (ALMT1 or Ma1) is the fruit acidity determining-gene in the Ma locus [89]. ALMT1 encodes a membrane-associated protein that is targeted to the tonoplast and actively transports malic acid molecules from the cytosol to the vacuole, which serves as major subcellular repository for organic acids, hence contributing to its cellular accumulation [90]. The low acidity *ma1* allele still localizes to the tonoplast but exhibits reduced malate transport functionality as compared to the pseudo-dominant high acidity *Ma1* allele due to a 84-AA truncation in the conserved C-terminal end domain [90]. Diversity studies in apple also showed that expression of Ma1 is significantly correlated with the fruit titratable acidity at harvest [89], making it a highly suitable candidate for direct modulation of fruit acidity through gene editing approaches. Recent studies provided more insights into the regulation of these tonoplast transporters in apple fruit and identified several MYB transcription factors, including MdMYB1 and MdMYB73, as important regulators of vacuolar accumulation of malate. MdMYB1 promotes the expression of two genes encoding B subunits of vacuolar H^+-ATPase (VHA), MdVHA-B1 and MdVHA-B2, and thereby transcriptionally activates its H+ pumping activity and enhances the transport of malate into the vacuoles [91]. Similarly, MdMYB73 transcriptionally activates MdALMT9, MdVHA-A and MdVHP1 (vacuolar pyrophosphatase 1) to enhance their activity, leading to increased concentrations of malate and vacuolar pH [92]. The activity of MdMYB73

towards its downstream targets was thereby found to be positively influenced by the interaction with MdCIbHLH1. This transcriptional cascade (that promotes malate accumulation in the vacuole) is antagonized by the BTB-BACK-TAZ domain protein MdBT2 which targets MdCIbHLH1 and MdMYB73 for ubiquitination and subsequent degradation by the 26S proteasome pathway, hence negatively regulating accumulation of malate and vacuolar acidification [93]. Recently, another R2R3 − MYB transcription factor, namely *MdMYB44,* was found to control the fruit malate content and acidity in apple, though in a negative manner. MdMYB44 represses the promoter activity of the malate-associated genes *Ma1* (*Aluminum-Activated Malate Transporter 9*), *Ma10* (*P-type ATPase 10*), *MdVHA-A3* (*V-type ATPase A3*), and *MdVHA-D2* (*V-type ATPase D2*), and thus suppresses vacuolar import and accumulation of malate. Importantly, specific SNPs in the promotor region of MdMYB44 thereby showed strong association with fruit malate content, i.e. either through their effect on basal activity or by altering affinity towards the basic-helix–loop–helix TF MdbHLH49 [94]. Parallel to MdMYB44, the protein phosphatase MdPP2CH also negatively regulates accumulation of malate in the fruit by post-transcriptionally suppressing the activity of the vacuolar H^+-ATPases MdVHA-A3, MdVHA-B2 and MdVHA-D2 as well as the malate transporter MdALMTII through dephosphorylation [95]. As MdSAUR37 was thereby found to promote malate accumulation in the apple fruit by negatively regulating the MdPP2HC phosphatase activity, the MdSAUR37/MdPP2CH/MdALMTII chain was found to precisely determine apple fruit malate contents through hierarchical epistatic genetic effects [95]. Overall, multiple genetic factors that contribute to metabolism and vacuolar accumulation of organic acids, and particularly malic acid, in apple fruit have been retrieved with identification of both positive and negative regulators and associated characterization of allelic effects. Despite the absence of concrete examples, these genetic insights provide a strong basis for precisely modulating the titratable acidity as well as the overall organoleptic appreciation of the apple fruit through targeted gene editing approaches such as CRISPR and TALENs.

3.2.2 Nutritional Quality and Food Functionality

During the last decade there has been a paradigm shift regarding consumer acceptance towards fruits. Due to the general awareness of the impact of food consumption on personal health and overall increased welfare, consumers now also take into account the nutritional, functional, and physio-chemical factors of fruits, in particular for fresh produce. Also for pome fruit, such as apple and pear, there is an increasing preference for varieties that have high levels of health-promoting compounds, such as essential vitamins, minerals, dietary fibers, antioxidants and other key phytochemicals. The visual characteristics (e.g. skin characteristics), eating quality (e.g. texture and flavour), and storability are among the main fruit quality traits being targeted in apple breeding programs, but the enhancement of phytochemicals is now gaining traction to select “bio-fortified” apple cultivars. However, despite

their relevance for public health, almost no directed selection for specific fruit biochemicals or overall augmented nutritional value has yet been performed. As a result, current apple varieties generally do not have high food functionality, and instead, for most nutritional parameters, have reduced contents or values as compared to their wild counterparts. For example, several studies reported that modern apple varieties have drastically reduced polyphenol content (particularly stilbenes, hydroxycinnamic acids, and dihydrochalcones) compared with the ancestral heritage, wild progenitors (*Malus sieversii*) and germline cultivars [96, 97]. Similar observations were made for organic acids, including malic and ascorbic acid, indicating significant counter-selection during domestication and breeding, most likely as indirect effect of selection against bitterness [96]. In contrast, some relevant flavonoids (flavonols and flavan-3-ols) and triterpenoids (ursolic, oleanolic, and betulinic acids) did not show this selection-induced reduction in modern apple varieties [96]. Moreover, the few incentives that projected to increase the nutritional fruit quality in apple via conventional ways failed or only had limited success, mainly due to the complexity of the underlying biochemical pathways and adverse side effects on other agronomic or consumer-related attributes.

Now, gene editing approaches allow specific enhancement of the health-related nutritional composition and food functionality of commercial apple cultivars, without affecting their typical flavor and fruit quality attributes, and therefore form an easy and straightforward method to improve general public health. Germplasm characterization studies have revealed dramatic variation in the content of various nutritional compounds in apple, including polyphenols, vitamin C, etc., indicating that the biochemical composition of the fruit is predominantly genetically determined. However, genetic studies have shown that both the content and composition of these phytochemicals is generally under polygenetic control with multiple small effect genetic loci. This diluted genetic control largely impairs the genetic enhancement of these compounds through single gene editing approaches. Though, for some biochemicals, like polyphenols, the polygenetic control is mainly determined by a small number of genetic loci that have a small effect [98]. In these cases, knowledge of these genes (the relatively simple genetic architecture) may provide a basis to significantly optimize the content or composition of the specific compound of interest through targeted gene editing approaches. For many fruit bio-chemicals, underlying genetic loci (QTLs) and associated candidate genes have been identified in apple through genetic linkage mapping or association studies [63, 99, 100]. Using a combined genomics-metabolomics approach, both Khan et al. [100] and Bilbrey et al. [99] hereby detected a large number of metabolite quantitative trait loci (mQTL) spread along all chromosomes, with hot spots on the linkage groups 16 and 17 for apple phytochemicals. However, up till now, only a few regulatory genes have actually been identified and were validated to play a functional role in the determination of the biochemical composition of apple fruit.

For polyphenols, candidate genes for the production of quercetin, epicatechin, catechin, chlorogenic acid, 4-*O*-caffeoylquinic acid and procyanidins B1, B2, and C1 have been retrieved via mapping [98], however, no further functional validation has yet been performed and actual genetic regulators have not yet been identified.

Specifically for the dihydrochalcone phloridzin (phloretin 2′-O-glucoside), i.e. the most abundant phenolic compound in apple trees (Malus × domestica), regulatory enzymes have been identified via genomics and *in vitro* studies with validation through transgenic approaches. In particular, six glucosyltransferases (UGTs) have been identified which are able to selectively glucosylate phloretin, i.e. the direct precursor of phloridzin [101]. As a follow up, one recent study used genetic approaches, including RNAi and CRISPR, to analyze the function of one of these UDP-2′-O-glucosyltransferases, namely MdPGT1, in phenol metabolism, and thereby demonstrated that PGT1 stimulates the production of phloridzin in the leaves of apple with distinct morphological differences between knock-down and genome-edited mutant lines [102].

Anthocyanins form an important group of phenolic compounds, as they confer health-related benefits due to their antioxidant activity but also contribute to sensorial fruit acceptance due to their role as a pigment. Several molecular regulators of anthocyanin metabolism have been identified, though most act in the biosynthesis and thus have a promotive effect on anthocyanin accumulation. For example, the anthocyanin biosynthesis pathway is transcriptionally regulated by the MYB-bHLH-WD40 (MBW) complex, with allelic variants of the enclosed MYB10/MdMYB1 TF [103] determining tissue-specific expression and thus controlling apple fruit (peel and flesh) as well as foliage color. Overexpression of *MYB10* leads to a significant increase in foliar, flower and fruit anthocyanins, especially in the fruit peel, with no negative impact on sensorial quality and other consumer-related quality traits. However, due to their promotive effect, these regulatory genes do not form suitable candidates for CRISPR-based gene editing for enhancing anthocyanin contents. Besides these promotive proteins, two other MYBs have recently been identified as transcriptional inhibitors of anthocyanin biosynthesis; namely MdMYB6 and MdMYB306. Xu et al. (2020) showed that MdMYB6 inhibits anthocyanin synthesis by directly inhibiting MdANS and MdGSTF12, i.e. two positive regulators of anthocyanin production, and by reducing contents of the precursors UDP-glucose and UDP-galactose by regulating the monosaccharide transporter MdTMT1 [104]. In addition, a second R2R3-MYB TF, namely MYB306-like, was found to act as an anthocyanin repressor gene. More specifically, the MdMYB306-like protein activates the expression of an anthocyanin repressor gene, MdMYB17, and inhibits the expression of the anthocyanin structural gene MdDFR through direct promotor binding, and additionally interacts with MdbHLH33 and MdMYB17 to enhance its TF regulatory activities [105]. In line with this, transient silencing of MdMYB6, MdBY306-like and MdMYB17 leads to increased anthocyanin concentrations, indicating that these genes form interesting targets for CRISPR-based mutagenesis to obtain increased anthocyanin contents in apple fruit [105]. Besides these antagonistic regulators of anthocyanin identified in apple, genetic studies in various other fruit crop systems, i.e. in particular tomato, have also identified several other factors that operate in the flavonoid metabolic pathways to suppress or reduce anthocyanin production. These include the *ATROVIOLACEA* (*ATV*) R3-MYB protein [106] and the nuclear protein DE-ETIOLATED1 (DET1) [107]. Genetic loss of function of these proteins is associated with a significant increase in

anthocyanins in the mature fruit, making them excellent candidates for CRISPR based mutagenesis of their orthologous proteins in pome fruit for increasing anthocyanin contents in the peel and the pulp.

Another bio-chemical component with strong nutritional value as antioxidant is vitamin C or ascorbic acid (AsA). Besides its general role in oxidative stress mitigation, dietary L-AsA also has various other important health benefits. Increased intake of vitamin C has been associated with a decreased incidence of several important human diseases, such as cataract, cardiovascular diseases, and cancers. Vitamin C also promotes the uptake of iron and zinc, which is particularly relevant in meat-poor diets. Humans cannot synthesize Vitamin C due to the absence of the gene encoding L-guluronic acid-1,4-lactone oxidase, which catalyzes the last step in the AsA synthesis pathway, and therefore completely rely on dietary intake of AsA to meet their daily requirements [108]. Fruits are the main source of human AsA intake, though fruit AsA levels in commercial pome fruit cultivars are generally quite low as compared to other fruit species, such as lemon, orange and strawberry, with apple and pear only containing 0.05–1.0 and 5–10 mg AsA per 100 g fresh weight, respectively [109]. The ascorbic acid metabolic pathway has been extensively studied in plants, and has been found to be rather complex involving several parallel pathways that include multiple enzymatic steps [108], with total AsA accumulation being regulated by transcription factors, protein interactions, phytohormones, and environmental factors. Despite the fact that most of the regulators have already been identified in model systems, their functional role and contribution in the AsA metabolism of pome fruit has not yet been resolved, although there are a few exceptions. For example paralogs of GDP-l-Galactose Phosphorylase (GGP) have been found to act as a major determinant of Vitamin C concentration in apple fruit, with specific alleles leading to a significantly higher level of Vitamin C content in the pulp [110]. However, as these alleles do not confer a functional GGP knock-out, but instead promote AsA biosynthesis through formation of specific protein variants, these GGP paralogs do not form suitable candidate genes to boost AsA levels in the pome fruit via CRISPR mutagenesis. Therefore, it is still unclear which genetic factors can be used for the engineering of increased fruit AsA levels in pome fruit through CRISPR editing.

3.3 Agronomic Traits

3.3.1 Disease and Pest Resistance

Apple and pear orchards are routinely plagued by insect pests, including aphids, mites, and caterpillars. Also many bacterial and fungal diseases are prominent in pome fruits. By far the most destructive bacterial disease is fire blight (*Erwinia amylovora*). Major fungal diseases include apple scab (*Venturia inaequalis*), pear scab (*Venturia pirina* and *Venturia nashicola*) and powdery mildew (*Podosphaera*

leucotricha). Viral diseases can generally be sufficiently controlled through the use of certified virus-free plant material. Economic losses due to these and other pests and diseases include loss of produce, reduced fruit quality, loss of trees or even orchards, disruption of orchard production, management costs, and costs related to stringent quarantine and international trade regulations. In addition, preventive and curative treatments through chemical fungal sprays or antibiotic sprays raise important environmental, biosafety and health concerns [111–114]. Disease and pest resistance is therefore an important breeding objective in pome fruit species.

A first approach to improve disease resistance is through the introgression of resistance genes into elite cultivars. There are two main types of disease resistance, namely quantitative and qualitative resistance. Quantitative resistance is conferred by many small-effect genes and results in partial resistance to multiple pathogen strains making it highly durable [111]. On the other hand, qualitative resistance is usually based on a gene-for-gene interaction between the pathogen avirulence (Avr) gene and the plant resistance (R) gene and often leads to a hypersensitive response against the pathogen. This form of single major gene disease resistance is easier to obtain for breeders, but can also be more easily broken by newly evolving virulent pathogen strains [111, 115]. It is therefore preferred to stack disease resistance genes or to combine qualitative and quantitative resistance [111, 114]. Resistance genes have been identified for several important pear and apple diseases. For fire blight, the only functionally characterized resistance gene is FB_MR5, however resistance conferred by this gene has regrettably already been overcome [116, 117]. Several other putative candidate resistance genes include FB_Mfu10, NBS-LRR genes of ornamental cultivar Evereste, and several genes with disease-related domains in the FB_Mar12 region [117–119]. For apple scab resistance, around 20 R genes are known, but not all confer equally durable resistance. Through worldwide monitoring of *Rvi* breakdown, the genes *Rvi5, Rvi11, Rvi12, Rvi14 and Rvi15* were identified as rarely overcome, possibly because of an associated fitness cost to the pathogen [120]. In pear, identified resistance genes for *Venturia nashicola* include *RVnk* [121], *Rvn2* [122] and *Rvn3* [123]. For *V. pirina*, *Rvp1* was identified [124]. Powdery mildew is mainly studied in apple where several resistance genes are known: *Pl1, Pl2, Plw, Pld* [125]. However, current advanced breeding material generally lacks major resistance genes and therefore, wild *Malus* and *Pyrus* species remain the most important source of disease resistance alleles. This hampers application of conventional breeding because of problems with the long life cycle of pome fruit species and linkage drag [111], especially when pyramiding multiple resistance genes. This process can be sped up through transgenic approaches. There are several examples of genetic transformation to bring resistance genes into apple and pear [126–128]. However, this approach is more difficult to apply using gene-editing techniques which are more suited for knock-out of negative regulators of resistance.

Alternatively, disease resistance in plants may be achieved through the silencing or knock-out of susceptibility (S) genes. Several studies have successfully obtained resistance cultivars through genetic transformation with S genes as target. For

example silencing of the *HIPM* gene (HrpN-interacting protein from *Malus*) through RNA interference resulted in increased fire blight resistance in apple cv. "Galaxy" [129]. Knockout of susceptibility genes is well suited for genome editing applications and has already been applied in apple cultivars "Gala" and "Golden Delicious" to significantly reduce bacterial fire blight symptoms through inactivation of *MdDIPM4* which interacts with pathogen effector protein DspA/E [37, 130]. Similarly, another study targeted *DIPM1, DIPM2* and *DIPM4* in "Golden Delicious" to increase resistance to fire blight [49]. Susceptibility genes don't always have to encode proteins that directly interact with plant effectors, but may also be more broadly involved in plant immunity. For example, targeted silencing of a frequently used susceptibility gene *MdMLO* increased powdery mildew resistance in "Gala" [131]. MLO is a transmembrane protein located in the plasma membrane and is presumed to be involved in plant defense and immunity responses [132].

Plant susceptibility genes may also be found for insect pests. For example, such genes may be involved in the induction of defense signaling pathways, food accessibility and food quality [133]. For example, in *A. thaliana*, the transcription factor *WRKY22* is involved in pathogen-triggered immunity and knock-out mutants were more difficult to colonize by aphid populations [134]. However, knowledge of S-genes against insect pests is limited and to our knowledge there have been no applications in pome fruit species to date.

Finally, another alternative approach is to target pathogen or microbial genomes using CRISPR/Cas9 technologies as compared to focusing solely on the host. For example, the gene drive system, which was shown to be successful in mosquito [135] may be adapted and applied in sexually inheriting plant pathogens [136]. Original gene drive systems are based on homing endonuclease genes (HEGs) which encode proteins that cleave a recognition site of around 20–30 nucleotides on the genome. The HEG itself is inserted in the middle of its own recognition site thereby protecting itself from further cleavage. When the HEG comes in contact with the target site on the wild-type homologous chromosome it will induce a DNA DSB. The DSB is repaired using the homologous chromosome as a template and the HEG allele is copied into the broken chromosome. As a result, these HEGs can spread rapidly through populations [137]. The gene drive system can be made more accurate and flexible using CRISPR-based adaptations [138] and there are many agricultural applications, including the sensitization of a population to pesticides (local sensitizing gene drive), manipulating plant pathogen-vector relationships, and knock-out of virulence genes [139]. The latter has already been shown successful under *in vitro* conditions for the wheat pathogen *Fusarium graminearum* [140]. Similarly, the CRISPR/Cas9 gene drive approach may also be applied to enhance performance of biocontrol agents or beneficial organisms which can heighten plant immunity [136]. However, this system is subject to major concerns regarding biosafety and bioethics and should not be applied without extensive risk assessment [141].

3.3.2 Abiotic Stress Tolerance

As sessile organisms, plants continuously endure a whole range of environmental stressors including drought, waterlogging, extreme temperatures, soil salinity, wind, and hail, which can severely affect growth and production. Improved abiotic stress tolerance allows fruit trees to be grown in suboptimal environments which is especially relevant in light of climate change and the associated occurrence of more extreme weather events worldwide. Important breeding objectives include tolerance to drought, cold, and soil salinity.

Drought tolerance in apple and pear trees is often tackled through root stock breeding in an attempt to achieve enhanced uptake of water through a better-developed root system. A recent study describes the knock-out of GRETCHEN HAGEN3.6 (*MdGH3.6*), an indole-3-acetic acid conjugating enzyme, in apple rootstocks through RNAi to increase tolerance to prolonged drought periods without impacting fruit quality. Knocking out this gene increased IAA content, adventitious root number and root length [142, 143]. Other potential drought tolerance related targets for CRISPR genome editing that were successful in other species include genes involved in stomatal density (VvEPFL9-1) [144], stomatal response (AtOST2) [145, 146], genes involved in ABA signaling (AtAREB1, SlMAPK3, OsSAPK2), and leaf rolling (OsSRL1 and OsSRL2) [145].

Although adult pome fruit trees are generally quite cold-hardy, severe production losses can still occur due to frost damage to flower buds, flowers, young shoots and fruits. Cold hardiness can differ between cultivars [147], but little is known about the genetic and biochemical basis. Several genes for general cold-hardiness have been identified in apple. Putative positive regulators of cold hardiness in apple include *MdHYL1, Mdm-miR172* [148], and *CBF* genes (C-repeat binding factors) like *MdCBF1-5* [149]. Knock-out of negative regulators, such as *Mdm-miR156*, can cause increased cold-hardiness, which is more interesting for genome editing application [148]. Many of the genes and pathways involved in cold-tolerance are also involved in other (a)biotic stress responses. For example, the previously mentioned *CBF* genes are considered hub genes that are involved in drought, salinity and cold responses [149, 150]. Targeting these genes may result in simultaneously increased tolerance to other abiotic stress factors, however caution must be taken to avoid undesired side effects.

Heat stress in pome fruits can lead to sunburn of leaves and fruits, scorching of leaves and in extreme cases early fruit drop and even leaf drop [151, 152]. Damage can be especially severe in combination with drought. In high temperature conditions, damage may occur in plant cells due to protein misfolding and denaturation, damage to membranes and accumulation of ROS species [153]. Gene targets to enhance heat tolerance in pome fruits are likely to be found in plant antioxidant defense systems or osmotic adjustment pathways [154]. Heat stressed plants will also generally synthesize a variety of heat-shock proteins (HSP). Such HSPs are also found in apple and have been identified as important regulators of temperature stress responses. Members of the *HSP20* gene family appear to be especially

important and are highly upregulated in response to heat stress [151]. Another heat-stress related gene is *MdPRP6* which was shown to enhance heat stress tolerance when overexpressed in transgenic tobacco plants [153]. Possible genome editing targets are negative regulators of these heat shock related genes.

Finally, soil salinity is detrimental to plants due to osmotic stress and salt toxicity [155]. It mainly occurs due to irresponsible irrigation and fertilizer application. The plant may try to overcome salt stress by inducing osmoregulating and antioxidant systems [156]. In apple, *MdINT1* has been shown to confer salinity tolerance by regulating antioxidant systems, and homeostasis of ions and osmosis [156]. Several other genes involved in response to salt stress have been identified in other plants, but relatively little knowledge is available in apple and pear. For example, knockout of *AITR* (ABA induced transcription repressors) gene family members in *A. thaliana* increased drought and salinity resistance [157]. Also in *A. thaliana*, a nucleoporin gene *NUP85* seems to modulate the response to salt stress [158]. Improved salt tolerance was also achieved in rice by CRISPR/Cas9-targeted mutagenesis of the *OsRR22* gene involved in cytokinin transduction and metabolism [159], and in tomato through down regulation of Auxin Response factor 4 (ARF4) [160]. Homologous gene targets may be explored in pome fruit species.

3.3.3 Tree Architecture

Tree architecture is influenced by four main factors: primary growth, branching patterns, flowering location and meristem and shoot mortality [161]. In commercial pome fruit production, tree architecture can influence important factors such as fruit quality, yield and orchard management requirements including planting density, pesticide application efficiency, harvesting efficiency, and requirements for thinning, pruning, branch-bending and tying [161]. The ideal tree form would allow high density plantings and maximum automatization [162]. The main breeding goals regarding tree architecture in pear and apple are dwarfing (mainly achieved through dwarfing root stocks) and optimal branch orientation [163]. Causative genes have been identified for the dwarfing trait, branch orientation and the columnar growth habit in apple [162].

Dwarfing root stocks limit tree size, enable high density plantings, increase flower density and allow more efficient mechanization [164]. Mainly genes associated with growth-related plant hormones are linked to the dwarfism trait and may be identified as putative targets of gene editing. For example, in peach, a nonsense mutation in the GA receptor *GID1c* was found to result in dwarfism [165]. In apple, overexpression of the transcription factors *WRKY9* and *NAC1*, which are negatively involved in the brassinosteroid biosynthesis pathway, was shown to result in dwarfing [162, 164, 166].

Several gene families are associated with branch orientation, namely *WEEP*, and IGT family genes *TAC1* and *LAZY1* [167–169]. Tiller Angle Control1 (TAC1) promotes lateral shoots to grow outward and reduced or eliminated expression of this gene causes more upright growth habits [162]. In contrast, LAZY1 promotes

upward shoot orientation. Plants with reduced or no *LAZY1* expression show wide shoot angles [170, 171]. A weeping growth habit was found in birch and plum with a defect or silenced *LAZY1* gene [162, 172]. In theory, a desired branching phenotype may be obtained in trees by balancing the effects of targeted editing of one or several of these genes.

One of the most ideal branching habits in apple is the columnar type. These trees grow upwards as a column with very short branches and an increased number of spurs [162]. They are ideally suited for high density plantings and automatization, although they are generally very susceptible to biennial bearing and produce lower quality fruits [173–175]. Columnar growth habits have been achieved in apple through mutation of the *MdCoL* gene, which encodes a putative 10G-FEII oxygenase [161, 176], and overexpression of *LEAFY* [177].

3.4 Pollination and Fertilization

3.4.1 Self-Incompatibility

Generally, fruit set in pome fruit species is dependent on cross-pollination between two cross-compatible cultivars due to the S-RNase dependent gametophytic self-incompatibility system (GSI) which prevents self-fertilization [178–180]. This system is genetically controlled by the S-locus which carries a pistil-expressed *S-RNase* gene and multiple pollen-expressed *SFBBs* (S-locus F-box brothers) [178, 181–183]. According to the currently accepted non-self-recognition mechanism, SFBB proteins expressed by the pollen haplotype recognize non-self *S*-RNases in the style, but not self-S-RNases, and mediate their degradation through the ubiquitin-26S proteasome [184, 185]. Problems with this innate self-incompatibility arise when cross-pollination is hampered. To ensure sufficient cross-pollination and thus economically viable yields, several conditions must apply including (1) adequate presence of a diverse pollinating insect population, (2) presence of well-distributed trees of a cross-compatible pollen donor cultivar with overlapping flowering period in the orchard, and (3) optimal weather conditions during flowering. However, these conditions cannot always be met and even then, fruit set after self-pollination is expected to be more stable compared to cross-pollination [186]. Therefore, self-compatibility (SC) has become an important breeding objective [5].

Spontaneous self-compatible (SC) mutant apple and pear varieties are rare and hardly ever suitable for commercial fruit production. One exception is "Osanijisseiki" which is a natural SC mutant of the *Pyrus pyrifolia* cultivar "Nijisseiki". "Osanijisseiki" was released as cultivar in 1979 and has been used as a parent to breed new SC cultivars using conventional breeding strategies [5]. Spontaneous SC mutants in pome fruit species are either the result of pistil-function breakdown, essentially meaning knock-out of the *S-RNase* gene [181, 187, 188] or alternatively of competitive interaction due to polyploidy or segmental duplications in the S-locus [189, 190]. The latter approach is less suited as genome-editing application,

however pistil-function breakdown of self-incompatibility has already been successfully applied in apple to create a transgenic SC Elstar mutant using a co-suppression approach resulting in *S-RNase* gene silencing [186]. Knock-out of the *S-RNase* gene could be performed relatively easily using genome-editing approaches to introduce the self-fertility trait into established commercial cultivars.

3.4.2 Parthenocarpy

Parthenocarpy refers to natural or artificially induced fruit development without fertilization of the ovule [191]. In commercial pome fruit production, this is a very interesting trait because it can alleviate problems with pollination, self-incompatibility, biennial bearing and spring frost. In addition, parthenocarpic fruits are seedless and often have more edible pulp and less core which is often preferred by consumers and can be an advantage for industrial food processing applications [192, 193]. Parthenocarpy is mainly genetically determined and can vary between species and cultivars. In pear, natural parthenocarpy is more common in *Pyrus communis* cultivars compared to the Asian pear species, such as *Pyrus pyrifolia* [194]. *Pyrus communis* parthenocarpic cultivars include "Conference" [195] and "Bartlett" [196]. In apple, some parthenocarpic fruit can develop on "Cox's Orange Pippin", "Wellington Bloomless" and "Spencer Seedless" [197].

Natural parthenocarpic fruit set in pome fruit species is usually relatively low, but can be stimulated using plant growth regulators (PGR) during early fruit development to obtain economically viable yields. These PGRs generally impact the auxin, cytokinin or gibberellin pathways that are involved in fruit set and development [193, 198, 199]. Correspondingly, parthenocarpic mutations in a variety of species are generally found in the synthesis and metabolism pathways of these hormones [193]. Overexpression of auxin biosynthesis or receptor genes and silencing of auxin signal repressor genes or negative regulators of auxin signaling are associated with parthenocarpy in tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*) [193, 200–203]. In the gibberellin pathway, overexpression of GA biosynthesis genes, such as gibberellin 20-oxidase genes (*GA20ox*), and suppression of GA respressor genes like *DELLA* and *GA2ox* genes have been shown to lead to parthenocarpy in *A. thaliana* and tomato [204–208].

Alternatively, floral homeotic genes are also often associated with parthenocarpy. For example, the silencing of genes responsible for stamen identity has been associated with parthenocarpy in tomato, possibly because stamens act as negative regulators to restrict ovary development before pollination and fertilization have occurred [209, 210]. These genes include, amongst others, several class B MADS-box genes such as TOMATO APETALA3 (*TAP3*), DEFICIENS (*sl*DEF), TOMATO MADS BOX GENE6 (*TM6*), and TOMATO PISTILLATA (*TPI*) [209, 211–213]. In apple, two parthenocarpic cultivars showed similar splicing variants of the *AtPI* homolog which were causally related to their parthenocarpic trait [214, 215]. In pear, several transcriptomic studies on induced parthenocarpic fruit development in

pear showed possible involvement of homologs of many of the above listed tomato genes. For example, key GA-associated genes related to parthenocarpy in pear include *GA20ox, GA3ox, GA2ox*, GA receptor *GID1*, and *DELLA* [216, 217], as well as MADS-box class B gene *DEF*. However, the genetic basis for parthenocarpic fruit set is still largely unclear in woody fruit tree species and mutations in many of the mentioned genes cause undesired pleiotropic effects. In addition, pathways leading to parthenocarpy in pome fruits may be different compared to *A. thaliana* which produces siliques or tomato which produces botanical fruits compared to accessory fruits. Therefore necessary caution must be taken when choosing possible gene targets for parthenocarpy in pome fruit trees based on studies in these model species.

3.5 Tree Phenology

As perennial species, pome fruit trees require immaculate regulation of their phenology to survive and reproduce in temperate climates with seasonally changing climatic conditions. In order to anticipate these seasonal changes, temperate tree species take climatic cues to regulate important transitions in their life-cycle, including dormancy, bud burst, flowering, fruit development, and leaf-drop. Phenology characteristics greatly determine the success of a cultivar in a given location due to their impact on the tree life-cycle, including many reproductive traits that are essential for fruit production. As a result, considerable effort has been devoted to elucidate the molecular mechanisms that underly important phenological transitions. In the following paragraphs three aspects of tree phenology are briefly discussed which have major relevance to pome fruit production: juvenility, dormancy and biennial bearing. We also discuss potential applications of gene editing techniques based on current knowledge.

3.5.1 Juvenility

Juvenility in most pome fruit species can take 5–6 years which significantly extends the breeding cycle and delays research on reproductive biology [218]. Therefore, a shortened juvenile period is of great interest to breeders and researchers, and several attempts have been made to obtain early flowering apple and pear mutants with the goal of accelerating conventional pome fruit breeding and research. For example, apple and European pear *TFL1-1* mutants showing an early flowering phenotype were successfully obtained using CRISPR-Cas9 technology [38]. These mutants flower continuously *in vitro*, do not require cold accumulation to induce flowering and completely by-pass the juvenile period. However, the benefits of cultivars with a short juvenile period are limited in the context of fruit production, since mature tissue can be clonally propagated onto rootstocks once juvenility is broken.

3.5.2 Dormancy

To survive the harsh winter conditions, temperate fruit trees go through both endo- and ecodormancy. Dormancy can be defined as the "temporary suspension of visible growth of any plant structure containing a meristem" [219]. In pome fruits, both mixed and vegetative buds are formed during the flowering period [220]. In autumn, low temperatures and short day conditions induce a state of endodormancy in these buds which can only be broken by the fulfillment of the cultivar-dependent chilling requirement [221]. Once this chilling requirement is reached, buds transition into a state of ecodormancy. Ecodormancy is broken by higher temperatures, initiating bud break. This two-factor regulatory system prevents early bud break on a warm day in late autumn or during cold days in early spring [222]. Ideally, this break of two dormancy states initiates uniform flowering, which is advantageous to both the plant and the grower. Dormancy cycles can be disturbed when pome fruit trees cannot reach the required amount of chilling after a mild winter. This can lead to abnormal bud break, resulting in extended flowering periods, delayed leaf formation and asynchronous fruit development [223]. Currently, problems associated with abnormal bud break are mainly observed in orchards grown in subtropical climates, but may also start to occur in more temperate regions as a consequence of climate change. Several chemical treatments can be used to break dormancy in pome fruits including Dormex, potassium nitrate, and mineral oil [224]. However, these products are associated with environmental and health concerns and pose significant costs to growers. Therefore, the development of commercial apple and pear cultivars with low chilling requirements would be beneficial.

Several key hormones and some regulatory genes involved in the maintenance and release of endodormancy have been identified [222]. In pear, abscisic acid (ABA) levels increase during endodormancy induction and remain high during endodormancy. During cold accumulation, transcription of *PpCYP707A-3*, which encodes an ABA 8-hydrolase enzyme, sharply increases. Simultaneously, ABA levels in the buds decrease initiating transition from endodormancy to ecodormancy in pear [225]. The onset of this transition is believed to result from the release of inhibition on GA biosynthesis, regulated by *PpGAST1*. Transcription of *PpGAST1* is inhibited by ABA and decreasing ABA levels during dormancy transition allow *PpGAST1* levels to rise. Increased *PpGAST1* transcription levels were accompanied by increased transcription of *PpGA20OX2*, a GA biosynthesis gene. Additionally, high ABA levels also indirectly induce the GA catabolism gene *PpGA2OX1*, resulting in decreasing GA levels [226]. Another important gene group associated with dormancy regulation is the *dormancy-associated MADS-box* (*DAM*) gene family. Their expression is tightly regulated by ABA through several transcription factors including ABA response element (ABRE)-binding transcription factor 1 (AREB1), which represses *PpDAM1* transcription in pear. *PpDAM* genes promote ABA biosynthesis by upregulating the expression of *PpNCED3*, an ABA biosynthesis gene [227]. In transgenic pear calli, *DAM3* was found to also inhibit cell division and cell growth, supporting their role in pear bud dormancy. Interestingly, two Asian pear cultivars (*P. pyrifolia*) "Suli" and "Cuiguan" with respectively a high and a low

chilling requirement, showed different expression patterns of *PpDAM3* during endodormancy [228]. Additionally, epigenetic regulation is also found to be involved in bud dormancy. In peach (*Prunus persica*) and sweet cherry (*Prunus avium*), *DAM* genes were found to be under epigenetic regulation by histone modifications and/or DNA methylations during the dormancy process [222]. In apple and pear, bud break was associated with a decrease in DNA methylation under ideal high chill conditions [229, 230]. However, the precise mechanisms underlying epigenetic regulation of dormancy in pome fruits remain unknown.

As negative regulators of dormancy release, *DAM* genes appear to be good candidates for targeted gene editing. *DAM* gene expression may be altered through CRISPR transcriptional repression to adapt the cold requirement of various pome fruit cultivars. Alternatively, CRISPR-Cas9 may be used for targeted mutagenesis of the *DAM* genes. To our knowledge, no previous work has targeted *DAM* genes in perennial fruit trees to alter dormancy. However, there is a known, natural *DAM evergrowing* (*evg*) peach mutant which is the result of a genomic deletion of four *DAM* genes and which does not enter dormancy when exposed to low temperatures or shortening days [231]. More research is needed to determine the effects of targeted *DAM* mutagenesis on dormancy in pome fruits and its applicability in commercial fruit production. Also, alternative targets need to be identified to more precisely fine tune chilling requirements.

It is predicted that winter warming and resulting disturbed dormancy will be an issue for temperate fruit production in increasingly larger areas of the world [232]. For example, due to rising winter temperatures in Japan, flowering disorders occur more frequently in *P. pyrifolia* "Hosui" trees which as a result show erratic flowering, asynchronous bud-break and bud loss due to inadequate chilling during the dormancy phase [223]. However, increased temperatures are not always negative for pome fruit production. For example, European pear (*P. communis*) production of the cultivar "Conference" in Belgium is predicted to be at a lower risk of frost damage-related production losses because of the decreased occurrence of frost days during the flowering period [233]. These two examples show that the consequences of climate change on pome fruit phenology trees are complex and region- and cultivar-specific. In given examples, a decreased chilling requirement may improve uniform bud break in "Hosui" pears grown in Japan, but could expose "Conference" pears grown in Belgium to frost damage due to early flowering. So, when adapting pome fruit tree phenology by breeding or gene editing, it is recommended to obtain an accurate view of the specific challenges present in the crop and region of interest.

3.5.3 Biennial Bearing

Biennial bearing (BB) occurs when a fruit tree has an alternating pattern of low and high fruit production over consecutive years. This is caused by the inhibition of flower induction (FI) in the meristems by growing fruits, since FI occurs simultaneously with fruit development during 4–8 weeks after full bloom [234]. In pome fruit trees, each individual spur is biannual and can only fruit every other year, commonly named

'ON' and 'OFF' years. During 'ON' years there is abundant flowering and potentially a heavy fruit set, followed by an 'OFF'-year with mostly vegetative growth and thus less potential for fruit set. Ideally, a fruit tree will have a balanced proportion of 'OFF' spurs and 'ON' spurs each year, resulting in a predictable and constant fruit production. If a certain event triggers low fruit set or causes early loss of fruitlets, all spurs on a tree will be synchronized to an 'OFF' status and, consequently, an 'ON' status next year. This starts a cycle of BB which causes variability in yield and fruit size over the years and is therefore undesired by the grower. A frequently applied measure to avoid the continuation of the BB cycle is early crop thinning in 'ON' years by removing flowers or young fruitlets. This can be done chemically by using compounds that damage flower organs and so inhibit fruit set or by mechanically removing the young fruitlets [235, 236]. During 'OFF' years gibberellic acids such as GA_7 can be applied to the trees after bloom to repress excess FI [237]. In addition to crop thinning, optimal pruning of the fruit trees will maintain young spurs which are less susceptible to BB than older spurs [235]. But despite these precautions and good management, there are still differences in susceptibility of different pome fruit cultivars to BB, especially in apple. This implies that there is a genetic basis determining susceptibility or resistance to BB in certain cultivars [238].

BB was long thought to be the result of hormonal signaling from the developing fruits to nearby developing buds. In 1998, diffusible auxins were shown to be present in the seeds of developing fruits during the period of FI, and the levels of these auxins in fruits increased with an increased number of seeds [239]. A recent study compared the apple cultivars "Gala" (a regular bearer) and "Fuji" (a biennial bearer) and found that "Fuji" had a higher average seed number per fruit compared to "Gala" [240]. The "Fuji" fruits did not only have a higher number of seeds per fruit, each seed also had higher levels of cytokinins and auxins than seeds from "Gala" fruits. On the contrary, "Gala" seeds had increased levels of GA_3 and GA_{19} compared to "Fuji". These plant hormones were also found to be exported by diffusion through the stem of the apple fruit in both cultivars. These differences in hormone production in seeds could explain the susceptibility of "Fuji" to BB, since some GAs repress FI in apple trees trough currently unknown mechanisms [241]. Several QTL's were identified for biennial bearing in apple, some included flowering genes like *BFTa*, *SOC1-like* and *COL1*, others included hormonal factors such as genes involved in GA biosynthesis *GIBBERELLIN 2-OXIDASE* (*GA2ox*), *GIBBERELLIN 20-OXIDASE* (*GA20ox*) and *GA3ox-like-b* and auxin related genes such as *AFB6* [242].

A study in "Gala" apple trees identified many differentially expressed genes (DEGs) between 'ON' and 'OFF' buds in trees by artificially inducing biennial bearing through manual flower removal during bloom. The authors found many DEGs among the flowering genes, including flowering repressor genes such as *TEMPRANILLO1* (*TEM1*) and *MAF2* (*MADS AFFECTING FLOWERING 2*). Also flowering promoting transcription factors, *SQUAMOSA PROMOTER BINDING-LIKE 5* (*SPL5*) and *SPL9* and *FLORAL TRANSITION AT MERISTEM* (*FTM1*) were upregulated in 'OFF' trees, simultaneously with *APELLATA1* (*AP1*) genes. Additionally, secondary metabolism genes were downregulated in the shoot apical meristem (SAM) of 'ON' trees, possibly due to the developing fruits causing

carbohydrate depletion in the SAM. This downregulation of secondary metabolism genes is an indication of reduced cell division in the apical tissues and is supported by the observed up-regulation of *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA* (*KNAT1*) and *NO APICAL MERISTEM* (*NAM*) genes during 'ON' years. *KNAT1* and *NAM* are both genes which prevent meristematic tissue differentiation and thus maintain the SAM, preventing FI and differentiation of floral tissues under low-carbon conditions. Also axillary meristem (AM) regulating genes were upregulated in 'ON' buds, such as *MORE AXILLARY BRANCHES 1* (*MAX1*) and *BRANCHED1* (*BRC1*), inhibiting AM formation and axillary bud outgrowth, respectively [243, 244]. This reduction in AM formation and axillary bud outgrowth results in reduced vegetative growth during 'ON' years with heavy fruit bearing. The study also found DEGs related to auxin, abscisic acid, brassinosteroid and ethylene. The Gibberellic Acid (GA) biosynthesis genes *GA2ox* and *GA20ox* were shown to be upregulated in 'ON' years, confirming the previously mentioned studies that showed these genes as QTLs for BB [242, 244]. This is not surprising, since GA treatments during 'OFF' years are used in horticulture to inhibit FI [245].

Also epigenetic modifications are involved in regulation of BB, as it was shown that differentially methylated regions (DMRs) are present between 'ON' and 'OFF' trees in buds of the apple cultivar "Fuji" [246]. Many flowering genes showed to be differentially methylated such as *MADS-box*, *COL*, *B-box*, *NFY* and *SPL*. Also genes involved in hormonal signaling such as gibberellin, auxin and jasmonic acid showed to be DMRs, again highlighting the importance of hormonal regulation of FI in BB in apple.

Differences in gene expression in developing buds during 'ON' and 'OFF' years are very valuable in understanding FI and the genes involved, but it does not provide evidence to identify the genetic factors responsible for susceptibility of a cultivar to BB. Since the exact molecular mechanisms of BB are not yet well characterized, it is difficult to put forward a good candidate gene for CRISPR gene editing. Since all genes that were identified so far in BB are involved in crucial plant functions as flowering and hormonal signaling, the generation of knock-out (KO) mutants may not be a suitable approach due to the possible occurrence of undesirable side effects in tree phenology and/or flower morphology [247]. A better understanding of the genetic control of BB is needed to apply innovative gene editing techniques such as CRISPR base editing. Since there are observations that GA and auxin production in the seeds play a role in BB, CRISPR transcriptional activation could be used to mimic expression patterns of hormone biosynthesis genes of resistant cultivars in susceptible cultivars.

4 Concluding Remarks

Apple and pear are among the most cultivated temperate fruit crops, but the threats to their production remain significant. Many of the currently grown cultivars still suffer from high susceptibility to pests, diseases and abiotic stress, low fruit quality,

biennial bearing, suboptimal yields and other problems. Effective breeding strategies are necessary to tackle these problems. However, current breeding approaches are too time-consuming and expensive to profitably develop improved cultivars and to meet the rapidly evolving breeding requirements, especially in light of impending climate change. In addition, apple and pear production are traditionally dominated by just a handful of cultivars with a high market recognition, such as “Gala”, “Golden Delicious” and “Fuji” for apple, and “Conference”, “Bartlett” and “Doyenné du Comice” for pear. It has proven difficult to introduce new cultivars in the market, therefore, enhancing important traits in established cultivars is a very effective strategy, albeit extremely difficult using conventional breeding techniques. There is therefore a great need for faster and more accurate breeding approaches.

The recent advancement of gene-editing techniques such as CRISPR/Cas related technologies can help address these issues. Although successful gene-editing applications in pome fruit are still limited compared to many model crops, several proof-of-concept studies in apple, pear and some other perennial tree species have shown that gene-editing is feasible in pome fruits and that there are numerous potential breeding applications. This chapter discussed the current state-of-the-art, challenges and opportunities regarding gene-editing in pome fruit, as well as potential gene-targets for new applications in light of important breeding objectives. Several technical challenges remain to be solved in order to successfully apply gene-editing in pome fruit breeding including the high recalcitrant nature of pome fruit plantlets to *in vitro* propagation and regeneration, the low transformation efficiencies, and the complex removal of transgenes by crossing due to the long life cycle and high heterozygosity of pome fruit tree species. Improvements to the original gene-editing approaches that are tailored to the specific problems in pome fruit and other perennial tree species will help improve the application potential in pome fruit breeding.

References

1. FAO: World Food and Agriculture – Statistical Yearbook 2021. ROME (2021)
2. FAOSTAT, FAO: Crops and livestock products. License:CC BY-NC-SA 3.0 IGO (2023)
3. Penna, S., Jain, S.M.: Fruit crop improvement with genome editing, in vitro and transgenic approaches. Horticulturae. **9**(58), 1–20 (2023)
4. Kumar, S., Kirk, C., Deng, C., Wiedow, C., Knaebel, M., Brewer, L.: Genotyping-by-sequencing of pear (Pyrus spp.) accessions unravels novel patterns of genetic diversity and selection footprints. Hortic. Res. **4**, 17015 (2017)
5. Saito, T.: Advances in Japanese pear breeding in Japan. Breed. Sci. **66**(1), 46–59 (2016)
6. Li, J., et al.: Pear genetics: recent advances, new prospects, and a roadmap for the future. Hortic. Res. **9**, uhab040 (2022)
7. Kumar, S., Hilario, E., Deng, C.H., Molloy, C.: Turbocharging introgression breeding of perennial fruit crops: a case study on apple. Hortic. Res. **7**, 47 (2020a)
8. Chagné, D., et al.: The draft genome sequence of European pear (Pyrus communis L. ‘Bartlett’). PLoS One. **9**(4), e92644 (2014)
9. Velasco, R., et al.: The genome of the domesticated apple (Malus × domestica Borkh.). Nat. Genet. **42**(10), 833–839 (2010)

10. Montanari, S., et al.: Development of a highly efficient Axiom™ 70 K SNP array for Pyrus and evaluation for high-density mapping and germplasm characterization. BMC Genomics. **20**(1), 1–18 (2019)
11. Chagné, D., et al.: Validation of SNP markers for fruit quality and disease resistance loci in apple (Malus × domestica Borkh.) using the OpenArray® platform. Hortic. Res. **6**, 30 (2019)
12. Alvarez, D., et al.: Fruit crops in the era of genome editing: closing the regulatory gap. Plant Cell Rep. **40**(6), 915–930 (2021)
13. Peer, R., et al.: Targeted mutagenesis using zinc-finger nucleases in perennial fruit trees. Planta. **241**(4), 941–951 (2015)
14. Wyman, C., Kanaar, R.: DNA double-strand break repair: All's well that ends well. Annu. Rev. Genet. **40**, 363–383 (2006)
15. Kim, S., Kim, J.S.: Targeted genome engineering via zinc finger nucleases. Plant Biotechnol. Rep. **5**(1), 9–17 (2011)
16. Kim, Y.G., Cha, J., Chandrasegaran, S.: Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc. Natl. Acad. Sci. U. S. A. **93**(3), 1156–1160 (1996)
17. Ramirez-Torres, F., et al.: Genome editing in fruit, ornamental, and industrial crops. Transgenic Res. **30**(4), 499–528 (2021)
18. Bhoi, A., Yadu, B., Chandra, J., Keshavkant, S.: Mutagenesis: A coherent technique to develop biotic stress resistant plants. Plant Stress. **3**, 100053 (2022)
19. Osakabe, K., Osakabe, Y., Toki, S.: Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proc. Natl. Acad. Sci. U. S. A. **107**(26), 12034–12039 (2010)
20. Lloyd, A., Plaisier, C., Carroll, D., Drews, G.: Targeted mutagenesis in Arabidopsis using zinc-finger nucleases. Proc. Natl. Acad. Sci. **102**(6), 2232–2237 (2004)
21. Curtin, S.J., et al.: Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. Plant Physiol. **156**(2), 466–473 (2011)
22. Jung, Y.J., Nogoy, F.M., Lee, S.K., Cho, Y.G., Kang, K.K.: Application of ZFN for site directed mutagenesis of Rice SSIVa gene. Biotechnol. Bioprocess Eng. **23**(1), 108–115 (2018)
23. Lu, H., Klocko, A.L., Dow, M., Ma, C., Amarasinghe, V., Strauss, S.H.: Low frequency of zinc-finger nuclease-induced mutagenesis in Populus. Mol. Breed. **36**(9), 1–13 (2016)
24. Zhang, Y., Massel, K., Godwin, I.D., Gao, C.: Applications and potential of genome editing in crop improvement. Genome Biol. **19**(1), 1–11 (2018)
25. Christian, M., et al.: Targeting DNA double-strand breaks with TAL effector nucleases. Genetics. **186**(2), 756–761 (2010)
26. Son, S., Park, S.R.: Challenges facing CRISPR/Cas9-based genome editing in plants. Front. Plant Sci. **13**(902413), 1–20 (2022)
27. Bortesi, L., Fischer, R.: The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. **33**(1), 41–52 (2015)
28. Li, T., Liu, B., Spalding, M.H., Weeks, D.P., Yang, B.: High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat. Biotechnol. **30**(5), 390–392 (2012a)
29. Shan, Q., Zhang, Y., Chen, K., Zhang, K., Gao, C.: Creation of fragrant rice by targeted knockout of the OsBADH2 gene using TALEN technology. Plant Biotechnol. J. **13**(6), 791–800 (2015)
30. Wang, Y., et al.: Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. **32**(9), 947–951 (2014)
31. Char, S.N., et al.: Heritable site-specific mutagenesis using TALENs in maize. Plant Biotechnol. J. **13**(7), 1002–1010 (2015)
32. Kannan, B., Jung, J.H., Moxley, G.W., Lee, S.M., Altpeter, F.: TALEN-mediated targeted mutagenesis of more than 100 COMT copies/alleles in highly polyploid sugarcane improves saccharification efficiency without compromising biomass yield. Plant Biotechnol. J. **16**(4), 856–866 (2018)
33. Jung, J.H., Altpeter, F.: TALEN mediated targeted mutagenesis of the caffeic acid O-methyltransferase in highly polyploid sugarcane improves cell wall composition for production of bioethanol. Plant Mol. Biol. **92**(1–2), 131–142 (2016)

34. Nishitani, C., et al.: Efficient genome editing in apple using a CRISPR/Cas9 system. Sci. Rep. **6**, 31481 (2016)
35. Jiang, F., Doudna, J.A.: CRISPR – Cas9 structures and mechanisms. Annu. Rev. Biophys. **46**, 505–529 (2017)
36. Qin, G., et al.: Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. Cell Res. **17**(5), 471–482 (2007)
37. Pompili, V., Dalla Costa, L., Piazza, S., Pindo, M., Malnoy, M.: Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system. Plant Biotechnol. J. **18**(3), 845–858 (2020)
38. Charrier, A., Vergne, E., Dousset, N., Richer, A., Petiteau, A., Chevreau, E.: Efficient targeted mutagenesis in apple and first time edition of pear using the CRISPR-Cas9 system. Front. Plant Sci. **10**(40), 1–12 (2019)
39. Zhang, Y., Zhou, P., Bozorov, T.A., Zhang, D.: Application of CRISPR/Cas9 technology in wild apple (Malus sieverii) for paired sites gene editing. Plant Methods. **17**(1), 1–9 (2021a)
40. Schröpfer, S., Flachowsky, H.: Tracing CRISPR/Cas12a mediated genome editing events in apple using high-throughput genotyping by PCR capillary gel electrophoresis. Int. J. Mol. Sci. **22**, 12611 (2021)
41. Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., et al.: Cpf1 Is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. **163**, 759–771 (2015)
42. An, Y., et al.: Efficient genome editing in populus using CRISPR/Cas12a. Front. Plant Sci. **11**, 1–9 (2020)
43. Jia, H., Orbović, V., Wang, N.: CRISPR-LbCas12a-mediated modification of citrus. Plant Biotechnol. J. **17**(10), 1928–1937 (2019)
44. Bandyopadhyay, A., Kancharla, N., Javalkote, V.S., Dasgupta, S., Brutnell, T.P.: CRISPR-Cas12a (Cpf1): a versatile tool in the plant genome editing tool box for agricultural advancement. Front. Plant Sci. **11**, 584151 (2020)
45. Malabarba, J., Chevreau, E., Dousset, N., Veillet, F., Moizan, J., Vergne, E.: New strategies to overcome present CRISPR/Cas9 limitations in apple and pear: efficient Dechimerization and base editing. Int. J. Mol. Sci. **22**, 319 (2021)
46. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., Liu, D.R.: Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. **533**(7603), 420–424 (2016)
47. Reed, K.M., Bargmann, B.O.R.: Protoplast regeneration and its use in new plant breeding technologies. Front. Genome Ed. **3**, 734951 (2021)
48. Osakabe, Y., et al.: CRISPR–Cas9-mediated genome editing in apple and grapevine. Nat. Protoc. **13**(12), 2844–2863 (2018)
49. Malnoy, M., et al.: DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci. **7**, 1904 (2016)
50. Chen, J., et al.: Significant improvement of apple (Malus domestica Bork.) transgenic plant production by pre-transformation with a Baby boom transcription factor. Hortic. Res. **9**, uhab014 (2022)
51. Lobato-Gómez, M., Hewitt, S., Capell, T., Christou, P., Dhingra, A., Girón-Calva, P.S.: Transgenic and genome-edited fruits: background, constraints, benefits, and commercial opportunities. Hortic. Res. **8**, 166 (2021)
52. Voss-Fels, K.P., Stahl, A., Hickey, L.T.: Q&A: modern crop breeding for future food security. BMC Biol. **17**(1), 18 (2019)
53. Lakso, A., Robinson, T.: Sunlight, yield, and productivity of apples. Tree Physiol. **117**(4), 551–557 (2013)
54. Anthony, B., Serra, S., Musacchi, S.: Optimizing crop load for new apple cultivar: "WA38". Agronomy. **9**, 107 (2019)
55. Devoghalaere, F., et al.: A genomics approach to understanding the role of auxin in apple (Malus x domestica) fruit size control. BMC Plant Biol. **12**, 7 (2012)

56. Liu, W., et al.: Research progress on genetic basis of fruit quality traits in apple (Malus × domestica). Front. Plant Sci. **13**, 918202 (2022)
57. Theron, K.I.: Size matters: Factors influencing fruit size in pear. Acta Hortic. **909**, 545–556 (2011)
58. Subbaraya, U., Rajendran, S., Simeon, S., Suthanthiram, B., Marimuthu Somasundram, S.: Unravelling the regulatory network of transcription factors in parthenocarpy. Sci. Hortic. (Amsterdam). **261**, 108920 (2020)
59. Dang, Q., Sha, H., Nie, J., Wang, Y., Yuan, Y., Jia, D.: An apple (Malus domestica) AP2/ERF transcription factor modulates carotenoid accumulation. Hortic. Res. **8**(223), 1–12 (2021)
60. Mignard, P., Beguería, S., Giménez, R., Font I Forcada, C., Reig, G., Moreno, M.Á.: Effect of genetics and climate on apple sugars and organic acids profiles. Agronomy. **12**(4), 827 (2022)
61. Amyotte, B., Bowen, A.J., Banks, T., Rajcan, I., Somers, D.J.: Mapping the sensory perception of apple using descriptive sensory evaluation in a genome wide association study. PLoS One. **12**(2), e0171710 (2017)
62. Jung, M., et al.: Genetic architecture and genomic predictive ability of apple quantitative traits across environments. Hortic. Res. **9**, uhac028 (2022)
63. Kumar, S., et al.: GWAS provides new insights into the genetic mechanisms of phytochemicals production and red skin colour in apple. Hortic. Res. **9**, uhac218 (2022)
64. Mcclure, K.A., et al.: A genome-wide association study of apple quality and scab resistance. Plant Genome. **11**(1), 170075 (2018)
65. Ikegaya, A., Toyoizumi, T., Ohba, S., Nakajima, T., Kawata, T., Ito, S., Arai, E.: Effects of distribution of sugars and organic acids on the taste of strawberries. Food Sci. Nutr. **7**, 2419–2426 (2019)
66. Aprea, E., et al.: Sweet taste in apple: the role of sorbitol, individual sugars, organic acids and volatile compounds. Nat. Publ. Gr. **7**, 44950 (2017a)
67. Larsen, B., et al.: Genome-wide association studies in apple reveal loci for aroma volatiles, sugar composition, and harvest date. Plant Genome. **12**(2), 180104 (2019)
68. Nishio, S., et al.: Genome-wide association study of individual sugar content in fruit of Japanese pear (Pyrus spp.). BMC Plant Biol. **21**(1), 378 (2021)
69. Guan, Y., et al.: QTLs detected for individual sugars and soluble solids content in apple. Mol. Breed. **35**(135), 1–13 (2015)
70. Miller, B.A., Kostick, S.A., Luby, J.J.: Large-effect QTLs for titratable acidity and soluble solids content validated in 'honeycrisp'-derived apple germplasm. Agronomy. **12**(7), 1703 (2022)
71. Kyung Hyun, T., Hee Eom, S., Kim, J.-S.: Genomic analysis and gene structure of the two invertase families in the domesticated apple (Malus × domestica Borkh.). Plant Omi. J. **4**(7), 391–399 (2011)
72. Li, M., Feng, F., Cheng, L.: Expression patterns of genes involved in sugar metabolism and accumulation during apple fruit development. PLoS One. **7**(3), 33055 (2012b)
73. Jiang, S., Li, S., Luo, J., Wang, X., Shi, C.: QTL mapping and transcriptome analysis of sugar content during fruit ripening of Pyrus pyrifolia. Front. Plant Sci. **14**, 13 (2023)
74. Zhen, Q., et al.: Developing gene-tagged molecular markers for evaluation of genetic association of apple SWEET genes with fruit sugar accumulation. Hortic. Res. **5**, 14 (2018)
75. Nie, P., Xu, G., Yu, B., Lyu, D., Xue, X., Qin, S.: Genome-wide identification and expression profiling reveal the potential functions of the SWEET gene family during the sink organ development period in apple (Malus × domestica Borkh.). Agronomy. **12**(8), 1747 (2022)
76. Ma, B., et al.: Determination of predominant organic acid components in Malus species: correlation with apple domestication. Meta. **8**(74), 1–20 (2018)
77. Zhang, Y., Li, P., Cheng, L.: Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in "Honeycrisp" apple flesh. Food Chem. **123**(4), 1013–1018 (2010)
78. Wu, J., et al.: Chemical compositional characterization of some apple cultivars. Food Chem. **103**(1), 88–93 (2007)

79. Aprea, E., et al.: Sweet taste in apple: the role of sorbitol, individual sugars, organic acids and volatile compounds. Nat. Publ. Gr. **7**(44950), 1–11 (2017b)
80. Xu, K., Wang, A., Brown, S.: Genetic characterization of the Ma locus with pH and titratable acidity in apple. Mol. Breed. **30**, 899–912 (2012)
81. Kenis, K., Keulemans, J., Davey, M.W.: Identification and stability of QTLs for fruit quality traits in apple. Tree Genet. Genomes. **4**(4), 647–661 (2008)
82. Ma, B., et al.: Genes encoding aluminum-activated malate transporter II and their association with fruit acidity in apple. Plant Genome. **8**(3), 1–20 (2015)
83. Ban, S., Xu, K.: Identification of two QTLs associated with high fruit acidity in apple using pooled genome sequencing analysis. Hortic. Res. **7**, 171 (2020)
84. Visser, T., Verhaegh, J.J.: Inheritance and selection of some fruit characters of apple. I. Inheritance of low and high acidity. Euphytica. **27**(3), 753–760 (1978)
85. Liebhard, R., Koller, B., Gianfranceschi, L., Gessler, C.: Creating a saturated reference map for the apple (Malus x domestica Borkh.) genome. Theor. Appl. Genet. **106**(8), 1497–1508 (2003)
86. Verma, S., et al.: Two large-effect QTLs, Ma and Ma3, determine genetic potential for acidity in apple fruit: breeding insights from a multi-family study. Tree Genet. Genomes. **15**(18), 1–20 (2019)
87. Maliepaard, C., et al.: Aligning male and female linkage maps of apple (Malus pumila Mill.) using multi-allelic markers. Theor. Appl. Genet. **97**, 60–73 (1998)
88. Kumar, S., Chagné, D., Bink, M., Volz, R.K., Whitworth, C., Carlisle, C.: Genomic selection for fruit quality traits in apple (Malus×domestica Borkh.). PLoS One. **7**(5), e36674 (2012)
89. Bai, Y., Dougherty, L., Li, M., Fazio, G., Cheng, L., Xu, K.: A natural mutation-led truncation in one of the two aluminum-activated malate transporter-like genes at the Ma locus is associated with low fruit acidity in apple. Mol. Gen. Genomics. **287**(8), 663–678 (2012)
90. Li, C., et al.: Apple ALMT9 requires a conserved C-terminal domain for malate transport underlying fruit acidity. Plant Physiol. **182**, 992–1006 (2020)
91. Hu, D.G., Sun, C.-H., Ma, Q.-J., You, C.-X., Cheng, L., Hao, Y.-J.: MdMYB1 regulates anthocyanin and malate accumulation by directly facilitating their transport into vacuoles in apples. Plant Physiol. **170**, 1315–1330 (2016)
92. Hu, D.G., et al.: The R2R3-MYB transcription factor MdMYB73 is involved in malate accumulation and vacuolar acidification in apple. Plant J. **91**(3), 443–454 (2017)
93. Zhang, Q.-Y., et al.: BTB-BACK-TAZ domain protein MdBT2-mediated MdMYB73 ubiquitination negatively regulates malate accumulation and vacuolar acidification in apple. Hortic. Res. **7**(151), 1–11 (2020)
94. Jia, D., et al.: Genetic variation in the promoter of an R2R3–MYB transcription factor determines fruit malate content in apple (Malus domestica Borkh.). Plant Physiol. **186**(1), 549 (2021)
95. Jia, D., et al.: Apple fruit acidity is genetically diversified by natural variations in three hierarchical epistatic genes: Mdsaur37, mdpp2ch and mdalmtii. Plant J. **95**(3), 427–443 (2018a)
96. Farneti, B., et al.: Is there room for improving the nutraceutical composition of apple? J. Agric. Food Chem. **63**, 2750–2759 (2015)
97. Davies, T., Watts, S., McClure, K., Migicovsky, Z., Myles, S.: Phenotypic divergence between the cultivated apple (Malus domestica) and its primary wild progenitor (Malus sieversii). PLoS One. **17**(3), e0250751 (2022)
98. McClure, K.A., et al.: Genome-wide association studies in apple reveal loci of large effect controlling apple polyphenols. Hortic. Res. **6**(1), 107 (2019)
99. Bilbrey, E.A., Williamson, K., Hatzakis, E., Miller, D.D., Fresnedo-Ramírez, J., Cooperstone, J.L.: Integrating genomics and multiplatform metabolomics enables metabolite quantitative trait loci detection in breeding-relevant apple germplasm. New Phytol. **232**(5), 1944–1958 (2021)
100. Khan, S.A., et al.: Genetic analysis of metabolites in apple fruits indicates an mQTL hotspot for phenolic compounds on linkage group 16. J. Exp. Bot. **63**(8), 2895–2908 (2012)

101. Gosch, C., et al.: Substrate specificity and contribution of the glycosyltransferase UGT71A15 to phloridzin biosynthesis. Trees Struct. Funct. **26**(1), 259–271 (2012)
102. Miranda, S., Piazza, S., Nuzzo, F., Li, M., Lagrèze, J., Mithöfer, A., Cestaro, A., et al.: CRISPR/Cas9 genome-editing applied to MdPGT1 in apple results in reduced foliar phloridzin without impacting plant growth. Plant J. **113**, 92–105 (2023)
103. Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S., Allan, A.C.: Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J. **49**, 414–427 (2007)
104. Xu, H., et al.: MdMYB6 regulates anthocyanin formation in apple both through direct inhibition of the biosynthesis pathway and through substrate removal. Hortic. Res. **7**, 72 (2020)
105. Wang, S., et al.: Apple MdMYB306-like inhibits anthocyanin synthesis by directly interacting with MdMYB17 and MdbHLH33. Plant J. **110**(4), 1021–1034 (2022)
106. Colanero, S., Perata, P., Gonzali, S.: The atroviolacea gene encodes an R3-MYB protein repressing anthocyanin synthesis in tomato plants. Front. Plant Sci. **9**, 830 (2018)
107. Davuluri, G.R., et al.: Manipulation of DET1 expression in tomato results in photomorphogenic phenotypes caused by post-transcriptional gene silencing. Plant J. **40**(3), 1–15 (2004)
108. Valpuesta, V., Botella, M.A.: Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. Trends Plant Sci. **9**(12), 573–577 (2004)
109. Zheng, X., et al.: Metabolism and regulation of ascorbic acid in fruits. Plants. **11**(12), 1602 (2022)
110. Mellidou, I., Chagné, D., Laing, W.A., Keulemans, J., Davey, M.W.: Allelic variation in paralogs of GDP-l-galactose phosphorylase is a major determinant of vitamin C concentrations in apple fruit. Plant Physiol. **160**(3), 1613 (2012)
111. Khan, A., Korban, S.S.: Breeding and genetics of disease resistance in temperate fruit trees: challenges and new opportunities. Theor. Appl. Genet. **135**(11), 3961–3985 (2022)
112. Kellerhals, M., et al.: European pome fruit genetic resources evaluated for disease resistance. Trees Struct. Funct. **26**(1), 179–189 (2012)
113. Zhao, Y.Q., et al.: Fire blight disease, a fast-approaching threat to apple and pear production in China. J. Integr. Agric. **18**(4), 815–820 (2019)
114. Höfer, M., Flachowsky, H., Schröpfer, S., Peil, A.: Evaluation of scab and mildew resistance in the gene bank collection of apples in Dresden-pillnitz. Plan. Theory. **10**(6), 1–16 (2021)
115. Papp, D., Singh, J., Gadoury, D., Khan, A.: New North American isolates of Venturia inaequalis can overcome apple scab resistance of Malus floribunda 821. Plant Dis. **104**(3), 649–655 (2020)
116. Fahrentrapp, J., et al.: A candidate gene for fire blight resistance in Malus × robusta 5 is coding for a CC–NBS–LRR. Tree Genet. Genomes. **9**, 237–251 (2013)
117. Emeriewen, F., Richter, K., Flachowksy, H., Malnoy, M., Peil, A.: Genetic analysis and fine mapping of the fire blight resistance locus of Malus × arnoldiana on linkage group 12 reveal first candidate genes. Front. Plant Sci. **12**, 667133 (2021)
118. Durel, C.E., Denancé, C., Brisset, M.N.: Two distinct major QTL for resistance to fire blight co-localize on linkage group 12 in apple genotypes “Evereste” and Malus floribunda clone. Genome. **52**, 139–147 (2009)
119. Emeriewen, O.F., et al.: Towards map-based cloning of FB_Mfu10: identification of a receptor-like kinase candidate gene underlying the Malus fusca fire blight resistance locus on linkage group 10. Mol. Breed. **38**, 106 (2018)
120. Patocchi, A., et al.: Ten years of VINQUEST: first insight for breeding new apple cultivars with durable apple scab resistance. Plant Dis. **104**(8), 2074–2081 (2020)
121. Shoda, S.T.M., Gonai, Y.A.T., Sawamura, M.K.Y., Kotobuki, H.I.K., Gessler, A.P.C., Yamamoto, T.H.T.: Genetic mapping of the pear scab resistance gene Vnk of Japanese pear cultivar Kinchaku. Theor. Appl. Genet. **113**, 743–752 (2006)
122. Cho, K.H., Shin, I.S., Kim, K.T., Suh, E.J., Hong, S.S., Lee, H.J.: Development of AFLP and CAPS markers linked to the scab resistance gene, Rvn2, in an inter-specific hybrid pear (Pyrus spp.). J. Hortic. Sci. Biotechnol. **84**(6), 619–624 (2015)

123. Oh, S., Han, H., Kim, D.: A novel pear scab (Venturia nashicola) resistance gene, Rvn3, from interspecific hybrid pear (Pyrus pyrifolia × P. communis). Plants. **10**, 2632 (2021)
124. Bouvier, L., et al.: A new pear scab resistance gene Rvp1 from the European pear cultivar “Navara” maps in a genomic region syntenic to an apple scab resistance gene cluster on linkage group 2. Tree Genet. Genomes. **8**, 53–60 (2012)
125. Caffier, V., Laurens, F.: Breakdown of Pl2, a major gene of resistance to apple powdery mildew, in a French experimental orchard. Plant Pathol. **54**, 116–124 (2005)
126. Kost, T.D., Gessler, C., Jänsch, M., Flachowsky, H., Patocchi, A., Broggini, G.A.L.: Development of the first cisgenic apple with increased resistance to fire blight. PLoS One. **10**(12), e0143980 (2015)
127. Broggini, G., et al.: Engineering fire blight resistance into the apple cultivar “Gala” using the FB_MR5 CC-NBS-LRR resistance gene of. Plant Biotechnol. **12**, 728–733 (2014)
128. Perchepied, L., et al.: Successful intergeneric transfer of a major apple scab resistance gene (Rvi6) from apple to pear and precise comparison of the downstream molecular mechanisms of this resistance in both species. BMC Genomics. **22**, 843 (2021)
129. Campa, M., et al.: HIPM is a susceptibility gene of Malus spp.: reduced expression reduces susceptibility to Erwinia amylovora. Mol. Plant-Microbe Interact. **32**(2), 167–175 (2019)
130. Malnoy, M., Meng, X., Bonasera, J.M., Nissinen, R.M.: Silencing of apple proteins that interact with DspE, a pathogenicity effector from Erwinia amylovora, as a strategy to increase resistance to fire blight. Acta Hortic. **663**, 469–474 (2004)
131. Pessina, S., et al.: The knock-down of the expression of MdMLO19 reduces susceptibility to powdery mildew (Podosphaera leucotricha) in apple (Malus domestica). Plant Biotechnol. J. **14**, 2033–2044 (2016)
132. Kusch, S., Panstruga, R.: mlo-based resistance: an apparently universal “weapon” to defeat powdery mildew disease. Mol. Plant-Microbe Interact. **30**(3), 179–189 (2017)
133. Åhman, I., Kim, S., Zhu, L.: Plant genes benefitting aphids — potential for exploitation in resistance breeding. Front. Plant Sci. **10**, 1452 (2019)
134. Kloth, K.J., et al.: AtWRKY22 promotes susceptibility to aphids and modulates salicylic acid and jasmonic acid signalling. J. Exp. Bot. **67**(11), 3383–3396 (2016)
135. Kyrou, K., et al.: Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. Nat. Biotechnol. **36**(11), 1062–1066 (2018)
136. Paul, N.C., et al.: Plant and fungal genome editing to enhance plant disease resistance using the CRISPR / Cas9 system. Front. Plant Sci. **12**, 700925 (2021)
137. Burt, A.: Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc. R. Soc. B Biol. Sci. **270**(1518), 921–928 (2003)
138. Bier, E.: Gene drives gaining speed. Nat. Rev. Genet. **23**(1), 5–22 (2022)
139. Tek, M.I., Budak, K.: A new approach to develop resistant cultivars against the plant pathogens: CRISPR drives. Front. Plant Sci. **13**, 889497 (2022)
140. Gardiner, D.M., Rusu, A., Barrett, L., Hunter, G.C., Kazan, K.: Can natural gene drives be part of future fungal pathogen control strategies in plants? New Phytol. **228**(4), 1431–1439 (2020)
141. Graeff, N., DeJongsma, K.R., Bredenoord, A.L.: Experts' moral views on gene drive technologies: a qualitative interview study. BMC Med. Ethics. **22**(25), 1–15 (2021)
142. Jiang, L., et al.: MdGH3.6 is targeted by MdMYB94 and plays a negative role in apple water-deficit stress tolerance. Plant J. **109**, 1271–1289 (2022a)
143. Jiang, L., et al.: Engineering drought-tolerant apple by knocking down six GH3 genes and potential application of transgenic apple as a rootstock. Hortic. Res. **9**, uhac122 (2022b)
144. Clemens, M., et al.: VvEPFL9-1 Knock-out via CRISPR / Cas9 reduces stomatal density in grapevine. Front. Plant Sci. **13**, 878001 (2022)
145. Kumar, R., Suhas, J., Bharat, S., Mishra, R.: Engineering drought tolerance in plants through CRISPR / Cas genome editing. Biotech. **10**, 400 (2020b)
146. Osakabe, Y., Watanabe, T., Sugano, S.S., Ueta, R., Ishihara, R.: Optimization of CRISPR / Cas9 genome editing to modify abiotic stress responses in plants. Nat. Publ. Gr. **6**, 26685 (2016)
147. Salazar-gutiérrez, M.R., Chaves, B., Hoogenboom, G.: Scientia Horticulturae Freezing tolerance of apple flower buds. Sci. Hortic. (Amsterdam). **198**, 344–351 (2016)

148. Shen, X., et al.: The RNA-binding protein MdHYL1 modulates cold tolerance and disease resistance in apple. Plant Physiol. **17**, 1–18 (2023)
149. Liang, Y., et al.: Transcriptional regulation of bark freezing tolerance in apple (Malus domestica Borkh.). Hortic. Res. **7**, 205 (2020)
150. Locatelli, F., Baldoni, Æ.E., Mattana, Æ.M.: Osmyb4 expression improves adaptive responses to drought and cold stress in transgenic apples. Plant Cell Rep. **27**, 1677–1686 (2008)
151. Bai, T., Zheng, X.: Genome-wide characterization of the HSP20 gene family identifies potential members involved in temperature stress response in apple. Front. Genet. **11**, 609184 (2020)
152. Torres, C.A., Sepúlveda, G., Kahlaoui, B.: Phytohormone interaction modulating fruit responses to photooxidative and heat stress on apple (Malus domestica Borkh.). Front. Plant Sci. **8**, 2129 (2017)
153. Zhang, X., et al.: Genome-wide identification of PRP genes in apple genome and the role of MdPRP6 in response to heat stress. Int. J. Mol. Sci. **22**, 5942 (2021b)
154. Dong, Q., et al.: MdVQ37 overexpression reduces basal thermotolerance in transgenic apple by affecting transcription factor activity and salicylic acid homeostasis. Hortic. Res. **8**, 220 (2021)
155. Zhu, J.: Abiotic stress signaling and responses in plants. Cell. **167**, 313–324 (2016)
156. Hu, L., et al.: MdINT1 enhances apple salinity tolerance by regulating the antioxidant system, homeostasis of ions, and osmosis. Plant Physiol. Biochem. **154**, 689–698 (2020)
157. Chen, S., et al.: Knockout of the entire family of AITR genes in Arabidopsis leads to enhanced drought and salinity tolerance without fitness costs. BMC Plant Biol. **21**, 137 (2021)
158. Zhu, Y., et al.: An Arabidopsis Nucleoporin NUP85 modulates plant responses to ABA and salt stress. Plos Genet. **13**(12), e1007124 (2017)
159. Zhang, A., Liu, Y., Wang, F., Li, T., Chen, Z., Kong, D.: Enhanced rice salinity tolerance via CRISPR / Cas9-targeted mutagenesis of the OsRR22 gene. Mol. Breed. **39**, 47 (2019)
160. Bouzroud, S., et al.: Down regulation and loss of Auxin Response Factor 4 function using CRISPR/Cas9 alters plant growth, stomatal function and improves tomato tolerance to salinity and osmotic stress. Genes (Basel). **11**, 272 (2020)
161. Okada, K., Honda, C.: Molecular mechanisms regulating the columnar tree architecture in apple. Forests. **13**, 1084 (2022)
162. Hill, J.L., Hollender, C.A.: Branching out: new insights into the genetic regulation of shoot architecture in trees. Curr. Opin. Plant Biol. **47**, 73–80 (2019)
163. Bryant, D.W., et al.: Building a foundation for gene family analysis in Rosaceae genomes with a novel work flow: a case study in Pyrus architecture genes. Front. Plant Sci. **13**, 975942 (2022)
164. Zheng, X., et al.: MdWRKY9 overexpression confers intensive dwarfing in the M26 rootstock of apple by directly inhibiting brassinosteroid synthetase MdDWF4 expression. New Phytol. **217**(3), 1086–1098 (2018)
165. Hollender, C.A., Hadiarto, T., Srinivasan, C., Scorza, R., Dardick, C.: A brachytic dwarfism trait (dw) in peach trees is caused by a nonsense mutation within the gibberellic acid receptor PpeGID1c. New Phytol. **210**(1), 227–239 (2016)
166. Jia, D., Gong, X., Li, M., Li, C., Sun, T., Ma, F.: Overexpression of a novel apple NAC transcription factor gene, MdNAC1, confers the Dwarf phenotype in transgenic apple (Malus domestica). Genes (Basel). **9**(5), 1–18 (2018b)
167. Johnson, J.M., Kohler, A.R., Haus, M.J., Hollender, C.A.: Arabidopsis weep mutants exhibit narrow root angles. Micro Publ. Biol. **2022** (2022). https://doi.org/10.17912/micropub.biology.000584
168. Waite, J.M., Dardick, C.: The roles of the IGT gene family in plant architecture: past, present, and future. Curr. Opin. Plant Biol. **59**, 101983 (2021)
169. Hollender, C.A., et al.: Loss of a highly conserved sterile alpha motif domain gene (WEEP) results in pendulous branch growth in peach trees. Proc. Natl. Acad. Sci. U. S. A. **115**(20), E4690–E4699 (2018)
170. Hollender, C.A., Hill, J.L., Waite, J., Dardick, C.: Opposing influences of TAC1 and LAZY1 on lateral shoot orientation in Arabidopsis. Sci. Rep. **10**(1), 1–13 (2020)

171. Taniguchi, M., et al.: The arabidopsis LAZY1 family plays a key role in gravity signaling within statocytes and in branch angle control of roots and shoots. Plant Cell. **29**(8), 1984–1999 (2017)
172. Salojärvi, J., et al.: Genome sequencing and population genomic analyses provide insights into the adaptive landscape of silver birch. Nat. Genet. **49**(6), 904–912 (2017)
173. Petersen, R., Krost, C.: Tracing a key player in the regulation of plant architecture: the columnar growth habit of apple trees (Malus × domestica). Planta. **238**(1), 1–22 (2013)
174. Otto, D., Petersen, R., Schmidt, E.R., Gypsy, Á.T., Leaf, I.Á.R.Á.: The columnar mutation ("'Co gene'") of apple (Malus x domestica) is associated with an integration of a Gypsy-like retrotransposon. Mol. Breed. **33**, 863–880 (2014)
175. Dougherty, L., Bai, T., Brown, S., Xu, K., Silva, H.: Exploring DNA variant segregation types enables mapping loci for recessive phenotypic suppression of Columnar growth in apple. Front. Plant Sci. **11**, 692 (2020)
176. Han, T., Yu, J., Zhuang, J., Wang, Z., Sun, X., Zhang, Y.: The characterization of columnar apple gene MdCoL promoter and its response to abscisic acid, brassinosteroid and gibberellic acid. Int. J. Mol. Sci. **23**(18), 1–15 (2022)
177. Flachowsky, H., Hättasch, C., Höfer, M., Peil, A., Hanke, M.V.: Overexpression of LEAFY in apple leads to a columnar phenotype with shorter internodes. Planta. **231**(2), 251–263 (2010)
178. Sassa, H., Hirano, H., Ikehashi, H.: Self-incompatibility-related RNases in styles of Japanese pear (Pyrus serotina Rehd.). Plant Cell Physiol. **33**(6), 811–814 (1992)
179. Okada, K., et al.: Related polymorphic F-box protein genes between haplotypes clustering in the BAC contig sequences around the S-RNase of Japanese pear. J. Exp. Bot. **62**(6), 1887–1902 (2011)
180. DeNettancourt, D.: Incompatibility in angiosperms. Sex. Plant Reprod. **10**(4), 185–199 (1997)
181. Sassa, H., Hirano, H., Nishio, T., Koba, T.: Style-specific self-compatible mutation caused by deletion of the S-RNase gene in Japanese pear (Pyrus serotina). Plant J. **12**(1), 223–227 (1997)
182. Sassa, H., et al.: S locus F-box brothers: multiple and pollen-specific F-box genes with S haplotype-specific polymorphisms in apple and Japanese pear. Genetics. **175**(4), 1869–1881 (2007)
183. Okada, K., et al.: Deletion of a 236 kb region around S4-RNase in a stylar-part mutant S4 sm-haplotype of Japanese pear. Plant Mol. Biol. **66**(4), 389–400 (2008)
184. Kubo, K., et al.: Collaborative non-self recognition system in S-RNase-based self-incompatibility. Science. **330**(6005), 796–799 (2010)
185. De Franceschi, P., Dondini, L., Sanzol, J.: Molecular bases and evolutionary dynamics of self-incompatibility in the Pyrinae (Rosaceae). J. Exp. Bot. **63**(11), 4015–4032 (2012)
186. Broothaerts, W., Keulemans, J., Van Nerum, I.: Self-fertile apple resulting from S-RNase gene silencing. Plant Cell Rep. **22**(7), 497–501 (2004)
187. Li, M.F., Li, X.F., Han, Z.H., Shu, H.R., Li, T.Z.: Molecular analysis of two Chinese pear (Pyrus bretschneideri Rehd.) spontaneous self-compatible mutants, Yan Zhuang and Jin Zhui. Plant Biol. **11**(5), 774–783 (2009a)
188. Sanzol, J.: Pistil-function breakdown in a new S-allele of European pear, S21°, confers self-compatibility. Plant Cell Rep. **28**(3), 457–467 (2009)
189. Sawamura, Y., et al.: A self-compatible Pollen-part mutant of Japanese pear produced by crossing "Kosui" with Pollen from Gamma-irradiated "Kosui". J. Japanese Soc. Hortic. Sci. **82**(3), 222–226 (2013)
190. Mase, N., et al.: A segmental duplication encompassing S-haplotype triggers pollen-part self-compatibility in Japanese pear (Pyrus pyrifolia). Mol. Breed. **33**(1), 117–128 (2014)
191. Gustafson, F.G.: Parthenocarpy: natural and artificial. Bot. Rev. **8**(9), 599–654 (1942)
192. Moniruzzaman, M., Darwish, A.G., Ismail, A., El-kereamy, A., Tsolova, V., El-Sharkawy, I.: Seedlessness trait and genome editing—a review. Int. J. Mol. Sci. **24**(6), 5660 (2023)
193. Joldersma, D., Liu, Z.: The making of virgin fruit: the molecular and genetic basis of parthenocarpy. J. Exp. Bot. **69**(5), 955–962 (2018)

194. Nishitani, C., et al.: Parthenocarpic genetic resources and gene expression related to parthenocarpy among four species in pear (Pyrus spp.). Sci. Hortic. (Amsterdam). **136**, 101–109 (2012)
195. Quinet, M., Buyens, C., Dobrev, P.I., Motyka, V., Jacquemart, A.L.: Hormonal regulation of early fruit development in European pear (Pyrus communis cv. "conference"). Horticulturae. **5**(1), 1–18 (2019)
196. Griggs, W.H., Iwakiri, B.T.: Pollination and parthenocarpy in the production of Bartlett pears in California. Hilgardia. **22**(19), 643–678 (1954)
197. Pauwels, E., Eyssen, R., Keulemans, J.: Parthenocarpy and apple breeding. Acta Hortic. **484**, 55–59 (1999)
198. Lordan, J., Vilardell, P., Peris, M., Torres, E., Alegre, S., Asín, L.: Post petal fall applications of gibberellins improve fruit set on pear. Sci. Hortic. (Amsterdam). **252**, 149–155 (2019)
199. Cong, L., et al.: CPPU may induce gibberellin-independent parthenocarpy associated with PbRR9 in "Dangshansu" pear. Hortic. Res. **7**(1), 1–14 (2020)
200. Ren, Z., Li, Z., Miao, Q., Yang, Y., Deng, W., Hao, Y.: The auxin receptor homologue in Solanum lycopersicum stimulates tomato fruit set and leaf morphogenesis. J. Exp. Bot. **62**(8), 2815–2826 (2011)
201. Mazzucato, A., et al.: A TILLING allele of the tomato Aux/IAA9 gene offers new insights into fruit set mechanisms and perspectives for breeding seedless tomatoes. Mol. Breed. **35**(22), 15 (2015)
202. Goetz, M., Hooper, L.C., Johnson, S.D., Rodrigues, J.C.M., Vivian-Smith, A., Koltunow, A.M.: Expression of aberrant forms of AUXIN RESPONSE FACTOR8 stimulates parthenocarpy in arabidopsis and tomato. Plant Physiol. **145**(2), 351–366 (2007)
203. Molesini, B., Pandolfini, T., Rotino, G.L., Dani, V., Spena, A.: Aucsia gene silencing causes parthenocarpic fruit development in tomato. Plant Physiol. **149**(1), 534–548 (2009)
204. García-Hurtado, N., et al.: The characterization of transgenic tomato overexpressing gibberellin 20-oxidase reveals induction of parthenocarpic fruit growth, higher yield, and alteration of the gibberellin biosynthetic pathway. J. Exp. Bot. **63**(16), 5803–5813 (2012)
205. Martínez-Bello, L., Moritz, T., López-Díaz, I.: Silencing C 19 -GA 2-oxidases induces parthenocarpic development and inhibits lateral branching in tomato plants. J. Exp. Bot. **66**(19), 5897–5910 (2015)
206. Martí, C., Orzáez, D., Ellul, P., Moreno, V., Carbonell, J., Granell, A.: Silencing of DELLA induces facultative parthenocarpy in tomato fruits. Plant J. **52**(5), 865–876 (2007)
207. Mazzucato, A., Taddei, A.R., Soressi, G.P.: The parthenocarpic fruit (pat) mutant of tomato (Lycopersicon esculentum Mill.) sets seedless fruits and has aberrant anther and ovule development. Development. **125**(1), 107–114 (1998)
208. Fuentes, S., Ljung, K., Sorefan, K., Alvey, E., Harber, N.P., Østergaard, L.: Fruit growth in Arabidopsis occurs via DELLA-dependent and DELLA-independent gibberellin responses. Plant Cell. **24**(10), 3982–3996 (2012)
209. Mazzucato, A., Olimpieri, I., Siligato, F., Picarella, M.E., Soressi, G.P.: Characterization of genes controlling stamen identity and development in a parthenocarpic tomato mutant indicates a role for the DEFICIENS ortholog in the control of fruit set. Physiol. Plant. **132**(4), 526–537 (2008)
210. Medina, M., et al.: Early anther ablation triggers parthenocarpic fruit development in tomato. Plant Biotechnol. J. **11**(6), 770–779 (2013)
211. De Martino, G., Pan, I., Emmanuel, E., Levy, A., Irish, V.F.: Functional analyses of two tomato APETALA3 genes demonstrate diversification in their roles in regulating floral development. Plant Cell. **18**(8), 1833–1845 (2006)
212. Niu, S., He, Y., Yan, S., Sun, Z., Cai, R., Zhang, Y.: Histological, transcriptomic, and gene functional analyses reveal the regulatory events underlying gibberellin-induced parthenocarpy in tomato. Hortic. Plant J. **12**, 1–16 (2023)
213. Okabe, Y., et al.: Aberrant stamen development is associated with Parthenocarpic fruit set through up-regulation of gibberellin biosynthesis in tomato. Plant Cell Physiol. **60**(1), 38–51 (2019)

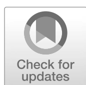
214. Tanaka, N., Wada, M.: Apple MADS genes are involved in Parthenocarpy and floral organ formation. Hortic. J. **91**(2), 131–139 (2022)
215. Yao, J.-L.: Parthenocarpic apple fruit production conferred by transposon insertion mutations in a MADS-box transcription factor. Proc. Natl. Acad. Sci. **98**(3), 1306–1311 (2001)
216. Cong, L., et al.: 2,4-D-induced parthenocarpy in pear is mediated by enhancement of GA 4 biosynthesis. Physiol. Plant. **166**(3), 812–820 (2019)
217. Liu, L., et al.: Histological, hormonal and transcriptomic reveal the changes upon gibberellin-induced parthenocarpy in pear fruit. Hortic. Res. **5**(1), 1–13 (2018)
218. Flachowsky, H., Hanke, M.V., Peil, A., Strauss, S.H., Fladung, M.: A review on transgenic approaches to accelerate breeding of woody plants: review. Plant Breed. **128**(3), 217–226 (2009)
219. Lang, G.A., Early, J.D., Martin, G.C., Darnell, R.L.: Endo-, Para-, and Ecodormancy: physiological terminology and classification for dormancy research. HortScience. **22**(3), 1–20 (1987)
220. Faust, M., Erez, A., Rowland, L.J.J., Wang, S.Y.Y., Norman, H.A.A.: Bud dormancy in perennial fruit trees. HortScience. **32**(4), 623–629 (1997)
221. Howe, G.T., et al.: Physiological and genetic approaches to studying endodormancy-related traits in Populus. HortScience. **34**(7), 1174–1184 (1999)
222. Yang, Q., Gao, Y., Wu, X., Moriguchi, T., Bai, S., Teng, Y.: Bud endodormancy in deciduous fruit trees: advances and prospects. Hortic. Res. **8**(1), 139 (2021)
223. Tominaga, A., Ito, A., Sugiura, T., Yamane, H.: How is global warming affecting fruit tree blooming? “Flowering (dormancy) disorder” in Japanese pear (Pyrus pyrifolia) as a case study. Front. Plant Sci. **12**(2), 1–17 (2022)
224. El-Yazal, M.A.S., Rady, M.M., Seif, S.A.: Foliar-applied dormancy-breaking chemicals change the content of nitrogenous compounds in the buds of apple (Malus sylvestris Mill. cv.Anna) trees. J. Hortic. Sci. Biotechnol. **87**(4), 299–304 (2012)
225. Li, J., Xu, Y., Niu, Q., He, L., Teng, Y., Bai, S.: Abscisic acid (ABA) promotes the induction and maintenance of pear (Pyrus pyrifolia white pear group) flower bud endodormancy. Int. J. Mol. Sci. **19**(1), 1–20 (2018)
226. Yang, Q., et al.: PpyGAST1 is potentially involved in bud dormancy release by integrating the GA biosynthesis and ABA signaling in “Suli” pear (Pyrus pyrifolia White Pear Group). Environ. Exp. Bot. **162**, 302–312 (2019)
227. Tuan, P.A., Bai, S., Saito, T., Ito, A., Moriguchi, T.: Dormancy-associated MADS-box (DAM) and the abscisic acid pathway regulate pear endodormancy through a feedback mechanism. Plant Cell Physiol. **58**(8), 1378–1390 (2017)
228. Yang, Q., et al.: ABA-responsive ABRE-BINDING FACTOR3 activates DAM3 expression to promote bud dormancy in Asian pear. Plant Cell Environ. **43**(6), 1360–1375 (2020)
229. Kumar, G., Rattan, U.K., Singh, A.K.: Chilling-mediated DNA methylation changes during dormancy and its release reveal the importance of epigenetic regulation during winter dormancy in Apple (Malus x domestica Borkh.). PLoS One. **11**(2), 1–25 (2016)
230. Saito, T., Bai, S., Imai, T., Ito, A., Nakajima, I., Moriguchi, T.: Histone modification and signalling cascade of the dormancy-associatedMADS-box gene, PpMADS13-1, in Japanese pear (Pyrus pyrifolia) during endodormancy. Plant Cell Environ. **38**(6), 1157–1166 (2015)
231. Li, Z., Reighard, G.L., Abbott, A.G., Bielenberg, D.G.: Dormancy-associated MADS genes from the EVG locus of peach [Prunus persica (L.) Batsch] have distinct seasonal and photoperiodic expression patterns. J. Exp. Bot. **60**(12), 3521–3530 (2009b)
232. Salama, A.M., et al.: Temperate fruit trees under climate change: challenges for dormancy and chilling requirements in warm winter regions. Horticulturae. **7**(86), 1–14 (2021)
233. Drepper, B., Gobin, A., Van Orshoven, J.: Spatio-temporal assessment of frost risks during the flowering of pear trees in Belgium for 1971–2068. Agric. For. Meteorol. **315**, 108822 (2022)
234. Hanke, M.-V., Flachowsky, H., Peil, A., Hättasch, C.: No flower no fruit – genetic potentials to trigger flowering in fruit trees. Genes, Genomes and Genomics. **1**(1), 1–20 (2007)

235. Jackson, D., Looney, N., Morley-Bunker, M.: Temperate and Subtropical Fruit Production, pp. 327. CAB International, Oxford UK, ISBN: 978-1-84593-501-6 (2010)
236. Washington State University: Apple Chemical Thinning | WSU Production Guide. *WSU* (2023)
237. Turner, J.N.: Gibberellic acid for controlling fruit production of pears. Acta Hortic. **34**, 287–298 (1972)
238. Sharma, N., Singh, S.K., Mahato, A.K., Ravishankar, H., Dubey, A.K., Singh, N.K.: Physiological and molecular basis of alternate bearing in perennial fruit crops. Sci. Hortic. (Amsterdam). **243**, 214–225 (2019)
239. Callejas, R., Bangerth, F.: Is auxin export of apple fruit an alternative signal for inhibition of flower bud induction? Acta Hortic. **463**, 271–277 (1998)
240. Milyaev, A., Tandron-Moya, Y.A., von Wirén, N., Neuwald, D., Flachowsky, H., Wünsche, J.N.: What else don't we know about biennial bearing? Phytohormone profile of seeds and seed number per fruit differ between a biennial and a non-biennial apple cultivar. Acta Hortic. **1342**, 7–14 (2022a)
241. Milyaev, A., et al.: Profiling of phytohormones in apple fruit and buds regarding their role as potential regulators of flower bud formation. Tree Physiol. **42**(11), 2319–2335 (2022b)
242. Guitton, B., Kelner, J.J., Velasco, R., Gardiner, S.E., Chagné, D., Costes, E.: Genetic control of biennial bearing in apple. J. Exp. Bot. **63**(1), 131–149 (2012)
243. Wang, M., et al.: BRANCHED1: a key hub of shoot branching. Front. Plant Sci. **10**(2), 1–12 (2019)
244. Guitton, B., et al.: Analysis of transcripts differentially expressed between fruited and deflowered "gala" adult trees: a contribution to biennial bearing understanding in apple. BMC Plant Biol. **16**(1), 1–22 (2016)
245. Turner, J.N.: GA for controlling fruit production of pears. Acta Hortic. Symp. Growth Regul. Fruit Prod. **34**, 287–297 (1973)
246. Fan, S., et al.: Dynamic cytosine DNA methylation patterns associated with mRNA and siRNA expression profiles in alternate bearing apple trees. J. Agric. Food Chem. **67**(18), 5250–5264 (2019)
247. Singh, S., Chaudhary, R., Deshmukh, R., Tiwari, S.: Opportunities and challenges with CRISPR-Cas mediated homologous recombination based precise editing in plants and animals. Plant Mol. Biol. **111**, 1–20 (2023)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 20
Genome Editing in Forest Trees



Tobias Bruegmann, Alexander Fendel, Virginia Zahn, and Matthias Fladung

Abstract Since the first CRISPR/Cas-mediated genome editing of poplar in 2015, an increasing number of tree species are being genome-edited. Although the availability of genome sequences, tissue culture and transformation systems are limiting factors, research is ongoing on advanced methods such as DNA-free genome editing and gene targeting approaches in addition to the optimisation of single gene knockouts. These can be used to address ambitious issues and perform genome editing more accurately, which has implications for the legal assessment of edited trees. Once technically established, CRISPR/Cas can be used to circumvent specific challenges related to forest tree species, e.g., longevity and extended vegetative phases, and to modify traits relevant for breeding, whether for direct application or to elucidate the genetic basis of individual traits. Not least due to climate change, adaptation to abiotic stress such as drought stress as well as biotic stresses caused by pathogens are strongly in focus. For the use as a renewable resource and as a carbon sink, wood productivity in forest trees as well as wood properties are of interest. In biosafety assessments, tree-specific aspects have to be considered, which result, among other aspects, from the long lifespan.

1 Prerequisites to Use Genome Editing in Trees

After CRISPR/Cas was first used for genome editing in plants as published in 2013 [1, 2], the first genome editing of a tree species was published only a short time later. Fan et al. [3] described the knockout of the visual marker gene *PDS* (encoding for phytoene desaturase) in *Populus* × *tomentosa*, resulting in albino phenotypes. For this, a Cas9 expression vector including matching guide-RNAs (gRNAs) was used

Alexander Fendel and Virginia Zahn contributed equally to this publication.

T. Bruegmann (✉) · A. Fendel · V. Zahn · M. Fladung
Thünen Institute of Forest Genetics, Grosshansdorf, Germany
e-mail: tobias.bruegmann@thuenen.de

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_20

for stable *Agrobacterium*-mediated transformation of *in vitro* poplars. Even though innovative methods are being developed, in practice mostly functioning tissue culture systems remain an essential basis for successful editing in trees. This involves three steps with respective hurdles: (1) establishment of an *in vitro* culture system that is able to regenerate plant shoots, (2) establishment of a protocol for the transfer of the Cas/gRNA into living cells, i.e., by classical genetic transformation, and (3) establishment of a protocol for genome editing.

Many tree species are considered *in vitro*-recalcitrant. It is difficult to transfer them to the *in vitro* culture and, once this initial step is accomplished, to regenerate them in large quantities and within manageable time frames for biotechnological purposes. The term recalcitrance summarizes many problems, some of them are still unidentified because the physiological basis of recalcitrance is not fully understood to date [4]. A fundamental difficulty is the need for clean (often generalised as "sterile") cultivars, i.e., without overwhelming bacterial or fungal contamination. In a few tree species, the transfer from sterilised vegetative organs such as leaves into *in vitro* culture has worked, e.g., for poplars. Sterilised embryos or somatic meristems from shoots are more suitable for many tree species [4]. For this purpose, younger starting material seems to be more suitable than older trees if plant material is to be used for organogenesis by the cultivation of meristems (unpublished data, [5]). If organogenesis cannot be induced directly, somatic embryogenesis is often used for plant regeneration. Here, the development of complete embryos with radicle, shoot, and cotyledons is induced from somatic cells, e.g., callus. These somatic embryos are similar to zygotic embryos. They can be easily separated from the mother tissue, have all the necessary structures for regeneration into a whole plant [6].

The ability to regenerate single cells into complete plants is necessary for genetic transformation and the generation of genetically uniform regenerates. For many tree species, regeneration media with tuned hormone contents can be used to induce the totipotency of somatic cells and stimulate regenerating callus for organogenesis. Cytokinins, such as 6-Benzylaminopurine (BAP), and/or auxins, such as Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D), are usually used for this purpose. Their concentrations should be optimised for each species [7]. The use of protoplasts with appropriate regeneration medium is also suitable. At this point, brief attention should be drawn to somaclonal variation that can be induced by *in vitro* techniques [8]. Even though these mutations usually have no effect on the transformability or editability of the plants, it should be considered that, in practice, some genetic variability is unavoidable.

If an *in vitro* culture system is achievable, the basis for genetic transformation is given. However, establishing the transformation method is far from easy, as existing transformation protocols can only be utilised in tree species to a limited extent. If modifications are needed, an establishment process follows which, if at all, can lead to success over a certain amount of time. Three conventional methods of genetic transformation are commonly used, with ascending relevance for tree species: ballistic transformation by particle gun, polyethylene glycol (PEG)-mediated transformation of protoplasts, and *Agrobacterium*-mediated transformation. The use of a particle gun requires good mechanical tuning to determine the bombardment

parameters that produce many transformations and little damaged plant material [9]. In addition, special equipment is required, especially the particle gun itself. Provided protoplasts can be generated, PEG-mediated uptake of plasmid DNA works reliably. To give some examples, PEG-mediated transformation of protoplasts, partly combined with electroporation, works in *Eucalyptus* species [10, 11], a poplar hybrid (*Populus tremula* × *Populus alba* [12]) and rubber tree (*Hevea brasiliensis* [13]). The challenge of this method is the isolation of vital protoplasts and their regeneration via callus stages into whole plants. For *Agrobacterium*-mediated transformation, bacteria with a natural competence to transfer genetic material are used. Although the term *Agrobacterium*-mediated transformation continues to endure in the research community, the associated bacterial species have been renamed *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*) and *Rhizobium rhizogenes* (formerly known as *Agrobacterium rhizogenes*). This commonly used method works for many tree species including conifers such as *Abies koreana* [14], *Larix decidua* [15], *Picea abies* [16], *Picea glauca* and *Picea mariana* [17], *Pinus radiata* [18], *Pinus taeda* [16], *Pseudotsuga menziesii* [19], and broadleaf trees such as *Ailanthus altissima* [20], *Castanea dentata* [21], *Castanea sativa* [22], *Eucalyptus globulus* [23], *Fraxinus americana* [24], *Fraxinus excelsior* [25], *Ginkgo biloba* [26], Poplar and aspen hybrids (*Populus* spp.) [27–29], *Quercus robur* [30], *Quercus suber* [31], *Robinia pseudoacacia* [32], and *Ulmus americana* [33].

After the establishment of the transformation method, the genome editing technique needs to be established. For forest trees, only CRISPR/Cas actually plays a significant role. The three other genome editing mechanisms using transcription activator-like effector nucleases (TALEN), zinc finger nucleases (ZFN) and oligo-directed mutagenesis (ODM) are of minor importance in trees. TALEN and ODM have not been used to date. In poplars, ZFN was used experimentally before CRISPR/Cas technology was made accessible [34]. However, the results indicated that further technical improvements would be needed to increase the moderate mutation frequency.

For CRISPR/Cas-mediated genome editing, suitable and efficiently editable targets need to be identified based on gene sequencing, following the selection of a suitable Cas nuclease. Sequence information of the individual to be edited is required for accurate and reliable genome editing. Even though the number of genome-sequenced tree species is steadily increasing since the first tree genome of the poplar species *Populus trichocarpa* [35], reference genomes are still quite limited to tree species with a manageable genome size. In particular, sequencing the complex genomes of some conifers remains a hurdle [36]. The availability of a reference genome is a prerequisite for selecting an editing target and verifying the presence of the protospacer-adjacent motif (PAM), as well as for identifying loci in the genome that might be considered as off-target sites. Avoiding off-target editing is one of the determining factors for the reliability of genome editing and its safety assessment. Meanwhile, some online tools for predicting potential off-targets have emerged for plants such as Cas-OFFinder, available at http://www.rgenome.net/cas-offinder/ [37]. Furthermore, it should be remembered that trees are nearly

undomesticated plants and therefore have high genetic diversity. Thus, it cannot be assumed that the sequence of a target gene is identical in all individuals of a species. Although a reference genome can assist genome editing by providing evidence for targeting, the target sequence should be verified in the individual being targeted before the final design. This challenge is addressed by the concept of the pan-genome, but this is still in the distant future for trees [38].

Genome editing in forest trees, despite all the challenges described, is a promising technology that can bring benefits to plant molecular genetics research in particular. CRISPR/Cas and the other editing techniques are excellent for gene characterization studies. In these classical knockout approaches, genome editing serves as a tool to knock out genes as described by Fan et al. [3], Zhou et al. [39], and Bruegmann et al. [40]. With knockout approaches, the function of genes can simply be characterised which could subsequently be good starting points for conventional forest plant breeding. With corresponding genetic markers, individuals can be selected from natural populations and used as crossing partners to introgress favourable traits. If legal conditions permit, genome-edited trees could be used directly, too. The targeted genetic modifications can be regarded as optimised breeding. Although the breeding goal can be achieved with conventional breeding methods based on crossing and selection, genome editing can accelerate genetic adaptation because trees take up to several decades to flower and fruit, depending on the species. Accelerating the adaptation process offers the possibility of adapting trees to rapidly advancing climate change and associated environmental conditions in foreseeable time frames. Plants' natural adaptation mechanisms such as genetic adaptation by recombination, mutation, and selection, or migration are likely to be too slow for adaptation to the new environmental challenges associated with climate change [41, 42].

2 Genome-Edited Forest Tree Species

Forests have significant ecological and economic functions, so their preservation and vitality are of great importance. Thus, by any method, breeding forest trees is an important human mission. The term "tree" is indistinctly defined. In general, it refers to perennial plants that have wood formation and secondary thickening growth and - to distinguish them from shrubby woody plants – a single main stem. The tree forms a more or less definite crown [43]. Some definitions add the size: The stem grows to a height of at least six meters without external disturbance [44]. As previously indicated, TALEN and ODM have not been applied in trees to date. A ZFN was used experimentally in poplar hybrids (*P. tremula* × *P. alba* [referred to as *Populus* × *canescens*] and *P. tremula* × *P. tremuloides*) to mutagenize poplar orthologs of *LEAFY* and *AGAMOUS*. The editing rate in this *Agrobacterium*-based approach was among the lowest of all experiments with plants overall [34]. Due to the technical advantages offered by CRISPR/Cas and the boost of these techniques

in plant research throughout, from which woody plant research also benefited and still benefits, research work focused on this technique.

The first CRISPR/Cas-edited tree was a Chinese white poplar (*P.* × *tomentosa*) in which the phytoene desaturase gene (*PDS*) was knocked out by mutation [3]. In subsequent years, *PDS* continues to serve as a marker gene in different annual and perennial plant species, as *PDS*-deficient plants are albinos [45]. Table 20.1 lists the forest tree species genome-edited to date. In trees, even though alternative Cas nucleases such as Cas12a have been available in principle for several years, Cas9 is the tool of choice. To our knowledge, the first Cas12a editing of a tree species was performed in 2020 using the *PDS* knockout in the poplar hybrid *Populus alba* × *Populus glandulosa* [46].

3 Advanced Editing Technologies and Current Developments

Since the first genome editing experiments that resulted in Cas9-mediated knockouts, CRISPR-based methods have continuously improved and evolved, particularly in annual crop plants and model species. Due to the tree-specific bottlenecks described above, the development of novel editing techniques in trees is not progressing as rapidly as in other model plants.

3.1 Effecting CRISPR/Cas Editing During Transformation and Regeneration

Despite the expanding range of tree species that can undergo genome editing, most forest tree species still exhibit low transformation efficiencies [58, 59]. Much time can be spent obtaining a sufficient number of transgenic and genome-edited plants by scaling up transformation experiments or optimising the transformation method. Those optimisations include the transfer of the DNA as well as regeneration during tissue culture.

To enhance transformation efficiency in recalcitrant species by boosting regeneration, morphogenic regulator genes like *WUSCHEL* or *BABY BOOM* can be co-expressed with CRISPR/Cas components [60]. In poplar, Pan et al. [61] significantly increased callus and root initiation as well as shoot growth by co-activation of endogenous morphogenic genes *WUSCHEL* (*PtWUS*) or *WUSCHEL-RELATED HOMEOBOX 11* (*PtWOX11*). While morphogenic regulator genes are facing the bottleneck of regeneration, using nanoparticles can enhance the direct delivery of plasmid DNA into the target tissue, making transformation more efficient or even independent of expensive and complicated laboratory equipment [62]. In *Paulownia tomentosa*, the polysaccharide nanoparticle Chitosan was used as a carrier for direct plasmid transfection of nodular segments. Since Chitosan has a positive charge, a

Table 20.1 CRISPR/Cas-edited forest tree species. For poplar trees, only inventions are mentioned here. More editing events are described in the section on traits and breeding

Scientific species name	Common species name	Editing nuclease	Infiltration mechanism	Reference
Betula platyphylla	Asian white birch	Cas9 targeting unspecified regions	*Agrobacterium*-based	[47]
Castanea sativa	European chestnut	Cas9 targeting *PDS*	RNP editing	[45]
Cryptomeria japonica	Japanese cedar	Cas9 targeting *GFP* and *CjCHLI*	*Agrobacterium*-based	[48]
Eucalyptus grandis	Rose gum	Cas9 targeting *CCR1*	*Agrobacterium*-based	[49]
Hevea brasiliensis	Rubber tree	Cas9 targeting *FT* and *TFL1*	RNP editing	[50]
Juglans regia	English walnut	Cas9 targeting *PDS*	*Agrobacterium*-based	[51]
Larix gmelinii	Dahurian larch	SpRY targeting three genomic sites	PEG-mediated protoplast transformation	[52]
Parasponia andersonii	w/o common name	Cas9 targeting *PanHK4*, *PanEIN2*, *PanNSP1*, and *PanNSP2*	*Agrobacterium*-based	[53]
Picea glauca (*Picea engelmannii*)	Silver spruce / Engelmann spruce	Cas9 targeting *DXS1*	*Agrobacterium*-based	[54]
Pinus radiata	Monterey pine	Cas9 targeting *GUX1*	RNP editing	[55]
Populus alba × *Populus glandulosa*	Poplar hybrid	Cas12a targeting *PDS*	*Agrobacterium*-based	[46]
Populus alba × *Populus glandulosa*	Poplar hybrid	Cas9 targeting *SAP1*	RNP editing	[56]
Populus davidiana × *Populus bolleana*	Poplar hybrid	Cas9 targeting unspecified regions	*Agrobacterium*-based	[47]
Populus × *tomentosa*	Chinese white poplar	Cas9 targeting *PDS*	*Agrobacterium*-based	[3]
Populus tremula × *Populus alba (P.* × *canescens)*	Grey poplar (hybrid)	Cas9 targeting *4CL*	*Agrobacterium*-based	[39]
Populus trichocarpa	Black cottonwood	Cas9 targeting *PtrADA2b-3*	*Agrobacterium*-based	[57]

RNP — ribonucleoprotein

complex was formed with negatively charged DNA improving the uptake through the negatively charged cell membrane [63].

Because a shoot often develops from more than one single transformed cell, regenerated transgenic plants are often chimeric resulting in possibly non-uniformly

edited regenerates in CRISPR/Cas approaches. This can affect the resulting phenotype and experiments based on genetically uniform tissue. A second step of regeneration decreased those chimeras in poplar and increased the frequency of homozygous CRISPR/Cas-mediated mutations [64].

Additionally, the activity of Cas9 and Cas12a can be enhanced by including a heat treatment during regeneration after transformation. This way, the editing efficiencies in poplar as well as birch have been increased [46, 47].

3.2 Optimising CRISPR/Cas Vectors for Forest Tree Editing

Many aspects influence the efficiency of CRISPR/Cas editing. On the one hand, editing efficiency depends on the accessibility of the genomic target sequence to the Cas-enzyme, a factor depending on chromosome structure [65]. On the other hand, it depends on the expression of the Cas-gene as well as the activity of the Cas-enzyme and the transcription of gRNA, which can be influenced by proper vector design.

In reports of CRISPR/Cas experiments in non-model forest trees like birch, chestnut, or walnut, the 35S promoter is the promoter of choice to drive Cas expression [45, 47, 51]. Because of its broad host range and well-studied functionality, as in classical gene technique experiments, this promoter is often used for the establishment of methods. In CRISPR/Cas attempts with the model tree genus *Populus*, Cas expression under control of a synthetic 35S-MAS fusion promoter increased editing efficiency by 11% compared to the 35S promoter, which still is one of the standard promoters in poplars to date [66]. Driving the Cas expression under heterologous ubiquitin promoters resulted in editing efficiencies of up to 95% in poplar [61, 67]. Those examples show the potential of optimisation by promoter choice in non-model forest tree species.

To enhance the translation of the Cas endonuclease in forest trees, codon-optimised variants like the plant codon optimised and the *Arabidopsis thaliana* codon-optimised Cas9 are routinely used [3, 40, 51, 53]. The use of a poplar codon-optimised AsCas12a, the first report of target organism specific codon optimisation for forest trees, resulted in editing efficiencies of up to 70% [46]. For this kind of optimisation, knowledge about the codon usage of the target tree species must be available. In the Codon Usage Database, codon usage tables for different forest trees like poplar (*Populus* spp.), beech (*Fagus* spp.), chestnut (*Castanea* spp.), pine (*Pinus* spp.) or eucalyptus (*Eucalyptus* spp.) are available [68]. These tables can be used as a query for a codon usage analysis and optimisation of Cas genes. To predict Cas expression levels based on codon usage, the Codon Adaptation Index (CAI) can be calculated [69]. A value of one implements an optimal translation rate. If values are low, the online tool Optimizer can be used to create a sequence with a maximum CAI [70]. If only the rare codons that limit translation are to be identified, the graphical codon usage analyser can be used to predict relative adaptiveness of each codon [71].

The most used promoter to drive gRNA transcription in forest trees is the U6-26 promoter from *A. thaliana* (*At*U6-26) [40, 51, 53], which is known for expressing high levels of gRNA in various plant species [72, 73]. In pine, a U6 promoter from *Pseudotsuga menziesii,* another member of the conifer family, is used [55]. Contrastively, in rubber tree (*Hevea brasiliensis*) endogenous U6 promoters were used for gRNA transcription resulting in editing efficiencies of *HbPDS* of up to 67% [49], representing higher efficiency compared to reports of *PDS* editing with *A. thaliana* U6 promoters in forest trees [3, 51]. These results correspond with reports in other tree species and plants indicating that endogenous polymerase III promoters can enhance editing efficiency in general [74, 75].

To obtain a knockout mutant or to edit multiple genes at once, it is often required to target multiple sites in the genome. In consequence, the ability to express multiple gRNAs at once is desired. In chestnut, poplar or eucalyptus, individual transcription units were used to drive sgRNA transcription for Cas9 under the same or different polymerase III promoters [3, 49, 76]. To avoid usage of repetitive sequences, and therefore recombination, silencing and large vector size, multiple gRNAs separated by tRNAs can be combined in one transcription unit [77]. In this way, five crRNAs for Cas12a were transcribed by a single *At*U6-26 promoter in poplar [46]. Pan et al. [61] in turn, used a polymerase II promoter to drive gRNA transcription for Cas9 in poplar, which is recommended for more than two gRNAs and offers the opportunity of inducible gRNA transcription.

In plant species having a long regeneration time like forest trees, the proper choice of spacer sequence and, therefore, the functionality of the corresponding gRNA is essential due to time-consuming and ecological reasons. In poplar, spacer sequences and secondary structures of multiple gRNAs were associated with Cas9 editing efficiencies resulting in recommendations for favourable gRNA structure and spacer sequence. These recommendations can be used to design gRNA candidates for Cas9-mediated genome editing by *in silico* prediction of secondary structure to avoid non-functional sgRNAs [40]. For Cas12a, the effect of secondary structure and spacer sequence on editing efficiency has been analysed in human cell lines, *E. coli* and maize, but not yet specifically for forest tree species [78–80]. Potential gRNAs for different Cas variants and their efficiencies can also be predicted *in silico* using online tools. But it must be considered that most of them like CRISPOR or CHOPCHOP are based on editing efficiency data from mammalian cells or zebrafish [81]. Although some tools, e.g., CHOPCHOP, use reference genome data from tree species, they are not specifically designed for forest tree species [82].

To evaluate gRNAs in the target organism, transient expression systems can be used prior to stable transformations. In rubber tree, protoplast transfection and amplicon deep sequencing of the target region were combined to check editing efficiency of Cas9 before stable transformation [83]. In poplar and birch, *Agrobacterium* inoculation of whole *in vitro* plantlets and quantitative PCR of the target locus are used [47]. It is likely that these transient systems can easily be adapted to other Cas nucleases, such as Cas12a. However, especially when using protoplasts, the cell type dependence on editing efficiency must be considered [65].

If not being able to test in advance, a second (or even more) gRNA targeting the same genomic region can be used as backup to increase the probability of inducing at least one mutation [40, 67, 76]. Moreover, a 1.3 kb deletion at the target site was obtained using two sgRNAs for Cas9-mediated genome editing in Monterey pine (*P. radiata*, [55]). This large deletion is more likely to completely destroy the gene function.

3.3 DNA-Free Editing

In conventional CRISPR/Cas9 approaches, the Cas9 nuclease and gRNA are stably transferred into the target organism as genetic information. However, integration of CRISPR/Cas-related transgenes can be disadvantageous. Continuous cleavage activity of the Cas nuclease can increase formation of chimeric plants and off-target cleavage. In addition, transgenes are a limiting factor in terms of legal regulation.

Since the outcrossing of transgenes in tree species is not an option in practice due to long reproduction cycles, it could be promising to obtain transgene-free edited plants in the first generation by avoiding transgene integration. Therefore, recombinant Cas-ribonucleoproteins (RNPs) can be used instead of plasmid-encoded CRISPR components. Since RNPs do not depend on gene expression and effective promoters, vector optimisation is omitted. However, the use of RNPs has its own parameters to be optimised, such as RNP concentration, protein-gRNA ratio and incubation temperatures. For proof of principle, RNPs for CRISPR/Cas editing were introduced into protoplasts of poplar (*P. alba* × *P. glandulosa*), chestnut (*C. sativa*) and rubber tree (*H. brasiliensis*) using PEG [45, 50, 56]. A biolistic approach was used to co-deliver Cas-RNPs and a plasmid-encoded selection marker to somatic embryos of Monterey pine (*P. radiata*). Editing efficiencies of up to 33% were observed in selection marker resistant plantlets [55].

3.4 Gene Sequence Modification

CRISPR/Cas-knockout mutants are primarily based on random indels obtained by error-prone non-homologous end joining (NHEJ) during the repair of DNA double-strand breaks (DSBs) [84]. But if interested in specific insertions, deletions, or exchanges of DNA, a more precise and accurate way of DNA modification is needed. Such ambitious techniques are at the very beginning in forest trees.

For the conversion of single base pairs, CRISPR base editors (BEs) have been invented. BEs combine DNA binding domains with a nucleotide base deaminase that chemically modifies certain nucleotide bases. Using cytosine (CBEs) or adenine base editors (ABEs), conversions of C•G to T•A or A•T to G•C are possible, respectively [85]. Expression of Cas9 nickase-based BE in poplar (*P. tremula* × *P. alba*) led to the precise conversion efficiency of up to 100% for CBE and 95% for

ABE. Thereby indicating that efficiency depends on the target and can be improved by using a U3 instead of a U6 *A. thaliana* promoter for sgRNA transcription [67].

To enable insertion or replacement of sequences larger than single base pairs and up to several kilobases, gene targeting based on homology-directed repair (HDR) is applied. Here, a donor DNA containing the intended DNA modification as well as flanking sequences homologous to the target site can be co-delivered with the CRISPR/Cas components. With the simultaneous inhibition of NHEJ by knocking *XRCC4* out and the enhancement of HDR by overexpressing *CtIP* and *MRE11* in poplar (*P. trichocarpa*), a bleomycin resistance gene was seamlessly integrated in frame of an endogenous promoter by Cas9 with knock-in efficiency of up to 48% [86].

3.5 CRISPR Activation

CRISPR/Cas can be used for activation of target genes by recruiting transcription activators, independent of CRISPR/Cas-mediated changes to the DNA sequence [87]. Because a DSB is not required, CRISPR activation is achieved by nuclease-inactive deadCas9 (dCas9). For gene activation in poplar (*P. alba* × *P. tremula*), a CRISPR-Combo system based on CRISPR-Act3.0 was used to enable gene editing and activation at the same time. Therefore, Cas9 endonuclease activity was deactivated by using short protospacer sequences of 14 to 16 nucleotides. Activators were acquired by gRNA using MS2-SunTags. Editing efficiencies of 100% and gene activation of up to 100-fold expression were achieved [61].

4 Forest Tree Relevant Traits as Breeding Objectives

Forest trees are important sequesters of CO_2 into biomass and components of terrestrial biodiversity. As sessile organisms with prolonged growth, forest trees are frequently exposed to diverse stresses derived from the abiotic and biotic environment. Climate change-related weather conditions contribute to novel and increasingly severe environmental stresses for forest trees, such as drought periods or increased soil and water salinisation in certain climate zones. Although trees are evolutionarily adapted to local environments, fast-changing fluctuations of local climate conditions strongly affect their viability. Moreover, the establishment of novel tree pathogens caused by climate fluctuations and the increased vulnerability of already stressed forest trees to domestic pathogens also put forest trees under stress [88]. Trees are increasingly required to withstand specific stresses and to remain upright, providing, in part, irreplaceable both ecological and economic value for countries and their people. The ability of trees to assimilate CO_2 in great quantities displays a natural mechanism to mitigate global warming effects. However, climate change-derived stresses may reduce carbon fixation due to reduced photosynthesis rates when water is scarce or temperatures are too high [89].

Forest tree tolerances, resistances, and the refined breeding of commercial traits display the present-day breeding objectives. Improved and especially accelerated breeding strategies and genetic research on traits need more attention if species or products are to be sustained in the near future. Even though implementing genome editing in forest trees to improve and accelerate breeding purposes is still young, some research has displayed this mechanism's fast potential, especially on functional analyses of single genes and their correlation with desired traits.

4.1 Abiotic Stress Tolerances

Abiotic stress tolerance-related research deals with elucidating and improving traits for tolerance of abiotic environmental factors, especially drought or salinity, that trees are increasingly confronted with. The continuing incorporation of genome editing mechanisms (particularly CRISPR/Cas) in this research field drastically improves the understanding of single gene functions and their impact on tolerance traits by subsequently isolated phenotype analyses. Even though, to date, the research on abiotic stress tolerance-related traits is still limited in trees, an increase is observable and will gain more attention in future. However, some research has been done regarding single or multiple stresses and their higher-ordered adaption mechanisms.

4.1.1 Drought Stress

Drought stress describes the stress caused by the absence of water supply, which can reduce biomass production and the energy-providing mechanism of photosynthesis. While the research on annual model plants already revealed important mechanisms and genes involved in drought stress tolerance, the research on forest trees (especially under the application of genome editing) is in its infancy. The research of recent years mainly applied CRISPR/Cas-mediated knockouts in the model genus *Populus* to verify observable phenotypes derived from overexpression of putative drought tolerance-related genes. Even though CRISPR/Cas-mediated knockout mutants did not improve traits of drought stress tolerance, the precise knockouts of candidate genes helped insights into gene functions and their further use for tree tolerance breeding purposes. As a trait of putative drought stress tolerance, Zhou et al. [90] analysed the mechanism of root growth under drought stress. Overexpression and CRISPR/Cas9 knockouts of the root-specific *NUCLEAR FACTOR-Y* transcription factor (TF) *NF-YB21* were analysed in the poplar hybrid 84 K (*P. alba* × *P. glandulosa*). Comparative analyses of one-month-old *nf-yb21* mutants and WT poplars revealed a reduced drought stress tolerance of the mutants by significantly reduced overall root growth and biomass, as well as thinner xylem vessels with tyloses and lower lignin contents, which reduced the hydraulic conductivity, an important indicator of water transport from soils [90].

As stomata regulate the flow of gases and thereby a plants water status, Shen et al. [91] analysed the impact of the TF *Pd*GNC on stomatal aperture in *P.* × *canescens*. 60-days-old CRISPR/Cas9-mediated *gnc* mutants exhibited increased stomatal aperture and water loss with reduced drought stress tolerance under drought stressed experimental conditions of 75 days. Analyses explained the drought-susceptible phenotype with lower nitric oxide (NO) levels and hydrogen peroxide (H_2O_2) production in guard cells, increasing the stomatal aperture and, thereby water loss [91]. Similarly, Bai et al. [92] studied the function of the TF gene *OSMOTIC STRESS INDUCED C2H2 1* (*OSIC1*) in *P. alba* var. *pyramidalis* that is likewise involved in the pathway of stomatal aperture. CRISPR/Cas9-mediated knockout mutants showed significantly reduced performance under drought stress by greater stomatal aperture and water loss. This was explained by the involvement of *OSIC1* in a newly discovered transcriptional regulatory mechanism of H_2O_2 production in guard cells. The results enrich the understanding of how perennial woody plants respond to drought-induced osmotic stress, which can be further used for refined breeding approaches [92].

4.1.2 Salt Stress

The salinisation of forest soils describes the excessive accumulation of water-soluble salts in upper soil horizons. It mainly derives from extreme weather conditions, with dry climates and low precipitation. If prolonged over a long time or at high concentrations, the salinity of the soil results in reduced water and increased salt uptake of trees, resulting in stress through ionic, osmotic, oxidative, and other secondary changes. Salt stress can, when exposed for a long time, end in plant die-offs [93]. Here, damage severity depends on salt concentrations, the growth stage of trees or the tree species, with *Populus euphratica* known to tolerate specific salt concentrations and growing in saline semi-arid areas [94].

Efforts have been made to study the impact of single gene modifications on salt stress tolerance by using genome editing in *Populus*. CRISPR/Cas9-mediated knockout of the TF gene *WRKY77* in *P. alba* var. *pyramidalis* significantly improved the salt tolerance of poplars under *in vitro* salt stress conditions [95]. After growing for one month in liquid medium with 150 mM NaCl, wild-type (WT) poplars showed leaf chlorosis symptoms, while *palwrky77* mutant leaves remained green with little discolourations. Further, significantly higher electrolyte leakage measurements suggested a higher cell disruption in WT compared to mutant poplars. *In vivo* and *in vitro* assays revealed the differences in salt stress tolerance by the *Pal*WRKY77-induced repression of abscisic acid-related genes. Therefore, *Pal*WRKY77 was found to be a negative regulator of salt stress response in poplars, providing a potential basis for genetic modification to generate salt-tolerant poplars in saline habitats [95].

To further elucidate salt stress tolerance in trees, candidate genes could be selected by the orientation of promising genes in annual plants such as *A. thaliana*, *Oryza sativa,* or *Solanum lycopersicum*. Shelake et al. [96] illustrate the potential of

genome editing in crop plants to increase salinity tolerance. Here, CRISPR/Cas-mediated knockouts of *AtAITR* genes involved in abscisic acid signalling in *A. thaliana* [97], the *OsPQT3* gene, an E3 ubiquitin ligase involved in the regulation of oxidative stress in *O. sativa* [98], or the *SlARF4* gene, an auxin response factor in *S. lycopersicum* [99], resulted in improved salinity tolerance and may be promising candidate genes for genetic modifications in forest trees if sequence information and genetic transformability are given.

4.2 Biotic Stress Resistance

Plant pathogens, including viruses, fungi, bacteria, or oomycetes, are part of every functional ecosystem. However, globalisation or changing environmental factors contribute to the spread and mutation of pathogens, leading to pathogens harmful to single species or whole plant ecosystems. Resulting emerging infectious diseases can cause landscape-level mortality and, subsequently, ecosystem-wide changes [100]. The importance of tree pathogen control can be exemplified in the interference of the fungus *Hymenoscyphus fraxineus*, causing ash dieback with severe mortality of common ash trees (*F. excelsior*) in most parts of the ash distribution range in Europe [101]. Other prominent examples of forest tree pathogens are chestnut blight, Dutch elm disease, myrtle rust, white pine blister rust, poplar leaf rust, and sudden oak death [100].

To date, the research on forest pathogen resistance by genome editing mechanisms is in its infancy. In addition to technical limitations in working with forest trees, the lack of knowledge of tree-pathogen interactions may restrict genome editing applications, as it denotes the basis for advanced research. Wang et al. [102] analysed the involvement of the TF MYB115 on the production of proanthocyanidins (PAs), a class of defence phenolic compounds in the leaves of poplars (*Populus* spp.) in response to abiotic and biotic stresses. CRISPR/Cas9 knockouts of *MYB115* in *P.* × *tomentosa* revealed significantly reduced levels of PAs and decreased expression of PA biosynthesis genes, suggesting the positive contribution of MYB115 to PA biosynthesis. After infection with *Dothiorella gregaria,* leaves from the poplar mutant *myb115* showed significantly higher damage [102].

Widespread biotrophic rust fungi of the genus *Melampsora* can reduce the economic value of trees, such as for *Populus* in natural stands and plantations, by reducing significant amounts of biomass [103, 104]. To elucidate genes involved in *Melampsora* resistance, Jiang et al. [105] focused on the TFs WRKY18 and WRKY35. The WRKY group is well known for being involved in abiotic and biotic stress responses in plants. Constitutive overexpression of *WRKY18* and *WRKY35* in *P.* × *tomentosa* led to increased resistance to *Melampsora* by elevated expression levels of downstream genes and lower H_2O_2 accumulation. In contrast, CRISPR/Cas9-mediated knockout mutants did not differ from WT poplars regarding the expression levels of downstream genes and H_2O_2 accumulation, concluding an unaltered *Melampsora* resistance [105].

4.3 Commercial Traits: Productivity and Wood Properties

Trees provide biomaterials with benefits for human life. Especially wood production and wood composition are interesting traits for refined breeding purposes due to the broad range of applications, ranging from the area of building and other industries up to energy supply. Economically important tree species include *P. abies*, *Tectona grandis*, or *Cedrus deodara* for timber production or *Picea rubens*, *Abies balsamea* or *Populus tremuloides* for the production of pulp and paper [106]. Prominent representatives of trees for biofuel production are species of the genus *Populus* or *Salix* due to their fast growth, allowing the production of significant amounts of biomass [107, 108].

4.3.1 Wood Productivity

The productivity of a forest is defined by the standing forest volume at a specific time and referred to as yield, expressed by the accumulation of aboveground stem wood in standing trees. This biomass formation and correlated wood production exhibit an essential trait to improve due to the increasing wood utilisation demands, especially under increasing climate change-related disturbances on wood supply [109].

Genome editing mechanisms have been used to investigate the growth and development of woody plants. Thereby, conclusions could be made of genes involved in productivity, mainly in wood formation, to meet the demands of future wood production. CRISPR/Cas9-mediated knockouts of the *A. thaliana BRANCHED* orthologs *BRANCHED1-1* (*PcBRC1-1*) and *BRANCHED2-1* (*PcBRC2-1*) genes in *P.* × *canescens* strongly enhanced bud outgrowth [110]. *Pcbrc2-1* mutants revealed a significantly higher number of branches, whereas mutants of *Pcbrc1-1* revealed significantly higher shoots. As poplar trees are used for bioenergy production on short rotation coppices (SRCs), the enhanced sylleptic branching of the mutants may be an improved trait regarding the critical plantation establishment phase in the first year. It may increase the biomass yield through the early closure of the canopy and, subsequently, the reduction of competing weeds by shading [110]. However, long-term biomass evaluation is still needed.

Fladung [111] generated CRISPR/Cas9-mediated knockouts of the rice ortholog *TILLER ANGLE CONTROL 1* (*TAC1*) in *P.* × *canescens* to investigate the function of the gene on the pyramidal plant growth, as could be seen by reduced expression levels of *TAC1* in *Prunus* species [112]. After a growth period of 3 years in the greenhouse, mutated poplars showed an altered phenotype compared to WT trees, with leaves of a narrower angle and an upright growth of shoots [111]. Even though no increased biomass production was detectable, upright-grown poplar trees may be interesting for SRCs, as the erect leaf or shoot growth allows more trees per area and, therefore, higher yield per area.

Further experiments of CRISPR/Cas knockouts of poplars were conducted to investigate the function of genes on wood productivity and growth [113]. However, knockouts were not correlated with improved biomass production.

4.3.2 Wood Composition

Wood mainly comprises the polymers lignin and the polysaccharidic cellulose and hemicelluloses, enriched in secondary-thickened cell walls. Polysaccharides are the desired substances of industries producing paper and pulp. In contrast, even though increasingly used for aromatic building blocks in the chemical industry, lignin impairs the extraction of cellulose and hemicelluloses, therefore being declared as a factor of biomass recalcitrance [114].

To reduce the amount of lignin in the wood composition, several genome editing attempts were conducted targeting lignin biosynthesis, ranging from involved transcription factors and oxidative enzymes up to the lignin biosynthesis genes themselves. Early genome editing via the CRISPR/Cas9 system in the *P.* × *canescens* produced biallelic knockouts of the *4-COUMARATE:COA LIGASE 1* (*4CL1*) gene, which was shown to be involved in the lignin biosynthesis [39]. The poplar mutants revealed 23% less lignin in stem wood. Xu et al. [115] genetically modified the TF gene *PtoMYB170* in *P.* × *tomentosa* by creating knockout mutants generated by three target sites for CRISPR/Cas9 endonuclease. Knockout mutants of *PtoMYB170* displayed inability for upright growth, resulting in a pendant phenotype due to significantly reduced lignin deposition in the stem's secondary xylem growth. Expression analyses of lignin biosynthesis genes indicated strongly reduced expression levels in the knockout mutants, demonstrating that *Pto*MYB170 is strongly influential on the downstream genes and lignin deposition in *P.* × *tomentosa* [115].

Other strategies involved the manipulation of genes involved in the direct biosynthesis of lignin. Vries et al. [116] conducted CRISPR/Cas9 knockouts of the *CAFFEOYL SHIKIMATE ESTERASE 1* and *2* (*CSE1, CSE2*) genes in *P.* × *canescens*. After 4 months of growth in the greenhouse, double mutants *cse1cse2* showed a height reduction of 35%, with further reduced stem diameter by 14%, stem fresh weight (not debarked) by 52% and stem dry weight (debarked) by 69% as compared to WT trees [116]. However, lignin contents were decreased by 35%, which translated into a fourfold increase in cellulose-to-glucose conversion upon limited saccharification. That indicates that the saccharification efficiency (hydrolysis from polysaccharides to monosaccharides), positively affects the fermentation of monosaccharides to ethanol, a favourable trait of biofuel production [117]. Jang et al. [118] conducted a comparable CRISPR/Cas9 knockout approach of *CSE1* and *CSE2* in the closely related poplar hybrid 84 K (*P. alba* × *P. glandulosa*). In contrast to Vries et al. [116], they found a reduction of lignin deposition of up to 29.1% in either *cse1* or *cse2* single mutants, along with reduced expression levels of lignin biosynthesis genes. Simultaneously, the genome-edited lines showed no growth retardation and a morphologically indistinguishable phenotype to WT trees in a

long-term living modified organism field test covering four seasons [118]. In addition, mutant poplars showed up to 25% higher saccharification efficiency than the WT control. The difference between both conducted studies from Vries et al. [116] and Jang et al. [118] may rely on the different species (*P.* × *canescens; P. alba* × *P. glandulosa*, respectively) or the amount of lignin reduction (35%, 29.1%, respectively) and thereby a specific threshold, under which no phenotypic changes are observable [118].

Within several years, genome editing positively affected the understanding of lignin-related genes in the model tree genus *Populus* and successfully established poplar trees with limited amounts of lignin and no growth retardation, providing essential insights into the future breeding of lignin-reduced wood composition in trees.

Apart from the genetic modification of wood composition-related genes, parameters of wood anatomy, particularly xylem fibre and vessel length were analysed by genome editing of Fasciclin-like arabinogalactan proteins (FLAs) in *P. trichocarpa* [119]. Out of 50 *in vivo* characterised poplar FLAs, two genes (*PtrFLA40* and *PtrFLA45*) were selected due to their high expression in the developing xylem as well as their high similarity in amino acids of 95.2%. Selected *FLA* genes were knocked out by CRISPR/Cas9. Double mutants *ptrfla40ptrfla45* revealed significantly increased stem length and diameter and enlarged cell sizes of xylem fibres and vessels of 4-month-old grown greenhouse poplars compared to unmodified WT trees [119]. These findings may be relevant for the paper and pulp industry, as the fibre length is an important quality trait due to its positive effect on sheet strength [120].

5 Biosafety of Genome-Edited Trees

Trees differ from most agricultural crop plants in many characteristics, such as long lifespan and long generation cycles, complex habitat, and low degree of domestication. As with genetically modified (GM) trees, biosafety has to be considered before their deployment [121, 122], but well-documented knowledge on specific biosafety aspects is rare for genome-edited trees. Thus, information on biosafety protocols for genome-edited trees is required which provide a scientific basis for future European Union regulations on environmental risk assessment to ensure the safe development and use of genome-edited trees.

Biosafety-relevant aspects comprise four main technological issues that need to be discussed for genome-edited trees [123]: (i) Are the gene-edited and naturally emerged modifications in fact identical? (ii) If not, are the differences potentially hazardous? (iii) Are efficient containment strategies required to avoid possible adverse outcomes from vertical and horizontal gene transfer? (iv) Are off-target effects probable, and if yes, is the selective inclusion of “omics”-technologies needed to study cellular effects following the expression of the gene-edited gene(s)?

Like other plant species, genome-edited trees have the potential for gene flow when they flower. Thus, edited gene(s) could be spread to wild relatives through gene flow (vertical gene transfer). Many tree species are wind pollinators releasing pollen into the environment, which can sometimes be transported over very long distances. In case that the edited gene(s) have a developmental/evolutionary advantage, this could pose an invasive potential of these trees. Unintended ecological consequences could occur, such as the unintended spread of invasive genes or of the whole tree (by vegetative propagation, e.g., root suckers [124]) into natural populations. Thus, at least theoretically, the necessity of establishing containment strategies has to be considered. Therefore, the establishment of confinement systems, i.e., by making trees sterile by suppressing either pollen production (in male stamens) or female ovule development, is considered to prevent the uncontrolled spread of the edited genes [125].

In addition, possible unintended effects of genome-edited trees on non-target organisms must be considered. For example, if a tree is modified in a gene involved in the secondary metabolism and, as consequence, produces a new ingredient, this could act as a toxin to the tree-interacting organisms. Trees fulfil numerous ecosystem services, such as carbon sequestration, soil conservation, and water regulation. Again, genome-edited trees producing a new ingredient, could impact these services. For example, if a tree is modified to grow faster, it may sequester more carbon, but it could also deplete soil nutrients faster.

If a mutation has been detected in the plant genome without knowing whether it was natural or induced, to date, there are no detection methods to distinguish between gene-edited and natural mutations. In addition, if a cultivar carrying a natural mutation has been assessed as being safe in biosafety testing, there is no reason to assume a hazard if the cultivar has an induced mutation similar to a natural one. However, the mechanisms leading to the mutations are different, thus, because of the longevity of trees, long-term effects of genome-edited trees have to be considered, at least theoretically. This includes, for example, the long-term stability of the gene-edited modification or the epigenetics of the whole edited DNA region. To study the stability of the edited gene, the establishment of field trials under natural conditions are necessary. Such field trials could (i) deliver results about phenotypic effects resulting from expression of the gene edited genes, (ii) validate observations made under greenhouse conditions, and (iii) unravel putative non-target effects when the trees are grown within the range of natural variation. However, similar to GM trees, field trials with gene-edited trees will be the exception rather than the rule in Europe. Reasons for this are manifold. Firstly, a high level of public concern exists against genetic engineering technologies. Thus, many consumers are reluctant to accept products made from genetically modified organisms. In addition, regulatory hurdles which are based on the precautionary principle are high in Europe, making the regulatory process for field trials extremely long and making it difficult to obtain approval for a field trial with gene-edited trees. And finally, similar to GM plants in general, many companies and researchers fear that anti-GMO activists will destroy field trials with gene-edited trees.

Off-target effects are unintended changes in the DNA resulting from gene editing in "wrong" genes. This could occur, for example, when gene editing is aimed at duplicated genomes and the target genes are present in duplicated or even in multiple copies and are highly similar in their sequence. As a consequence, it could be that the gRNA was not specifically designed for the one gene to be edited and that also sequence-homologous genomic regions (e.g., paralogous genes) are targeted by the gRNA. This could lead to mutations in other parts of the genome, potentially causing gene knockouts or activation or silencing of genetic regulatory elements. However, such off-target modifications could simply be avoided through improvement in the gRNA design [40], based on reliable genome sequences of the tree species to be edited.

It is common silvicultural practice to perform a formal evaluation of the behaviour of new tree varieties under natural field conditions. Accordingly, a number of field trials have to be set up to assess the safety of gene-edited trees modified for different genes, similarly as it was performed with GM trees [126]. However, in contrast to classical GM-technology, genome editing modified genomic information is targeted and precise, thus, organisms (microbes, plants and animals) harbouring mutations created by genome editing are indistinguishable from organisms carrying an identical but naturally emerged mutation. This could lead to the question whether the biosafety of gene-edited trees needs to be tested in the field at all.

References

1. Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M., Sheen, J.: Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. Nat. Biotechnol. **31**(8), 688–691 (2013). https://doi.org/10.1038/nbt.2654
2. Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J.-L., Gao, C.: Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. **31**(8), 686–688 (2013). https://doi.org/10.1038/nbt.2650
3. Fan, D., Liu, T., Li, C., Jiao, B., Li, S., Hou, Y., Luo, K.: Efficient CRISPR/Cas9-mediated targeted mutagenesis in *Populus* in the first generation. Sci. Rep. **5**, 12217 (2015). https://doi.org/10.1038/srep12217
4. McCown, B.H.: Chapter 8 – Woody shrubs and trees. In: Park, S. (ed.) Plant tissue culture. Techniques and experiments, pp. 101–109. Academic, London (2021)
5. Pierik, R.L.M.: In vitro culture of higher plants. Springer, Netherlands, Dordrecht (1997)
6. Kadereit, J.W., Körner, C., Kost, B., Sonnewald, U.: Strasburger – Lehrbuch der Pflanzenwissenschaften, 37th edn. Springer, Berlin, Heidelberg (2014)
7. Park, S.: Callus induction. In: Park, S. (ed.) Plant tissue culture. Techniques and experiments, pp. 67–85. Academic, London (2021)
8. Häggman, H., Rusanen, M., Jokipii, S.: Cryopreservation of *in vitro* tissues of deciduous Forest trees. In: Reed, B.B.M. (ed.) Plant cryopreservation: a practical guide, pp. 365–386. Springer, New York (2010)
9. Russell, J.A., Roy, M.K., Sanford, J.C.: Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol. **98**(3), 1050–1056 (1992). https://doi.org/10.1104/pp.98.3.1050

10. Teulières, C., Grima-Pettenati, J., Curie, C., Teissie, J., Boudet, A.M.: Transient foreign gene expression in polyethylene/glycol treated or electropulsated *Eucalyptus gunnii* protoplasts. Plant Cell Tissue Organ Cult. **25**(2), 125–132 (1991). https://doi.org/10.1007/BF00042183
11. Manders, G., dos Santos, A.V.P., d'Utra Vaz, F.B., Davey, M.R., Power, J.B.: Transient gene expression in electroporated protoplasts of *Eucalyptus citriodora* Hook. Plant Cell Tissue Organ Cult. **30**(1), 69–75 (1992). https://doi.org/10.1007/BF00040003
12. Nietsch, J., Brügmann, T., Becker, D., Fladung, M.: Old methods rediscovered: application and improvement of two direct transformation methods to hybrid poplar (*Populus tremula* × *P. alba*). Plant Cell Tissue Organ Cult. **130**(1), 183–196 (2017). https://doi.org/10.1007/s11240-017-1214-7
13. Zhang, X., Wang, L., He, C., Luo, H.: An efficient transient mesophyll protoplast system for investigation of the innate immunity responses in the rubber tree (*Hevea brasiliensis*). Plant Cell Tissue Organ Cult. **126**(2), 281–290 (2016). https://doi.org/10.1007/s11240-016-0997-2
14. Lee, H., Moon, H.-K., Park, S.-Y.: *Agrobacterium*-mediated transformation via somatic embryogenesis system in Korean fir (*Abies koreana* Wil.), a Korean native conifer. Korean J. Plant Res. **27**(3), 242–248 (2014). https://doi.org/10.7732/kjpr.2014.27.3.242
15. Huang, Y., Diner, A.M., Karnosky, D.F.: *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix decidua*. In Vitro Cell Dev. Biol. Plant. **27**, 201–207 (1991)
16. Wenck, A.R., Quinn, M., Whetten, R.W., Pullman, G., Sederoff, R.: High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). Plant Mol. Biol. **39**(3), 407–416 (1999). https://doi.org/10.1023/A:1006126609534
17. Klimaszewska, K., Lachance, D., Pelletier, G., Lelu, M.-A., Séguin, A.: Regeneration of transgenic *Picea glauca*, *P. mariana*, and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In Vitro Cell. Dev. Biol. Plant. **37**(6), 748–755 (2001). https://doi.org/10.1007/s11627-001-0124-9
18. Cerda, F., Aquea, F., Gebauer, M., Medina, C., Arce-Johnson, P.: Stable transformation of *Pinus radiata* embryogenic tissue by *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult. **70**(3), 251–257 (2002). https://doi.org/10.1023/A:1016508031151
19. Dandekar, A.M., Gupta, P.K., Durzan, D.J., Knauf, V.: Transformation and foreign gene expression in micropropagated Douglas–Fir (*Pseudotsuga menziesii*). Nat. Biotechnol. **5**(6), 587–590 (1987). https://doi.org/10.1038/nbt0687-587
20. Park, Y.G., Huh, K., Choi, M.S.: Gene transformation of *Ailanthus altissima* Swingle by *Agrobacterium tumefaciens*. Curr. Res. Agric. Life Sci. **10**, 137–145 (1992)
21. Polin, L.D., Liang, H., Rothrock, R.E., Nishii, M., Diehl, D.L., Newhouse, A.E., Nairn, C.J., Powell, W.A., Maynard, C.A.: *Agrobacterium*-mediated transformation of American chestnut (*Castanea dentata* (Marsh.) Borkh.) somatic embryos. Plant Cell Tissue Organ Cult. **84**(1), 69–79 (2006). https://doi.org/10.1007/s11240-005-9002-1
22. Seabra, R.C., Pais, M.S.: Genetic transformation of European chestnut. Plant Cell Rep. **17**(3), 177–182 (1998). https://doi.org/10.1007/s002990050374
23. Moralejo, M., Rochange, F., Boudet, A.M., Teuliéres, C.: Generation of transgenic *Eucalyptus globulus* plantlets through *Agrobacterium tumefaciens* mediated transformation. Functional Plant Biol. **25**(2), 207 (1998). https://doi.org/10.1071/pp97041
24. Palla, K.J., Pijut, P.M.: *Agrobacterium*-mediated genetic transformation of *Fraxinus americana* hypocotyls. Plant Cell Tissue Organ Cult. **120**(2), 631–641 (2015). https://doi.org/10.1007/s11240-014-0630-1
25. Hebda, A., Liszka, A., Zgłobicki, P., Nawrot-Chorabik, K., Lyczakowski, J.J.: Transformation of European ash (*Fraxinus excelsior* L.) callus as a starting point for understanding the molecular basis of ash dieback. Plan. Theory. **10**(11), 2524 (2021). https://doi.org/10.3390/plants10112524

26. Shunan, L., Tianen, S., Genbao, L.: Transformation of ginkgo hairy root and establishment of its suspension culture clone. Wuhan Univ. J. Nat. Sci. **2**(4), 493–495 (1997). https://doi.org/10.1007/BF02830266
27. Fillatti, J.J., Sellmer, J., McCown, B., Haissig, B., Comai, L.: *Agrobacterium* mediated transformation and regeneration of *Populus*. Mol. Gen. Genet. **206**(2), 192–199 (1987). https://doi.org/10.1007/BF00333574
28. Fladung, M.: Gene stability in transgenic aspen (*Populus*). I. Flanking DNA sequences and T-DNA structure. Mol. Gen. Genet. **260**(6), 574–581 (1999). https://doi.org/10.1007/s004380050931
29. Leple, J.C., Brasileiro, A.C., Michel, M.F., Delmotte, F., Jouanin, L.: Transgenic poplars: expression of chimeric genes using four different constructs. Plant Cell Rep. **11**(3), 137–141 (1992). https://doi.org/10.1007/BF00232166
30. Roest, S., Brueren, H., Evers, P.W., Vermeer, E.: *Agrobacterium*-mediated transformation of oak (*Quercus robur* L.). Acta Hortic. **289**, 259–260 (1991). https://doi.org/10.17660/ActaHortic.1991.289.69
31. Alvarez, R., Alonso, P., Cortizo, M., Celestino, C., Hernández, I., Toribio, M., Ordás, R.J.: Genetic transformation of selected mature cork oak (*Quercus suber* L.) trees. Plant Cell Rep. **23**(4), 218–223 (2004). https://doi.org/10.1007/s00299-004-0810-2
32. Han, K.-H., Keathley, D.E., Davis, J.M., Gordon, M.P.: Regeneration of a transgenic woody legume (*Robinia pseudoacacia* L., black locust) and morphological alterations induced by *Agrobacterium rhizogenes*-mediated transformation. Plant Sci. **88**(2), 149–157 (1993). https://doi.org/10.1016/0168-9452(93)90086-F
33. Newhouse, A.E., Schrodt, F., Liang, H., Maynard, C.A., Powell, W.A.: Transgenic American elm shows reduced Dutch elm disease symptoms and normal mycorrhizal colonization. Plant Cell Rep. **26**(7), 977–987 (2007). https://doi.org/10.1007/s00299-007-0313-z
34. Lu, H., Klocko, A.L., Dow, M., Ma, C., Amarasinghe, V., Strauss, S.H.: Low frequency of zinc-finger nuclease-induced mutagenesis in *Populus*. Mol Breeding. **36**(9) (2016). https://doi.org/10.1007/s11032-016-0546-z
35. Tuskan, G.A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., Salamov, A., Schein, J., Sterck, L., Aerts, A., Bhalerao, R.R., Bhalerao, R.P., Blaudez, D., Boerjan, W., Brun, A., Brunner, A., Busov, V., Campbell, M., Carlson, J., Chalot, M., Chapman, J., Chen, G.-L., Cooper, D., Coutinho, P.M., Couturier, J., Covert, S., Cronk, Q., Cunningham, R., Davis, J., Degroeve, S., Déjardin, A., Depamphilis, C., Detter, J., Dirks, B., Dubchak, I., Duplessis, S., Ehlting, J., Ellis, B., Gendler, K., Goodstein, D., Gribskov, M., Grimwood, J., Groover, A., Gunter, L., Hamberger, B., Heinze, B., Helariutta, Y., Henrissat, B., Holligan, D., Holt, R., Huang, W., Islam-Faridi, N., Jones, S., Jones-Rhoades, M., Jorgensen, R., Joshi, C., Kangasjärvi, J., Karlsson, J., Kelleher, C., Kirkpatrick, R., Kirst, M., Kohler, A., Kalluri, U., Larimer, F., Leebens-Mack, J., Leplé, J.-C., Locascio, P., Lou, Y., Lucas, S., Martin, F., Montanini, B., Napoli, C., Nelson, D.R., Nelson, C., Nieminen, K., Nilsson, O., Pereda, V., Peter, G., Philippe, R., Pilate, G., Poliakov, A., Razumovskaya, J., Richardson, P., Rinaldi, C., Ritland, K., Rouzé, P., Ryaboy, D., Schmutz, J., Schrader, J., Segerman, B., Shin, H., Siddiqui, A., Sterky, F., Terry, A., Tsai, C.-J., Uberbacher, E., Unneberg, P., Vahala, J., Wall, K., Wessler, S., Yang, G., Yin, T., Douglas, C., Marra, M., Sandberg, G., van de Peer, Y., Rokhsar, D.: The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science. **313**(5793), 1596–1604 (2006). https://doi.org/10.1126/science.1128691
36. Rodríguez, S.M., Ordás, R.J., Alvarez, J.M.: Conifer biotechnology: an overview. Forests. **13**(7), 1061 (2022). https://doi.org/10.3390/f13071061
37. Bae, S., Park, J., Kim, J.-S.: Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics (Oxford, England). **30**(10), 1473–1475 (2014). https://doi.org/10.1093/bioinformatics/btu048
38. Bayer, P.E., Golicz, A.A., Scheben, A., Batley, J., Edwards, D.: Plant pan-genomes are the new reference. Nat. Plants. **6**(8), 914–920 (2020). https://doi.org/10.1038/s41477-020-0733-0

39. Zhou, X., Jacobs, T.B., Xue, L.-J., Harding, S.A., Tsai, C.-J.: Exploiting SNPs for biallelic CRISPR mutations in the outcrossing woody perennial *Populus* reveals *4-coumarate:CoA ligase* specificity and redundancy. New Phytol. **208**(2), 298–301 (2015). https://doi.org/10.1111/nph.13470
40. Bruegmann, T., Deecke, K., Fladung, M.: Evaluating the efficiency of gRNAs in CRISPR/Cas9 mediated genome editing in poplars. IJMS. **20**(15), 3623 (2019). https://doi.org/10.3390/ijms20153623
41. Kijowska-Oberc, J., Staszak, A.M., Kamiński, J., Ratajczak, E.: Adaptation of forest trees to rapidly changing climate. Forests. **11**(2), 123 (2020). https://doi.org/10.3390/f11020123
42. Loarie, S.R., Duffy, P.B., Hamilton, H., Asner, G.P., Field, C.B., Ackerly, D.D.: The velocity of climate change. Nature. **462**(7276), 1052–1055 (2009). https://doi.org/10.1038/nature08649
43. Global Forest Resources Assessment 2020: Terms and Definitions, Rome. https://www.fao.org/3/I8661EN/i8661en.pdf (2018). Accessed 20 February 2023
44. Schwedt, G.: Forstbotanik. Springer, Berlin, Heidelberg (2021)
45. Pavese, V., Moglia, A., Abbà, S., Milani, A.M., Torello Marinoni, D., Corredoira, E., Martínez, M.T., Botta, R.: First report on genome editing via ribonucleoprotein (RNP) in *Castanea sativa* Mill. Int. J. Mol. Sci. **23**(10), 5762 (2022). https://doi.org/10.3390/ijms23105762
46. An, Y., Geng, Y., Yao, J., Fu, C., Lu, M., Wang, C., Du, J.: Efficient genome editing in *Populus* using CRISPR/Cas12a. Front. Plant Sci. **11** (2020). https://doi.org/10.3389/fpls.2020.593938
47. Wang, Z., He, Z., Qu, M., Liu, Z., Wang, C., Wang, Y.: A method for determining the cutting efficiency of the CRISPR/Cas system in birch and poplar. For. Res. **1**(1), 1–8 (2021). https://doi.org/10.48130/FR-2021-0016
48. Nanasato, Y., Mikami, M., Futamura, N., Endo, M., Nishiguchi, M., Ohmiya, Y., Konagaya, K., Taniguchi, T.: CRISPR/Cas9-mediated targeted mutagenesis in Japanese cedar (*Cryptomeria japonica* D. Don). Sci. Rep. **11**(1), 16186 (2021). https://doi.org/10.1038/s41598-021-95547-w
49. Dai, Y., Hu, G., Dupas, A., Medina, L., Blandels, N., Clemente, H.S., Ladouce, N., Badawi, M., Hernandez-Raquet, G., Mounet, F., Grima-Pettenati, J., Cassan-Wang, H.: Implementing the CRISPR/Cas9 technology in *Eucalyptus* hairy roots using wood-related genes. Int. J. Mol. Sci. **21**(10) (2020). https://doi.org/10.3390/ijms21103408
50. Fan, Y., Xin, S., Dai, X., Yang, X., Huang, H., Hua, Y.: Efficient genome editing of rubber tree (*Hevea brasiliensis*) protoplasts using CRISPR/Cas9 ribonucleoproteins. Ind. Crop. Prod. **146**, 112146 (2020). https://doi.org/10.1016/j.indcrop.2020.112146
51. Walawage, S.L., Zaini, P.A., Mubarik, M.S., Martinelli, F., Balan, B., Caruso, T., Leslie, C.A., Dandekar, A.M.: Deploying genome editing tools for dissecting the biology of nut trees. Front. Sustain. Food Syst. **3** (2019). https://doi.org/10.3389/fsufs.2019.00100
52. Ren, Q., Sretenovic, S., Liu, S., Tang, X., Huang, L., He, Y., Liu, L., Guo, Y., Zhong, Z., Liu, G., Cheng, Y., Zheng, X., Pan, C., Yin, D., Zhang, Y., Li, W., Qi, L., Li, C., Qi, Y., Zhang, Y.: PAM-less plant genome editing using a CRISPR-SpRY toolbox. Nat. Plants. **7**(1), 25–33 (2021). https://doi.org/10.1038/s41477-020-00827-4
53. van Zeijl, A., Wardhani, T.A.K., Seifi Kalhor, M., Rutten, L., Bu, F., Hartog, M., Linders, S., Fedorova, E.E., Bisseling, T., Kohlen, W., Geurts, R.: CRISPR/Cas9-mediated mutagenesis of four putative symbiosis genes of the tropical tree *Parasponia andersonii* reveals novel phenotypes. Front. Plant Sci. **9** (2018). https://doi.org/10.3389/fpls.2018.00284
54. Cui, Y., Zhao, J., Gao, Y., Zhao, R., Zhang, J., Kong, L.: Efficient multi-sites genome editing and plant regeneration via somatic embryogenesis in *Picea glauca*. Front. Plant Sci. **12** (2021). https://doi.org/10.3389/fpls.2021.751891
55. Poovaiah, C., Phillips, L., Geddes, B., Reeves, C., Sorieul, M., Thorlby, G.: Genome editing with CRISPR/Cas9 in *Pinus radiata* (D. Don). BMC Plant Biol. **21**(1) (2021). https://doi.org/10.1186/s12870-021-03143-x

56. Park, S.J., Choi, Y.-I., Jang, H.A., Kim, S.-G., Choi, H., Kang, B.-C., Lee, H., Bae, E.-K.: Genome editing of hybrid poplar (*Populus alba* × *P. glandulosa*) protoplasts using Cas9/gRNA ribonucleoprotein. J. Plant Biotechnol. **48**(1), 34–43 (2021). https://doi.org/10.5010/JPB.2021.48.1.034
57. Li, S., Lin, Y.-C.J., Wang, P., Zhang, B., Li, M., Chen, S., Shi, R., Tunlaya-Anukit, S., Liu, X., Wang, Z., Dai, X., Yu, J., Zhou, C., Liu, B., Wang, J.P., Chiang, V.L., Li, W.: The *AREB1* transcription factor influences histone acetylation to regulate drought responses and tolerance in *Populus trichocarpa*. Plant Cell. **31**(3), 663–686 (2019). https://doi.org/10.1105/tpc.18.00437
58. Fladung, M., Schenk, T.M.H., Polak, O., Becker, D.: Elimination of marker genes and targeted integration via FLP/FRT recombination system from yeast in hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.). Tree Genet. Genomes. **6**(2), 205–217 (2010). https://doi.org/10.1007/s11295-009-0241-x
59. Igasaki, T., Mohri, T., Ichikawa, H., Shinohara, K.: *Agrobacterium tumefaciens*-mediated transformation of *Robinia pseudoacacia*. Plant Cell Rep. **5**, 448–453 (2000)
60. Lowe, K., Wu, E., Wang, N., Hoerster, G., Hastings, C., Cho, M.-J., Scelonge, C., Lenderts, B., Chamberlin, M., Cushatt, J., Wang, L., Ryan, L., Khan, T., Chow-Yiu, J., Hua, W., Yu, M., Banh, J., Bao, Z., Brink, K., Igo, E., Rudrappa, B., Shamseer, P.M., Bruce, W., Newman, L., Shen, B., Zheng, P., Bidney, D., Falco, C., Register, J., Zhao, Z.-Y., Xu, D., Jones, T., Gordon-Kamm, W.: Morphogenic regulators *Baby boom* and *Wuschel* improve monocot transformation. Plant Cell. **28**(9), 1998–2015 (2016). https://doi.org/10.1105/tpc.16.00124
61. Pan, C., Li, G., Malzahn, A.A., Cheng, Y., Leyson, B., Sretenovic, S., Gurel, F., Coleman, G.D., Qi, Y.: Boosting plant genome editing with a versatile CRISPR-combo system. Nat. Plants. **8**(5), 513–525 (2022). https://doi.org/10.1038/s41477-022-01151-9
62. Lv, Z., Jiang, R., Chen, J., Chen, W.: Nanoparticle-mediated gene transformation strategies for plant genetic engineering. Plant J. **104**(4), 880–891 (2020). https://doi.org/10.1111/tpj.14973
63. Hussien, E.T.: Production of transgenic *Paulownia tomentosa* (Thunb.) steud. Using chitosan nanoparticles to express antimicrobial genes resistant to bacterial infection. Mol. Biol. Res. Commun. **9**(2), 55–62 (2020). https://doi.org/10.22099/mbrc.2019.35331.1454
64. Ding, L., Chen, Y., Ma, Y., Wang, H., Wei, J.: Effective reduction in chimeric mutants of poplar trees produced by CRISPR/Cas9 through a second round of shoot regeneration. Plant Biotechnol. Rep. **14**(5), 549–558 (2020). https://doi.org/10.1007/s11816-020-00629-2
65. Miyaoka, Y., Berman, J.R., Cooper, S.B., Mayerl, S.J., Chan, A.H., Zhang, B., Karlin-Neumann, G.A., Conklin, B.R.: Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. Sci. Rep. **6**, 23549 (2016). https://doi.org/10.1038/srep23549
66. An, Y., Geng, Y., Yao, J., Wang, C., Du, J.: An improved CRISPR/Cas9 system for genome editing in *Populus* by using Mannopine synthase (MAS) promoter. Front. Plant Sci. **12**, 703546 (2021). https://doi.org/10.3389/fpls.2021.703546
67. Li, G., Sretenovic, S., Eisenstein, E., Coleman, G., Qi, Y.: Highly efficient C-to-T and A-to-G base editing in a *Populus* hybrid. Plant Biotechnol. J. **19**(6), 1086–1088 (2021). https://doi.org/10.1111/pbi.13581
68. Nakamura, Y., Gojobori, T., Ikemura, T.: Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res. **28**, 292 (2000)
69. Puigbò, P., Bravo, I.G., Garcia-Vallve, S.: CAIcal: a combined set of tools to assess codon usage adaptation. Biol. Direct. **3**, 38 (2008). https://doi.org/10.1186/1745-6150-3-38
70. Puigbò, P., Guzmán, E., Romeu, A., Garcia-Vallvé, S.: OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res. **35**(Web Server issue), W126–W131 (2007). https://doi.org/10.1093/nar/gkm219
71. McInerney, J.O.: GCUA: general codon usage analysis. Bioinformatics (Oxford, England). **14**, 372–373 (1998)

72. Dutt, M., Mou, Z., Zhang, X., Tanwir, S.E., Grosser, J.W.: Efficient CRISPR/Cas9 genome editing with citrus embryogenic cell cultures. BMC Biotechnol. **20**(1), 58 (2020). https://doi.org/10.1186/s12896-020-00652-9
73. Li, X., Jiang, D.-H., Yong, K., Zhang, D.-B.: Varied transcriptional efficiencies of multiple *Arabidopsis U6* small nuclear RNA genes. J. Integr. Plant Biol. **2**, 222–229 (2007)
74. Charrier, A., Vergne, E., Dousset, N., Richer, A., Petiteau, A., Chevreau, E.: Efficient targeted mutagenesis in apple and first time edition of pear using the CRISPR-Cas9 system. Front. Plant Sci. **10**, 40 (2019) https://doi.org/10.3389/fpls.2019.00040
75. Kor, S.D., Chowdhury, N., Keot, A.K., Yogendra, K., Chikkaputtaiah, C., Sudhakar Reddy, P.: RNA Pol III promoters-key players in precisely targeted plant genome editing. Front. Genet. **13**, 989199 (2023). https://doi.org/10.3389/fgene.2022.989199
76. Pavese, V., Moglia, A., Corredoira, E., Martínez, M.T., Torello Marinoni, D., Botta, R.: First report of CRISPR/Cas9 gene editing in *Castanea sativa* Mill. Front. Plant Sci. **12** (2021). https://doi.org/10.3389/fpls.2021.728516
77. Xie, K., Minkenberg, B., Yang, Y.: Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc. Natl. Acad. Sci. U. S. A. **112**(11), 3570–3575 (2015). https://doi.org/10.1073/pnas.1420294112
78. Creutzburg, S.C.A., Wu, W.Y., Mohanraju, P., Swartjes, T., Alkan, F., Gorodkin, J., Staals, R.H.J., van der Oost, J.: Good guide, bad guide: spacer sequence-dependent cleavage efficiency of Cas12a. Nucleic Acids Res. **48**(6), 3228–3243 (2020). https://doi.org/10.1093/nar/gkz1240
79. Lee, K., Zhang, Y., Kleinstiver, B.P., Guo, J.A., Aryee, M.J., Miller, J., Malzahn, A., Zarecor, S., Lawrence-Dill, C.J., Joung, J.K., Qi, Y., Wang, K.: Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize. Plant Biotechnol. J. **17**(2), 362–372 (2019). https://doi.org/10.1111/pbi.12982
80. Kim, H.K., Song, M., Lee, J., Menon, A.V., Jung, S., Kang, Y.-M., Choi, J.W., Woo, E., Koh, H.C., Nam, J.-W., Kim, H.: *In vivo* high-throughput profiling of CRISPR-Cpf1 activity. Nat. Methods. **14**(2), 153–159 (2016). https://doi.org/10.1038/nmeth.4104
81. Li, C., Chu, W., Gill, R.A., Sang, S., Shi, Y., Hu, X., Yang, Y., Zaman, Q.U., Zhang, B.: Computational tools and resources for CRISPR/Cas genome editing. Genomics Proteomics Bioinformatics. (2022). https://doi.org/10.1016/j.gpb.2022.02.006
82. Montague, T.G., Cruz, J.M., Gagnon, J.A., Church, G.M., Valen, E.: CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. **Nucleic Acids Res, 42**(Web Server issue), W401–W407 (2014). https://doi.org/10.1093/nar/gku410
83. Dai, X., Yang, X., Wang, C., Fan, Y., Xin, S., Hua, Y., Wang, K., Huang, H.: CRISPR/Cas9-mediated genome editing in *Hevea brasiliensis*. Ind. Crop. Prod. **164**, 113418 (2021). https://doi.org/10.1016/j.indcrop.2021.113418
84. Puchta, H.: The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. J. Exp. Bot. **56**(409), 1–14 (2005). https://doi.org/10.1093/jxb/eri025
85. Huang, T.-K., Puchta, H.: Novel CRISPR/Cas applications in plants: from prime editing to chromosome engineering. Transgenic Res. **30**(4), 529–549 (2021). https://doi.org/10.1007/s11248-021-00238-x
86. Movahedi, A., Wei, H., Zhou, X., Fountain, J.C., Chen, Z.-H., Mu, Z., Sun, W., Zhang, J., Li, D., Guo, B., Varshney, R.K., Yang, L., Zhuge, Q.: Precise exogenous insertion and sequence replacements in poplar by simultaneous HDR overexpression and NHEJ suppression using CRISPR-Cas9. Hortic. Res. **9**, uhac154 (2022). https://doi.org/10.1093/hr/uhac154
87. Lowder, L.G., Paul, J.W., Qi, Y.: Multiplexed transcriptional activation or repression in plants using CRISPR-dCas9-based systems. Methods Mol. Biol. (Clifton, N.J.). **1629**, 167–184 (2017). https://doi.org/10.1007/978-1-4939-7125-1_12
88. Hennon, P.E., Frankel, S.J., Woods, A.J., Worrall, J.J., Norlander, D., Zambino, P.J., Warwell, M.V., Shaw, C.G.: A framework to evaluate climate effects on forest tree diseases. For. Path. **50**(6) (2020). https://doi.org/10.1111/efp.12649

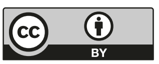
89. Sperry, J.S., Venturas, M.D., Todd, H.N., Trugman, A.T., Anderegg, W.R.L., Wang, Y., Tai, X.: The impact of rising CO_2 and acclimation on the response of US forests to global warming. Proc. Natl. Acad. Sci. U. S. A. **116**(51), 25734–25744 (2019). https://doi.org/10.1073/pnas.1913072116
90. Zhou, Y., Zhang, Y., Wang, X., Han, X., An, Y., Lin, S., Shen, C., Wen, J., Liu, C., Yin, W., Xia, X.: Root-specific NF-Y family transcription factor, *PdNF-YB21*, positively regulates root growth and drought resistance by abscisic acid-mediated indoylacetic acid transport in *Populus*. New Phytol. **227**(2), 407–426 (2020). https://doi.org/10.1111/nph.16524
91. Shen, C., Zhang, Y., Li, Q., Liu, S., He, F., An, Y., Zhou, Y., Liu, C., Yin, W., Xia, X.: *PdGNC* confers drought tolerance by mediating stomatal closure resulting from NO and H_2 O_2 production via the direct regulation of *PdHXK1* expression in *Populus*. New Phytol. **230**(5), 1868–1882 (2021). https://doi.org/10.1111/nph.17301
92. Bai, Q., Niu, Z., Chen, Q., Gao, C., Zhu, M., Bai, J., Liu, M., He, L., Liu, J., Jiang, Y., Wan, D.: The C_2H_2-type zinc finger transcription factor OSIC1 positively regulates stomatal closure under osmotic stress in poplar. Plant Biotechnol. J. **21**(5), 943–960 (2023). https://doi.org/10.1111/pbi.14007
93. Yang, Y., Guo, Y.: Elucidating the molecular mechanisms mediating plant salt-stress responses. New Phytol. **217**(2), 523–539 (2018). https://doi.org/10.1111/nph.14920
94. Chen, S., Li, J., Wang, S., Fritz, E., Hüttermann, A., Altman, A.: Effects of NaCl on shoot growth, transpiration, ion compartmentation, and transport in regenerated plants of *Populus euphratica* and *Populus tomentosa*. Can. J. For. Res. **33**(6), 967–975 (2003). https://doi.org/10.1139/X03-066
95. Jiang, Y., Tong, S., Chen, N., Liu, B., Bai, Q., Chen, Y., Bi, H., Zhang, Z., Lou, S., Tang, H., Liu, J., Ma, T., Liu, H.: The PalWRKY77 transcription factor negatively regulates salt tolerance and abscisic acid signaling in *Populus*. Plant J. **105**(5), 1258–1273 (2021). https://doi.org/10.1111/tpj.15109
96. Shelake, R.M., Kadam, U.S., Kumar, R., Pramanik, D., Singh, A.K., Kim, J.-Y.: Engineering drought and salinity tolerance traits in crops through CRISPR-mediated genome editing: targets, tools, challenges, and perspectives. Plant Commun. **3**(6), 100417 (2022). https://doi.org/10.1016/j.xplc.2022.100417
97. Chen, S., Zhang, N., Zhou, G., Hussain, S., Ahmed, S., Tian, H., Wang, S.: Knockout of the entire family of *AITR* genes in Arabidopsis leads to enhanced drought and salinity tolerance without fitness costs. BMC Plant Biol. **21**(1), 137 (2021). https://doi.org/10.1186/s12870-021-02907-9
98. Alfatih, A., Wu, J., Jan, S.U., Zhang, Z.-S., Xia, J.-Q., Xiang, C.-B.: Loss of rice *PARAQUAT TOLERANCE 3* confers enhanced resistance to abiotic stresses and increases grain yield in field. Plant Cell Environ. **43**(11), 2743–2754 (2020). https://doi.org/10.1111/pce.13856
99. Bouzroud, S., Gasparini, K., Hu, G., Barbosa, M.A.M., Rosa, B.L., Fahr, M., Bendaou, N., Bouzayen, M., Zsögön, A., Smouni, A., Zouine, M.: Down regulation and loss of *Auxin response factor 4* function using CRISPR/Cas9 alters plant growth, stomatal function and improves tomato tolerance to salinity and osmotic stress. Genes. **11**(3) (2020). https://doi.org/10.3390/genes11030272
100. Dort, E.N., Tanguay, P., Hamelin, R.C.: CRISPR/Cas9 gene editing: an unexplored frontier for forest pathology. Front. Plant Sci. **11**, 1126 (2020). https://doi.org/10.3389/fpls.2020.01126
101. Enderle, R., Stenlid, J., Vasaitis, R.: An overview of ash (*Fraxinus spp*.) and the ash dieback disease in Europe. CABI Rev. **2019**, 1–12 (2019). https://doi.org/10.1079/PAVSNNR201914025
102. Wang, L., Ran, L., Hou, Y., Tian, Q., Li, C., Liu, R., Fan, D., Luo, K.: The transcription factor MYB115 contributes to the regulation of proanthocyanidin biosynthesis and enhances fungal resistance in poplar. New Phytol. **215**(1), 351–367 (2017). https://doi.org/10.1111/nph.14569
103. Benetka, V., Černý, K., Pilařová, P., Kozlíková, K.: Effect of *Melampsora larici-populina* on growthand biomass yield of eight clones of *Populus nigra*. J. For. Sci. **57**(2), 41–49 (2011). https://doi.org/10.17221/51/2010-JFS

104. Eberl, F., Hammerbacher, A., Gershenzon, J., Unsicker, S.B.: Leaf rust infection reduces herbivore-induced volatile emission in black poplar and attracts a generalist herbivore. New Phytol. **220**(3), 760–772 (2018). https://doi.org/10.1111/nph.14565
105. Jiang, Y., Guo, L., Ma, X., Zhao, X., Jiao, B., Li, C., Luo, K.: The WRKY transcription factors PtrWRKY18 and PtrWRKY35 promote *Melampsora* resistance in *Populus*. Tree Physiol. **37**(5), 665–675 (2017). https://doi.org/10.1093/treephys/tpx008
106. Seth, M.K.: Trees and their economic importance. Bot. Rev. **69**(4), 321–376 (2003). https://doi.org/10.1663/0006-8101(2004)069[0321:TATEI]2.0.CO;2
107. Sannigrahi, P., Ragauskas, A.J., Tuskan, G.A.: Poplar as a feedstock for biofuels: a review of compositional characteristics. Biofuels Bioprod. Biorefin. **4**(2), 209–226 (2010). https://doi.org/10.1002/bbb.206
108. Labrecque, M., Teodorescu, T.I.: Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada). Biomass Bioenergy. **29**(1), 1–9 (2005). https://doi.org/10.1016/j.biombioe.2004.12.004
109. Kirilenko, A.P., Sedjo, R.A.: Climate change impacts on forestry. Proc. Natl. Acad. Sci. U. S. A. **104**(50), 19697–19702 (2007). https://doi.org/10.1073/pnas.0701424104
110. Muhr, M., Paulat, M., Awwanah, M., Brinkkötter, M., Teichmann, T.: CRISPR/Cas9-mediated knockout of *Populus BRANCHED1* and *BRANCHED2* orthologs reveals a major function in bud outgrowth control. Tree Physiol. **38**(10), 1588–1597 (2018). https://doi.org/10.1093/treephys/tpy088
111. Fladung, M.: Targeted CRISPR/Cas9-based Knock-out of the Rice Orthologs *TILLER ANGLE CONTROL 1* (*TAC1*) in poplar induces erect leaf habit and shoot growth. Forests. **12**(12), 1615 (2021). https://doi.org/10.3390/f12121615
112. Hollender, C.A., Waite, J.M., Tabb, A., Raines, D., Chinnithambi, S., Dardick, C.: Alteration of *TAC1* expression in *Prunus* species leads to pleiotropic shoot phenotypes. Hortic Res. **5**, 26 (2018). https://doi.org/10.1038/s41438-018-0034-1
113. Shen, Y., Li, Y., Xu, D., Yang, C., Li, C., Luo, K.: Molecular cloning and characterization of a brassinosteriod biosynthesis-related gene *PtoDWF4* from *Populus tomentosa*. Tree Physiol. **38**(9), 1424–1436 (2018). https://doi.org/10.1093/treephys/tpy027
114. Chanoca, A., de Vries, L., Boerjan, W.: Lignin engineering in forest trees. Front. Plant Sci. **10**, 912 (2019). https://doi.org/10.3389/fpls.2019.00912
115. Xu, C., Fu, X., Liu, R., Guo, L., Ran, L., Li, C., Tian, Q., Jiao, B., Wang, B., Luo, K.: PtoMYB170 positively regulates lignin deposition during wood formation in poplar and confers drought tolerance in transgenic Arabidopsis. Tree Physiol. **37**(12), 1713–1726 (2017). https://doi.org/10.1093/treephys/tpx093
116. de Vries, L., Brouckaert, M., Chanoca, A., Kim, H., Regner, M.R., Timokhin, V.I., Sun, Y., de Meester, B., van Doorsselaere, J., Goeminne, G., Chiang, V.L., Wang, J.P., Ralph, J., Morreel, K., Vanholme, R., Boerjan, W.: CRISPR-Cas9 editing of *CAFFEOYL SHIKIMATE ESTERASE 1* and *2* shows their importance and partial redundancy in lignification in *Populus tremula* × *P. alba*. Plant Biotechnol. J. **19**(11), 2221–2234 (2021). https://doi.org/10.1111/pbi.13651
117. Marriott, P.E., Gómez, L.D., McQueen-Mason, S.J.: Unlocking the potential of lignocellulosic biomass through plant science. New Phytol. **209**(4), 1366–1381 (2016). https://doi.org/10.1111/nph.13684
118. Jang, H.-A., Bae, E.-K., Kim, M.-H., Park, S.-J., Choi, N.-Y., Pyo, S.-W., Lee, C., Jeong, H.-Y., Lee, H., Choi, Y.-I., Ko, J.-H.: CRISPR-knockout of *CSE* gene improves saccharification efficiency by reducing lignin content in hybrid poplar. Int. J. Mol. Sci. **22**(18) (2021). https://doi.org/10.3390/ijms22189750
119. Zhen, C., Hua, X., Jiang, X., Tong, G., Li, C., Yang, C., Cheng, Y.: Cas9/gRNA-mediated mutations in *PtrFLA40* and *PtrFLA45* reveal redundant roles in modulating wood cell size and SCW synthesis in poplar. Int. J. Mol. Sci. **24**(1) (2022). https://doi.org/10.3390/ijms24010427

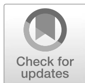
120. Seth, R.S.: Fibre quality factors in papermaking — II the importance of fibre coarseness. MRS Proc. **197** (1990). https://doi.org/10.1557/PROC-197-143
121. Fladung, M., Pasonen, H.-L., Walter, C.: Genetically modified trees and associated environmental concerns. In: El-Kassaby, Y.A., Prado, J.A. (eds.) Forests and Genetically Modified Trees, pp. 177–201. Food and Agriculture Organisation of the United Nations, Rome (2010)
122. Vettori, C., Gallardo, F., Häggman, H., Kazana, V., Migliacci, F., Pilate, G., Fladung, M. (eds.): Biosafety of Forest Transgenic Trees. Improving the Scientific Basis for Safe Tree Development and Implementation of EU Policy Directives. Forestry Sciences, vol. 82. Springer, Dordrecht (2016)
123. Aguilera, J., Nielsen, K., Sweet, J.: Risk assessment of GM trees in the EU: current regulatory framework and guidance. iForest Biogeosci. For. **6**(3), 127–131 (2013). https://doi.org/10.3832/ifor0101-006
124. Fladung, M., Nowitzki, O., Ziegenhagen, B., Kumar, S.: Vegetative and generative dispersal capacity of field released transgenic aspen trees. Trees. **17**(5), 412–416 (2003). https://doi.org/10.1007/s00468-003-0253-3
125. Hoenicka, H., Lehnhardt, D., Polak, O., Fladung, M.: Early flowering and genetic containment studies in transgenic poplar. iForest. **5**(1), 138–146 (2012). https://doi.org/10.3832/ifor0621-005
126. Walter, C., Fladung, M., Boerjan, W.: The 20-year environmental safety record of GM trees. Nat. Biotechnol. **28**(7), 656–658 (2010). https://doi.org/10.1038/nbt0710-656

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.



Chapter 21
Genome Editing for Reduction of Bitterness and for Production of Medicinal Terpenes in *Cichorium* Species



Katarina Cankar, Katrijn Van Laere, and Dirk Bosch

Abstract *Cichorium* varieties are cultivated both as leafy vegetables as well as industrial root crop for extraction of the food fibre inulin. *Cichorium* is a typical European crop and grown on a relatively small scale. However, due to its distinctive taste and health benefits and its capacity to produce multiple bioactive compounds, *Cichorium* has great potential if varieties could be optimised for these properties by breeding. In recent years it has been demonstrated in several laboratories that chicory is very amenable to genome editing. Different protocols were developed and implemented to adapt bitterness as well as to accumulate medicinal terpenes, generating potential socio-economic benefits over the entire value chain from farmers to consumers, as well as for the environment. In addition, scientific knowledge on chicory biology, particularly on the biosynthesis of secondary metabolites was significantly increased. This demonstrates how genome editing can contribute to breeding of niche crops such as *Cichorium*, which have relatively little investment leverage for extensive breeding programs.

1 *Cichorium* Species and Their Sesquiterpene Lactones

The genus *Cichorium* belongs to the Asteraceae family of plants. About 10 *Cichorium* species have been described, with *C. intybus* being the best known and being cultivated for different applications (Fig. 21.1).

C. intybus var. *foliosum* (e.g., witloof), *C. intybus* var. *latifolium* (e.g., radicchio) and the closely related *C. endivia* (e.g., endive), are well-known leafy vegetables appreciated for their characteristic bitter taste and richness in nutritionally relevant

K. Cankar (✉) · D. Bosch
Wageningen University & Research, Wageningen Plant Research, Wageningen, The Netherlands
e-mail: katarina.cankar@wur.nl

K. Van Laere
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Melle, Belgium

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_21

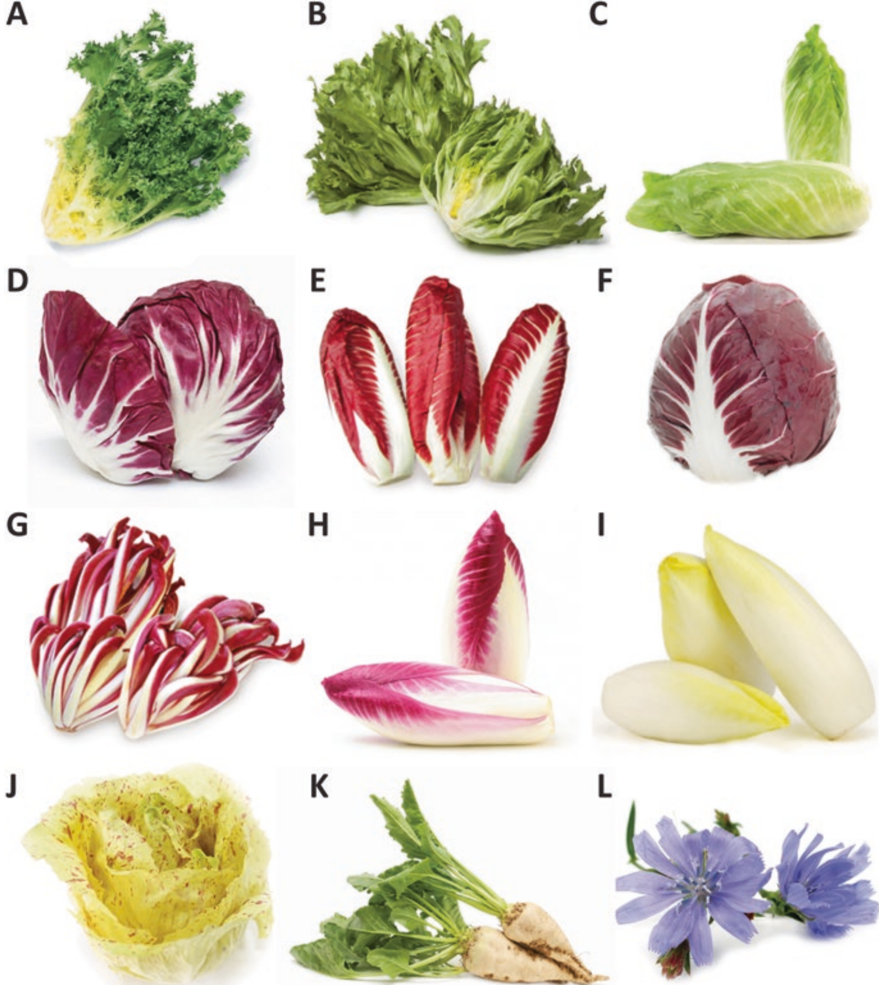


Fig. 21.1 Overview of different *Cichorium* species and varieties: (**a**) *C. intybus* var. *sylvestre* (Catalogne); (**b**) *C. endivia* (endive); (**c**) *C. intybus* var. *porphyreum* (sugerloaf); (**d**, **f**) *C. intybus* var. *latifolium* (radicchio); (**e**) *C. intybus* var. *latifolium* (radicchio early red of Treviso); (**g**) *C. intybus* var. *latifolium* (radicchio late red of Treviso); (**h**) *C. intybus* var. *foliosum* (redloof); (**i**) *C. intybus* var. *foliosum* (witloof); (**j**) *C. intybus* 'variegata di Castelfranco'; (**k**) *C. intybus* var. *sativum* (industrial chicory); (**l**) Flowers of *C. intybus*. (Pictures provided by Bram Van de Poel, KU Leuven, Belgium)

compounds with beneficial effects on human health. However, the bitter taste can have a negative influence on consumer acceptability, especially among young adults and children. Industrial chicory (*C. intybus* var. *sativum*) is grown, particularly in the south of the Netherlands, Belgium and north of France, for its large taproots from which inulin is extracted. *C. intybus* is grown on a relatively small area with a

total global acreage of around 14,500 ha [1, 2]. Inulin is a prebiotic food fibre which stimulates gut health [3] and is used as food ingredient in products such as yogurts and granola bars. Chicory is actually an ancient crop and its use as food and for its medicinal properties is documented already since Roman times [4]. Apart from inulin extraction, industrial chicory roots also have potential as raw material to make chicory flour for use in food industry as the flour will contain the nutritional benefits of inulin and does not contain gluten. But the use of this flour is still limited due to the high bitterness of the roots.

The bitter taste of the *Cichorium* crops is caused by the presence of sesquiterpene lactones (STLs) that accumulate both in leaves and roots. STLs represent a large and diverse group of terpenoids with more than 5000 different STLs being identified in Asteraceae [5]. STLs are specialized isoprenoid metabolites that contain 15 carbon atoms and which have a lactone function. The presence of a γ-lactone ring containing an α-methylene group, is a significant characteristic of STLs. They were shown to have a defence role in plants as protection against herbivorous insects and fungi [6–8].

Based on their skeleton, STLs are classified in six major groups namely germacranolides, eudesmanolides, eremophilanolides, guaianolides, pseudoguaianolides and hypocretenolides and many subtypes [8]. Leaves and especially roots of *Cichorium* species contain high concentrations of the bitter guaianolides lactucopicrin, lactucin, and 8-deoxylactucin. Eudesmanolides and germacranolides are present in smaller amounts [9]. 8-Deoxylactucin, lactucin, and lactucopicrin predominantly occur in the oxalated form [10], but also their derivatives such as 11,13-dihydro-analogues are present. Several health-related bioactivities of chicory STLs have been documented. Lactucin and lactucopicrin display analgesic and sedative effects [11] and have antimalarial activity [12] and natural extracts from chicory roots present antibacterial and antifungal properties [13]. Purified fractions from chicory roots containing a mixture of 8-deoxylactucin and dihydro-8-deoxylactucin possess promising anti-inflammatory properties [14]. Also dihydrolactucin, a lactucin derivative found in chicory, revealed anti-inflammatory potential [15]. It is not yet clear which STLs contribute the most to the bitterness perceived by consumers [5].

The biosynthesis of STLs in chicory has been partly elucidated (Fig. 21.2). The common sesquiterpene precursor farnesyl pyrophosphate (FPP) is first cyclized to germacrene A by the germacrene A synthase (CiGAS) [16]. The biosynthesis continues by oxygenation by presumably as many as six cytochrome P450 enzymes of which the genes encoding germacrene A oxidase (CiGAO), costunolide synthase (CiCOS), kauniolide synthase (CiKLS) and lactucin synthase (CiLCS) have been identified [17–23], all belonging to the CYP71 clan of cytochrome P450 enzymes. The STLs are further modified by conjugations with 4-hydroxyphenyl acetate and oxalate and by reduction of the 11,13-double bond. Enzymes involved in 4-hydroxyphenyl acetate and oxalate transfer and reduction of the double bond have not yet been identified. The basic STL structures in different chicory subspecies are identical but between varieties their relative abundance and the extent of modifications can differ. The downstream steps in the STL biosynthesis, from the tricyclic

Fig. 21.2 Biosynthetic pathway of chicory STLs. Enzymes to be inactivated for reduction of bitterness are indicated in red font and enzymes to be inactivated for accumulation of medicinal compounds are indicated by a blue arrow. Dashed arrows indicate uncharacterized enzymatic conversions. FPP – farnesyl pyrophosphate, CiGAS – germacrene A synthase, CiGAO – germacrene A oxidase, CiCOS – costunolide synthase, CiKLS – kauniolide synthase, CiLCS – lactucin synthase

kauniolide onward, appear to take place in the latex itself, as the *CiKLS* and *CiLCS* genes show latex-specific expression [17, 18].

In summary, chicory species are producing several compounds that are highly relevant for food, namely the dietary fibre inulin and the bitter tasting terpenes, with some of these terpenes displaying beneficial health properties. Generating varieties with an optimized composition of either of these compounds would therefore be highly interesting and open new markets for food industry with great economic impact. Today, for industrial chicory hot water extraction is used to extract inulin and the bitter tasting STLs are co-extracted in this process [24]. Since the presence of bitter compounds in inulin limits its use as a prebiotic dietary fibre and sweetener in food products, the STLs are removed in subsequent purification steps, increasing the costs and input for processing. Moreover, related to leafy *Cichorium* species as witloof and radicchio, product differentiation by creating a more diverse range of flavours would maximize witloof acceptance and benefit of its health promoting compounds. However, chicory species are relatively small crops with little investment leverage to enter lengthy breeding programs of about 10 years to produce a new cultivar. Although *Cichorium* species are self-incompatible, selfing at a low rate can still occur [25, 26]. Thanks to the high number of flower heads produced during a generative period, a quite high amount of selfed seeds can thus still be formed. However, creating true hybrids remains cumbersome. New breeding techniques, e.g. CRISPR/Cas genome editing, could make a difference to create chicory varieties with consumer traits in a faster and more efficient manner.

2 Genome Editing in *Cichorium* Species

Genome editing methods for *Cichorium* have first been described in 2019 [27]. Since then several studies describe the use of CRISPR/Cas-based genome editing in *Cichorium* species (Table 21.1). Protocols for four different delivery methods of CRISPR/Cas reagents have been established. High efficiency of genome editing using *Agrobacterium rhizogenes*-mediated stable transformation in hairy roots was first demonstrated for the chicory phytoene desaturase gene (*CiPDS*). This optimized protocol was later used to elucidate the function of biosynthetic enzymes involved in the synthesis of tetracoumaroyl spermine in chicory [28]. Introduction of CRISPR/Cas system via *Agrobacterium tumefaciens*-mediated stable transformation was first described targeting the *CiGAS* gene [29]. While stable transformation methods lead to high editing efficiency, thanks to the continuous editing after stable genome integration of CRISPR/Cas, they have a disadvantage for chicory breeding due to chicory self-incompatibility (although incomplete) and long time needed for outcrossing the T-DNA. Genome editing of the same gene in two compatible varieties is needed followed by segregation of transgenes after crossing. In addition, detailed genotyping of the resulting plants revealed that plants obtained by stable transformation often showed chimerism and a mixture of *CiGAS* genotypes in the same plant was observed.

In parallel, transient delivery methods for chicory protoplasts combined with whole plant regeneration were established. Transient delivery of plasmids encoding CRISPR/Cas reagents was used for editing of the *CiPDS* gene and genes involved in the biosynthesis of chicory STLs [17, 18, 21, 23, 27, 30]. Although efficient generation of biallelic mutants was described even for multi-gene families of the STL pathway, unintended integration of plasmid DNA into the chicory genome in up to 30% of plants was observed in several studies [17, 18, 27, 30]. Alternatively, delivery of preassembled ribonucleoprotein complexes (RNPs) was established for chicory protoplasts [17, 29, 30]. This DNA-free delivery method is efficient while at the same time eliminating the risk of unintended DNA integration. Therefore, it can be concluded that delivery by RNPs is the most suitable delivery method for genome editing in chicory.

When comparing the three methods, stable *Agrobacterium* transformation, transient plasmid delivery and RNP transfection, for off-target editing at six potential off-target sites in the chicory genome showing similarity to the target sequence in the *CiGAS* gene, no off-target editing was observed for either of the three delivery methods [29]. In conclusion, both stable transformation and transient transfection have successfully been used in chicory with high efficiency, also for editing multi-gene families (Table 21.1). This advance will facilitate functional analysis and genetic improvement of chicory.

Table 21.1 Studies describing use of genome editing in *Cichorium* sp.

Aim of study	Edited gene	Delivery system	Genome editing efficiency	Reference
Testing of genome editing efficiency	Phytoene desaturase (*CiPDS*)	*A. rhizogenes* - mediated stable transformation Plasmid transfection to protoplasts	31% 4.5%	[27]
Reducing bitterness	Germacrene A synthase (*CiGAS-S1, CiGAS-S2, CiGAS-S3, CiGAS-L*)	Plasmid transfection to protoplasts RNP transfection to protoplasts RNP transfection to protoplasts using modified sgRNA	14–16% 4–25% 20–40%	[30]
Reducing bitterness	Germacrene A synthase (*CiGAS-S1, CiGAS-S2)*	*A. tumefaciens* - mediated stable transformation Plasmid transfection to protoplasts RNP transfection to protoplasts	90% 51% 80%	[29]
Reducing bitterness	Germacrene A synthase (*CiGAS*) Germacrene A oxidase (*CiGAO*) Costunolide synthase (*CiCOS*)	Plasmid transfection to protoplasts	20–26%	[21]
Reducing bitterness	Germacrene A synthase (*CiGAS*), germacrene A oxidase (*CiGAO*), costunolide synthase (*CiCOS*), kauniolide synthase (*CiKLS1*, *CiKLS2*, *CiKLS3*)	Plasmid transfection to protoplasts; multiplexed gene targeting	24% (on average)	[22, 23]
Boosting of medicinal STL costunolide	Kauniolide synthase (*CiKLS1, CiKLS2, CiKLS3*)	Plasmid transfection to protoplasts RNP transfection to protoplasts	3–8% 3%	[17]
Boosting of anti-inflammatory STL 8-deoxylactucin	Lactucin synthase (*CiLCS*)	Plasmid transfection to protoplasts	21%	[18]
Elucidation of tetracoumaroyl spermine biosynthetic pathway	Spermidine hydroxycinnamoyl transferase-like proteins 1 and 2 (*CiSHT1* and *CiSHT2*)	*A. rhizogenes* - mediated stable transformation	nd[a]	[28]

[a]nd = not determined

3 Genome Editing for Reduction of Bitterness in *Cichorium* Crops

Although there is some natural variation in the STL content among *Cichorium* species [31, 32], variants with a considerable low STL content have not yet been identified. Using CRISPR/Cas targeting genes involved in the STL biosynthetic pathway would facilitate the generation of less or zero bitterness in *Cichorium*.

The first dedicated step of the pathway (Fig. 21.2) is catalysed by CiGAS enzyme which is encoded in the chicory genome in four gene copies of the terpene synthase gene family, i.e., *CiGAS-S1*, *CiGAS-S2*, *CiGAS-S3* and *CiGAS-L* [33]. Genome and transcriptome analysis identified two additional *CiGAS* paralogs belonging to the same gene clade [23]. Efficient editing of the *CiGAS* genes was achieved by several approaches (Table 21.1). The interruption of all *CiGAS-S1*, *CiGAS-S2*, *CiGAS-S3* and *CiGAS-L* alleles has been achieved and led to elimination of the STLs in chicory leaves and taproots [30]. The edited plants showed no other directly observable morphological phenotypic changes under greenhouse conditions.

To investigate if inactivation of downstream steps in the STL biosynthetic pathway could modulate bitterness, genes from the CYP71 clan of the cytochrome P450 gene family *CiGAO*, *CiCOS* and *CiKLS* were functionally characterized in *Cichorium* [17–20, 22, 23]. In conclusion, taken all these studies together, two paralogous *CiGAO* genes, three paralogous *CiCOS* genes, and three paralogous *CiKLS* genes displayed catalytic activity in the upstream STL pathway to produce costunolide and kauniolide. De Bruyn et al. [23] used CRISPR/Cas genome editing to simultaneously target multiple CYP71 genes in protoplasts of witloof, followed by regeneration of plants. A broad spectrum of plant genotypes containing different (loss of function) mutants in multiple genes were obtained. Several genotypes containing mutations in one or more paralogous CYP71 genes (*CiGAO* mutants, *CiCOS* mutants and *CiKLS* mutants) were profiled for their STL composition. The production of 14 out of 16 detectable STL metabolites [34] was eliminated in the mutant types containing a homozygous loss of function mutation in the three functional paralogous *CiKLS* genes [22, 23] as was also observed when the three *CiKLS* paralogs were edited in root chicory [17]. Other mutants only showed shifts in STL compounds concentrations, but no clear effects or decreases were detected. It is likely that the mutation effect on STL compound quantities is masked if at least one functional allele and/or paralog is still present.

Studies indicate that the perception of bitterness is not only related to the guaianolide STL metabolite quantities and composition. François et al. [35] showed a strong correlation between glucose and sucrose with crunchiness and bitterness and a correlation between fructose and sweetness. Also the balance between STL compounds and phenolic compounds is described to affect bitterness [36]. So STLs are probably not the only metabolites to consider when aiming to modify bitterness in *Cichorium*. Cellular assays and human sensory tests should be used to link bitterness to the STL concentrations in CRISPR/Cas mutants [37].

4 Genome Editing to Produce Medicinal Terpenes in Chicory

The use of industrial chicory for extraction of inulin fibre is well established, with the chicory STLs being discarded during the purification of inulin due to their bitterness. These terpenes could however be obtained from the chicory root as a secondary product via a procedure compatible with inulin processing, yielding both inulin and terpenes which can be used for e.g., health applications. Depending on the homogeneity and quality of the terpene fraction and on the application, this fraction might need further processing. Recently, a sustainable method of terpene extraction from chicory taproots using supercritical CO_2 was established [14]. The socio-economic impacts of a multi-product process compared to a reference inulin process indicate that a multi-product process leads to higher added value and increased socio-economic impact compared to the reference inulin process [24]. Therefore, it is interesting to increase not only the inulin content of the roots by breeding, but also the terpene amounts. Genome editing can be used to facilitate the generation of chicory varieties that accumulate higher amounts of medicinal terpenes.

A first example is a chicory variety with an increased accumulation of costunolide [17], which is a natural STL from the Asteraceae family and exhibits anticancer activity through its functional moiety α-methylene-γ-butyrolactone that reacts with the cysteine sulfhydryl group of various proteins [38]. In chicory, costunolide is an intermediate in the biosynthesis of STLs and does not accumulate since it is efficiently converted to downstream STLs. To accumulate costunolide the kauniolide synthase (CiKLS) that converts costunolide to kauniolide, needs to be inactivated. Genome editing of the three *CiKLS* paralogs in chicory was performed by transient transfection of both plasmids and RNPs to chicory protoplasts, both leading to small indel mutations but only the former sometimes to unintended DNA integration. As a result the biosynthesis of the chicory sesquiterpenes was interrupted and costunolide accumulation was observed [17]. Interestingly, next to free costunolide predominantly costunolide conjugates were found to be accumulating, presumably mitigating the toxicity of costunolide to chicory cells.

A second example of chicory varieties with increased medicinal compounds are chicory lines with increased amounts of anti-inflammatory 8-deoxylactucin and its derivatives [18]. 8-deoxylactucin is a natural STL of chicory that accumulates in chicory taproots next to lactucin and lactucopicrin, predominantly in oxalated form [10]. To increase 8-deoxylactucin accumulation in the taproots the gene encoding the lactucin synthase (*CiLCS*) that hydroxylates 8-deoxylactucin on position 8 to generate lactucin needs to be inactivated. Genome editing was used to inactivate three candidate cytochrome P450 genes with putative LCS activity resulting in the identification of one of the genes as *CiLCS* and generation of chicory lines that predominantly accumulate 8-deoxylactucin and its derivatives [18].

These two examples show that genome editing can be successfully applied in chicory to inactivate single genes or gene families, elucidating enzyme function and

resulting in novel terpene chemotypes. In both examples the target medicinal STLs in genome edited roots accumulated to the similar high amounts of total STLs as found in wild type roots, namely about 1.5 mg/g FW. The availability of the chicory lines with modified terpene composition can also be used to generate more knowledge on the natural role of terpenes in chicory development and defence.

5 Potential of Genome Editing for Chicory Breeding

Chicory is very amenable to genome editing. Highly efficient protocols have recently been developed in different laboratories. Using these protocols scientific knowledge on chicory biology, particularly of the biosynthesis of secondary metabolites was increased. Implementation of these protocols resulted in generation of multiple chicory lines with altered terpene profiles. These lines could have potential benefits for consumers (healthy terpenes, varieties with altered taste), food producers (lack of bitter compounds simplifies inulin extraction or alternatively, bitter terpenes currently discarded as waste can be used as secondary product), farmers (agricultural diversification), breeders (creation of new cultivars with added value), the environment (reduction in energy demand and greenhouse gas emissions due to avoiding bitter compound extraction), and the economy (higher added value and more jobs).

Using the now available genome editing protocols as breeding tool, also other important biological processes for *Cichorium* breeding, e.g., male sterility, haploid induction, inulin quality and yield, etc., can be investigated and used to facilitate the long breeding process and generate novel cultivars in relatively shorter time frame with commercial interesting traits related to e.g., root shape, root weight, inulin chain length, disease resistance, bolting resistance, etc. In a broader perspective, genome editing can be a powerful tool to help to stimulate agricultural biodiversity in Europe by improving niche crops, like chicory, which have relatively little investment leverage. In synergism with other breeding and farming methods, this is highly relevant for securing crop diversity and improving sustainable production while at the same time dealing with the challenges like that of climate change.

Acknowledgement This work has received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 760891 [H2020-NMBP-BIOTEC-07-2017: New Plant Breeding Techniques (NPBT) in molecular farming: Multipurpose crops for industrial bioproducts]. The authors want to thank all researchers and technicians that contributed to this work.

References

1. Wilson, R.G., Smith, J.A., Yonts, C.D.: Chicory root yield and carbohydrate composition is influenced by cultivar selection, planting, and harvest date. Crop Sci. **44**(3), 748–752 (2004)

2. FAOstat Chicory root production area and yield in 2017 in *Food and Agriculture Organization of the United Nations database* Downloaded on 1st April 2022. Available at: https://www.fao.org/faostat/en/#data/QCL. (2017)
3. Roberfroid, M.B.: Inulin-type fructans: functional food ingredients. J. Nutr. **137**(11 Suppl), 2493S–2502S (2007)
4. Puhlmann, M.L., de Vos, W.M.: Back to the roots: revisiting the use of the fiber-rich *Cichorium intybus L.* taproots. Adv. Nutr. **12**(4), 1598–1598 (2021)
5. Chadwick, M., Trewin, H., Gawthrop, F., Wagstaff, C.: Sesquiterpenoids lactones: benefits to plants and people. Int. J. Mol. Sci. **14**(6), 12780–12805 (2013)
6. Prasifka, J.R., Spring, O., Conrad, J., Cook, L.W., Palmquist, D.E., Foley, M.E.: Sesquiterpene lactone composition of wild and cultivated sunflowers and biological activity against an insect pest. J. Agric. Food Chem. **63**(16), 4042–4049 (2015)
7. Huber, M., Epping, J., Schulze Gronover, C., Fricke, J., Aziz, Z., Brillatz, T., Swyers, M., Kollner, T.G., Vogel, H., Hammerbacher, A., Triebwasser-Freese, D., Robert, C.A., Verhoeven, K., Preite, V., Gershenzon, J., Erb, M.: A latex metabolite benefits plant fitness under root herbivore attack. PLoS Biol. **14**(1), e1002332 (2016)
8. Padilla-Gonzalez, G.F., dos Santos, F.A., Da Costa, F.B.: Sesquiterpene lactones: more than protective plant compounds with high toxicity. Crit. Rev. Plant Sci. **35**(1), 18–37 (2016)
9. de Kraker, J.W., Franssen, M.C., de Groot, A., Konig, W.A., Bouwmeester, H.J.: (+)-Germacrene A biosynthesis. The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. Plant Physiol. **117**(4), 1381–1392 (1998)
10. Sessa, R.A., Bennett, M.H., Lewis, M.J., Mansfield, J.W., Beale, M.H.: Metabolite profiling of sesquiterpene lactones from *Lactuca* species. Major latex components are novel oxalate and sulfate conjugates of lactucin and its derivatives. J. Biol. Chem. **275**(35), 26877–26884 (2000)
11. Wesolowska, A., Nikiforuk, A., Michalska, K., Kisiel, W., Chojnacka-Wojcik, E.: Analgesic and sedative activities of lactucin and some lactucin-like guaianolides in mice. J. Ethnopharmacol. **107**(2), 254–258 (2006)
12. Bischoff, T.A., Kelley, C.J., Karchesy, Y., Laurantos, M., Nguyen-Dinh, P., Arefi, A.G.: Antimalarial activity of lactucin and lactucopicrin: sesquiterpene lactones isolated from *Cichorium intybus L.* J. Ethnopharmacol. **95**(2–3), 455–457 (2004)
13. Hakkinen, S.T., Sokovic, M., Nohynek, L., Ciric, A., Ivanov, M., Stojkovic, D., Tsitko, I., Matos, M., Baixinho, J.P., Ivasiv, V., Fernandez, N., Nunes Dos Santos, C., Oksman-Caldentey, K.M.: Chicory extracts and sesquiterpene lactones show potent activity against bacterial and fungal pathogens. Pharmaceuticals (Basel). **14**(9), 941 (2021)
14. Baixinho, J.P., Anastacio, J.D., Ivasiv, V., Cankar, K., Bosch, D., Menezes, R., de Roode, M., Santos, C.N.D., Matias, A.A., Fernandez, N.: Supercritical CO_2 extraction as a tool to isolate anti-inflammatory sesquiterpene lactones from *Cichorium intybus L.* roots. Molecules. **26**(9), 2583 (2021)
15. Matos, M.S., Anastacio, J.D., Allwood, J.W., Carregosa, D., Marques, D., Sungurtas, J., McDougall, G.J., Menezes, R., Matias, A.A., Stewart, D., Santos, C.N.D.: Assessing the intestinal permeability and anti-inflammatory potential of sesquiterpene lactones from chicory. Nutrients. **12**(11), 3547 (2020)
16. Bouwmeester, H.J., Kodde, J., Verstappen, F.W., Altug, I.G., de Kraker, J.W., Wallaart, T.E.: Isolation and characterization of two germacrene A synthase cDNA clones from chicory. Plant Physiol. **129**(1), 134–144 (2002)
17. Cankar, K., Hakkert, J.C., Sevenier, R., Campo, E., Schipper, B., Papastolopoulou, C., Vahabi, K., Tissier, A., Bundock, P., Bosch, D.: CRISPR/Cas9 targeted inactivation of the kauniolide synthase in chicory results in accumulation of costunolide and its conjugates in taproots. Front. Plant Sci. **13**, 940003 (2022)
18. Cankar, K., Hakkert, J.C., Sevenier, R., Papastolopoulou, C., Schipper, B., Baixinho, J.P., Fernandez, N., Matos, M.S., Serra, A.T., Santos, C.N., Vahabi, K., Tissier, A., Bundock, P., Bosch, D.: Lactucin synthase inactivation boosts the accumulation of anti-inflammatory

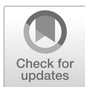
8-deoxylactucin and its derivatives in chicory (*Cichorium intybus L.*). J. Agric. Food Chem. **71**(15), 6061–6072 (2023)
19. Liu, Q., Majdi, M., Cankar, K., Goedbloed, M., Charnikhova, T., Verstappen, F.W., de Vos, R.C., Beekwilder, J., van der Krol, S., Bouwmeester, H.J.: Reconstitution of the costunolide biosynthetic pathway in yeast and *Nicotiana benthamiana*. PLoS One. **6**(8), e23255 (2011)
20. Nguyen, D.T., Gopfert, J.C., Ikezawa, N., Macnevin, G., Kathiresan, M., Conrad, J., Spring, O., Ro, D.K.: Biochemical conservation and evolution of germacrene A oxidase in Asteraceae. J. Biol. Chem. **285**(22), 16588–16598 (2010)
21. De Bruyn, C., Ruttink, T., Eeckhaut, T., Jacobs, T., De Keyser, E., Goosens, A., Van Laere, K.: Establishment of CRISPR/Cas9 genome editing in witloof (*Cichorium intybus* var. *foliosum*). Front. Genome Ed. **2**, 604876 (2020)
22. De Bruyn, C.: Unravelling of the sesquiterpene lactone biosynthetic pathway in *Cichorium Intybus* using CRISPR/Cas9 genome editing. Ghent University, Faculty of Sciences, Belgium (2022)
23. De Bruyn, C., Ruttink, T., Lacchini, E., Rombouts, S., Haegeman, A., De Keyser, E., Van Poucke, C., Desmet, S., Jacobs, T.B., Eeckhaut, T., Goosens, A., Van Laere, K.: Identification and characterization of CYP71 subclade cytochrome P450 enzymes involved in the biosynthesis of bitterness compounds in *Cichorium intybus*. Front. Plant Sci. **14**, 1200253 (2023)
24. Hingsamer, M., Kulmer, V., de Roode, M., Kernitzkyi, M.: Environmental and socio-economic impacts of new plant breeding technologies: a case study of root chicory for inulin production. Front. Genome Ed. **4**, 919392 (2022)
25. de Nettancourt, D.: The genetics of self-incompatibility. In: Incompatibility and Incongruity in Wild and Cultivated Plants. Springer, Berlin, Heidelberg (2001)
26. Lucchin, M., Varotto, S., Barcaccia, G.: Vegetables I: Asteraceae, brassicaceae, chenopodicaceae and cucurbitaceae. In: Handbook of Plant Breeding. Springer, New York (2008)
27. Bernard, G., Gagneul, D., Alves Dos Santos, H., Etienne, A., Hilbert, J.L., Rambaud, C.: Efficient genome editing using CRISPR/Cas9 technology in chicory. Int. J. Mol. Sci. **20**(5), 1155 (2019)
28. Bernard, G., Buges, J., Delporte, M., Molinie, R., Besseau, S., Bouchereau, A., Watrin, A., Fontaine, J.X., Mathiron, D., Berardocco, S., Bassard, S., Quero, A., Hilbert, J.L., Rambaud, C., Gagneul, D.: Consecutive action of two BAHD acyltransferases promotes tetracoumaroyl spermine accumulation in chicory. Plant Physiol. **189**(4), 2029–2043 (2022)
29. Salvagnin, U., Unkel, K., Sprink, T., Bundock, P., Sevenier, R., Bogdanovic, M., Todorovic, S., Cankar, K., Hakkert, J.C., Schijlen, E., Nieuwenhuis, R., Hingsamer, M., Kulmer, V., Kernitzkyi, M., Bosch, D., Martens, S., Malnoy, M.: A comparison of three different delivery methods for achieving CRISPR/Cas9 mediated genome editing in *Cichorium intybus L.* Front. Plant Sci. **14**, 1111110 (2023)
30. Cankar, K., Bundock, P., Sevenier, R., Hakkinen, S.T., Hakkert, J.C., Beekwilder, J., van der Meer, I.M., de Both, M., Bosch, D.: Inactivation of the germacrene A synthase genes by CRISPR/Cas9 eliminates the biosynthesis of sesquiterpene lactones in *Cichorium intybus L.* Plant Biotechnol. J. **19**(12), 2442–2453 (2021)
31. Foster, J.G., Cassida, K.A., Sanderson, M.A.: Seasonal variation in sesquiterpene lactone concentration and composition of forage chicory (*Cichorium intybus L.*) cultivars. Grass Forage Sci. **66**(3), 424–433 (2011)
32. Testone, G., Mele, G., Di Giacomo, E., Gonnella, M., Renna, M., Tenore, G.C., Nicolodi, C., Frugis, G., Iannelli, M.A., Amesi, G., Schiappa, A., Giannino, D.: Insights into the sesquiterpenoid pathway by metabolic profiling and de novo transcriptome assembly of stem-chicory (*Cichorium intybus* cultigroup "Catalogna"). Front. Plant Sci. **7**, 1676 (2016)
33. Bogdanović, M., Cankar, K., Todorović, S., Dragicević, M., Simonović, A., van Houwelingen, A., Schijlen, E., Schipper, B., Gagneul, D., Hendriks, T., Quillet, M., Bouwmeester, H., Bosch, D., Beekwilder, J.: Tissue specific expression and genomic organization of bitter sesquiterpene lactone biosynthesis in *Cichorium intybus L.* (Asteraceae). Ind. Crop. Prod. **129**, 253–260 (2019)

34. Kips, L.: Characterization and processing of horticultural byproducts: a case study of tomato and Belgian endive roots. Ghent University, Faculty of Bioscience Engineering, Belgium (2017)
35. Francois, I.M., Wins, H., Buysens, S., Godts, C., Van Pee, E., Nicolai, B., De Proft, M.: Predicting sensory attributes of different chicory hybrids using physico-chemical measurements and visible/near infrared spectroscopy. Postharvest Biol. Technol. **49**(3), 366–373 (2008)
36. D'Antuono, L.F., Ferioli, F., Manco, M.A.: The impact of sesquiterpene lactones and phenolics on sensory attributes: an investigation of a curly endive and escarole germplasm collection. Food Chem. **199**, 238–245 (2016)
37. Yanagisawa, T., Misaka, T.: Characterization of the human bitter taste receptor response to sesquiterpene lactones from edible Asteraceae species and suppression of bitterness through pH control. Acs Omega. **6**(6), 4401–4407 (2021)
38. Li, Q., Wang, Z., Xie, Y., Hu, H.: Antitumor activity and mechanism of costunolide and dehydrocostus lactone: two natural sesquiterpene lactones from the Asteraceae family. Biomed. Pharmacother. **125**, 109955 (2020)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 22
Engineering Phytonutrient Content in Tomato by Genome Editing Technologies



Aurelia Scarano and Angelo Santino

Abstract The application of precise genome editing represents an important step-forward in plant functional genomics research and crop improvement by generating tailored modifications within a target genome sequence. Among the genome editing technologies, the CRISPR/Cas9 system has been the most largely one applied in many crop species, thanks to its high customizable specificity. Tomato is one of the most cultivated and consumed horticultural crops worldwide and an ideal model plant for studying different physiological processes (e.g., plant development, response to biotic/abiotic stresses, fruit quality) by using different approaches, such as conventional breeding, classical transgenesis and genome editing technologies. In recent years, the number of studies on the genome editing application in tomato has increasingly grown, particularly for the improvement of fruit quality and nutritional value. In this chapter, we report about the main achievements provided by such technologies for engineering the content of nutritionally relevant compounds, such as polyphenols, carotenoids, vitamins, and other important phytonutrients in tomato fruit.

1 Introduction

Targeted genome engineering has emerged as an alternative to classical plant breeding and transgenic (GMO) methods to apply in both model and crop plants [1]. Precise genome editing provides great advantages in plant functional genomics research and crop improvement by generating tailored modifications at a target genome sequence [2]. Different genome-editing tools have been developed in the past decade, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced palindromic

A. Scarano (✉) · A. Santino (✉)
Institute of Science of Food Production, C.N.R. Unit of Lecce, Lecce, Italy
e-mail: aurelia.scarano@ispa.cnr.it; angelo.santino@ispa.cnr.it

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_22

repeat/CRISPR-associated protein 9 system (CRISPR/Cas9). Zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) can be used to mutagenize genomes at specific loci, and such systems require two different DNA binding proteins flanking a sequence of interest, each with a C-terminal FokI nuclease module. However, in plant research community CRISPR/Cas9 system has been the most largely preferred, thanks to its ability to reprogram the endonuclease specificity using customizable small noncoding RNAs, named single guide RNAs (sgRNAs), specific for a given targeted nucleic acid sequence [3, 4].

ZFNs, TALENs and CRISPR/Cas9 systems are able to induce site-specific double-strand breaks (DSBs) within the genome, generating modifications through homologous recombination (HR) or non-homologous end-joining (NHEJ) repair mechanisms [1]. HR can accurately repair DSBs using the homologous sequence flanking a DSB or an exogenously supplied DNA 'donor template' as the template. However, NHEJ repair is error-prone and frequently causes insertions or deletions (indels) around the sites of DNA breaks. In eukaryotic cells, and particularly in plant cells, DSBs are preferentially repaired by NHEJ mechanism, thus becoming the most used pathway to knockout genes in plants (e.g., via introducing frameshift mutation or a deletion) [5]. As preferential application, the gene knockouts can be implemented by using multiple sgRNAs easily assembled into a single delivery vector (for example using Golden Gate cloning, GoldenBraid, BioBrick technology) [6–8], thus allowing the knockout of all the members within a specific gene family. The specificity of CRISPR/Cas9 is guaranteed by a perfect match between the last 8–12 bases of the sgRNAs sequence, referred as the 'seed sequence' and the corresponding region of the target DNA (i.e., the region proximal to the 50 end of the PAM). Such specificity is particularly important for the cleavage efficiency target of Cas9 [4, 9, 10,] and to possibly limit potential off-target activities. Furthermore, the efficiency of CRISPR/Cas9 may be influenced by the sequence, location, context of the target (e.g., epigenetic factors, such as DNA methylation or histone modification, that might limit the DNA binding), beside the transformation efficiency. Unfortunately, not many crop plants are easily transformable, although new delivery systems (for instance, DNA-free in vitro systems consisting in the direct delivery of a ribonucleoprotein complex, formed by the Cas9 protein and the guide RNA) have been recently proposed [11, 12].

2 CRISPR-Cas9 System for the Nutritional Improvement in Tomato

Tomato is one of the most important commercial horticulture crops cultivated and consumed worldwide, and an ideal model plant for studying plant stress responses, plant reproductive development, functional genomics, and quality improvement [2]. Tomato fruit is a good source of lycopene, β-carotene, phenolic compounds, and micronutrients [13]. Most of these phytonutrients play an important role in human

nutrition and significant health benefits associated with their antioxidant activities, beside other potential ones, such as anticancer and anti-inflammatory activities [14]. Therefore, different metabolic pathways and several genes encoding important enzymatic activities or regulatory factors can be designated as genome editing targets, with the final aim of quality and nutritional improvement of tomato fruits.

Together with the nutritional improvement, tomato is also a relevant challenge for breeders, as fruit yield or (a)biotic stress tolerance are controlled by several genes acting independently or in concert [15]. In the past decades, many different approaches have been used for improvement of tomato fruit quality and nutritional value, including conventional breeding and genetic modifications [14]. Such approaches, together with the identification of genes and alleles and their rapid integration in elite cultivars, have been often laborious, time-consuming, and expensive, as the desired mutations or insertion of new allelic versions in the lines require several crossings along the generations [16]. The recent development of gene editing technologies using programmable nucleases appears therefore to be a promising and efficient approach for both applicative and basic research involving forward and reverse genetics in tomato [15]. Some examples of genome editing have been successfully applied in tomato, by using TALENs [17], CRISPR/Cas9 systems [18–20], CRISPR/Cpf1 [21], Cas9 variants like dCas9 [22], or base editing (Target-AID technology) [15].

3 Genome Editing for the Improvement of Carotenoid Content in Tomato Fruits

Several genes involved in carotenoid accumulation have been identified in tomato during the last two decades and used as target genes for genome editing applications. Improved lycopene content has been achieved inhibiting the conversion from lycopene to β- and α-carotene, by knocking down different genes associated with the carotenoid metabolic pathway (*SGR1*, *LCY-E*, *Blc*, *LCY-B1* and *LCY-B2* genes: *stay-green 1*, *lycopene δ-cyclase*, *β-lycopene cyclase*, *lycopene β-cyclase 1* and *2*, respectively) [18]. The tomato fruits from *SlSGR1* knockout null line obtained via CRISPR/Cas9 have showed a turbid brown color with significantly higher chlorophyll and carotenoid levels than in wild-type fruits. The strong changes in pigment and carotenoid content resulted in the accumulation of key primary metabolites, such as sucrose and its derivatives, and tricarboxylic acid cycle intermediates, such as malate and fumarate [23].

In another work, a series of tomato genotypes with different fruit colors, including yellow, brown, pink, light-yellow, pink-brown, yellow-green, and light green, has been generated by CRISPR/Cas9-mediated multiplex gene editing of three fruit color-related genes, two of them implicated in the lycopene biosynthesis in tomato (*PSY1* and *SGR1: phytoene synthase1* and *stay-green1*, respectively) [24].

The application of the CRISPR/Cas9 system further contributed to show the contribution of *PSY1* and *CRTR-B2* genes in fruit and flower colors changes in tomato fruit due to the carotenoid pigmentation. In fact, by targeting these genes, the obtained edited plants showed a loss-of function yellow-like phenotype caused by the suppression of lycopene biosynthesis and accumulation [25]. The CRISPR/Cas9 system has been also used to test the genome-editing efficiency by targeting the tomato *phytoene desaturase* gene (*slyPDS*), allowing to observe a severe albino/photobleached phenotype in most of transgenic tomato seedlings [18].

A Target activation-induced cytidine deaminase (Target-AID) base-editing technology has also been used to target genes related to carotenoid accumulation, such as tomato *DNA Damage UV Binding protein 1* (*SlDDB1*), *deetiolated1* (*SlDET1*), and *Lycopene beta cyclase* (*SlCYC-B*) [15]. This approach resulted in the generation of several lines showing substitutions in each target gene, resulting in lycopene, β-carotene, lutein and neoxanthin increased content. Multiplexed targeted plants in *SlDDB1*, *SlDET1* and *SlCYC-B* genes resulted in generation of several lines with improved lycopene accumulation via stacking of mutations [26].

4 Genome Editing for the Improvement of Polyphenol Content in Tomato Fruits

Different genome editing strategies have been applied in tomato targeting the polyphenol biosynthetic pathway at different levels and with different scopes. Some studies were aimed to better understand the regulatory genes encoding transcription factors modulating the polyphenol biosynthesis, in other cases the application of genome editing consisted in the improvement of such compounds associated with the enhancement of fruit quality and their benefits for human health. Zhi et al. [27] have investigated the anthocyanin 2 (SlAN2) transcription factor function conducing a CRISPR/Cas9- mediated targeted mutagenesis in the purple tomato cultivar "Indigo Rose". Although the fruit and the anthocyanin content were comparable with cv "Indigo Rose", the *SlAN2* tomato mutants resulted in lowered gene expression related to anthocyanins biosynthesis in the vegetative tissues. By using the genome editing approach, this study therefore provides a proof-of-concept that *SlAN2* regulates the anthocyanin biosynthesis in tomato vegetative tissues and that anthocyanin biosynthesis differs in terms of regulatory mechanisms between cotyledons and hypocotyls [27]. In another study, the CRISPR/Cas9 system was applied to generate pink-fruited tomato plants by disrupting the *SlMYB12* gene in different inbred lines [19]. Different homozygous, heterozygous, biallelic and chimeric mutants carrying the deletion in *SlMYB12* were obtained among the T0 regeneration plants and carrying different types of mutations, including an expected large deletion in the target site. T1 progeny obtained by self-pollination of three large-deletion mutants exhibited pink fruits and *y*-like colorless peels, with naringenin chalcone and transcript levels of two key biosynthetic enzymes (CHS1 and CHS2)

significantly reduced. Changes in the fruit color and naringenin chalcone levels have been also observed following a CRISPR/Cas9-mediated multiplex gene editing involving *SlMYB12* gene together with other genes implicated in carotenoid biosynthesis (*PSY* and *SGR1*). Among the different colored fruits obtained with this strategy, the tomato *myb12* single mutants exhibited the expected pink color, whereas the *psy myb12* double mutant displayed a light-yellow fruit color, *myb12 sgr1* double mutants a pink-brown color and the *psy myb12 sgr1* triple mutant a light green color, suggesting a key role for *SlPSY* and *SlSGR1* in the carotenoid metabolism and *SlMYB12* gene in polyphenol metabolism [24]. Different genome editing approaches have been applied to improve the polyphenol content in tomato, with a particular focus on anthocyanin biosynthesis. Gene targeting genes involving anthocyanins biosynthesis has implied a double advantage, represented by either a technical visual screen to facilitate the selection of targeting events, or the nutritional improvement of tomato fruits as anthocyanins carry benefits for human health. In their study, Čermák and co-workers [17] used TALENs and CRISPR/Cas9 systems to target *SlANT1* gene and introduce the 35S promoter by homologous recombination (HR) for inducing gene over-expression. In order to deliver sufficient donor template, geminivirus-based DNA replicons were generated to achieve high frequency, targeted modification in tomato plants. The obtained mutants showed dark purple coloration in flowers, fruit and foliage resulting from the expected targeted promoter insertion and from the boosted anthocyanin biosynthesis [17]. By using a multi-replicon system equipped with CRISPR/LbCpf1, Van Vu and collaborators [21] also applied a HR-base genome editing of tomato *ANT1* gene, obtaining purple plants and fruits enriched in anthocyanins. In another study, the tomato *DFR* gene was chosen for a targeted transgene insertion using CRISPR/Cas9 in a two-step strategy. The first step consisted of a targeted deletion within the endogenous *DFR* gene, resulting in green plantlets (instead of the usual purple color), and the second step consisting of an HR-mediated insertion in the *dfr* deletion to restore the original gene function. Following such insertion, the regenerating plantlets recovered a purple color phenotype, indicating that *DFR* function was regained [28].

5 Genome Editing for the Improvement in Vitamins and Other Phytonutrients in Tomato Fruits

Highly efficient CRISPR/Cas9 systems have been also employed for metabolic engineering of other important micronutrients in tomato. One example is represented by the *7-dehydrocholesterol reductase* (*7DR2)* gene targeting that allowed the increase the level of provitamin D3 in tomato. Leaves and fruits – especially the green ones – from the homozygous mutant lines showed increased levels of 7-dehydrocholesterol (7DHC), and reduced levels of α-tomatine. 7-DHC content was lower in ripe fruits but high enough compared to wild-type tomato fruits, and sufficient to be equivalent to that in two medium-sized eggs, if converted to vitamin

D3 by UVB treatment [20]. Genome editing can therefore support food nutritional security and help to develop novel foods enriched in vitamins to be easily consumed and directly address vitamin deficiency in susceptible individuals, as in this case of tomato fruits representing a good vitamin D source.

Another example in which genome editing can implement food nutritional quality is represented by the enhancement of soluble sugar content in tomato by targeting the *invertase inhibitor* and *vacuole processing enzyme* (*SlINVINH1* and *SlVPE5*) genes. By knocking down these two genes implicated in inhibition of soluble sugar accumulation, the glucose, fructose and total soluble solid contents were improved compared to wild-type tomato fruits, with a synergistic effect when the two genes were simultaneously knock-out and providing a key strategy to improve the sweetness in tomato fruits and related byproducts [29].

Finally, another important example of genome editing for phytonutrient improvement is the CRISPR/Cas9 application to increase GABA content in tomato fruits. By targeting five genes (*GABA-TP1, GABA-TP2, GABA-TP3, CAT9, SSADH: gamma-aminobutyrate transaminase subunit precursor isozyme 1, 2* and *3*, *cationic amino acid transporter 9*, *succinate semialdehyde dehydrogenase*, respectively), several editing events were obtained in different tomato mutants, displaying significant increases in GABA accumulation, especially in the leaves, and different glutamate contents significantly variable among the GABA mutants [30]. However, since GABA represent a signal molecule involved in various physiological processes, the GABA mutants presented vegetative growth and flower/fruit setting severely affected [30]. Similar results, including suppression of plant growth, flowering and fruit yield, were obtained by CRISPR/Cas9-mediated gene targeting of autoinhibitory domains in *SlGAS2* and *SlGAD3* genes [31]. By introducing a stop codon before such autoinhibitory domains, the GABA accumulation was dramatically increased in tomato fruits, with variable effects on the mutated plants [31].

6 Conclusions and Future Perspectives

The use of genome editing technologies offers an effective strategy to ameliorate agronomic and quality fruit traits bypassing the conventional breeding programs and transgenic approaches causing some ethical problems in the public acceptance [32]. Transgene-free genome editing provide a promising alternative to rapidly innovate germplasm and possibly introduce new traits for agrobiodiversity. The above presented studies in the tomato model represent examples of how different genome editing technologies can be easily applied for the improvement of carotenoids, polyphenols, and other important phytonutrients with valuable benefits for human health. Even though many progresses have been achieved in comparison to other crops (for example those recalcitrant to plant transformation), more efforts are still required to efficiently target other genes involved in tomato fruit quality and nutritional value, and to overcome the barrier of gene activation/overexpression, system deliveries, and high-throughput screening of the novel mutant lines.

References

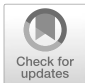
1. Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Nekrasov, V.: Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods. **9**, 39 (2013). https://doi.org/10.1186/1746-4811-9-39
2. Pan, C., Ye, L., Qin, L., Liu, X., He, Y., Wang, J., Chen, L., Lu, G.: CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. Sci. Rep. **6**, 24765 (2016). https://doi.org/10.1038/srep24765
3. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E.: A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. **337**, 816–821 (2012). https://doi.org/10.1126/science.1225829
4. Jiang, W., Bikard, D., Cox, D., Zhang, F., Marraffini, L.A.: RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat. Biotechnol. **31**, 233–239 (2013). https://doi.org/10.1038/nbt.2508
5. Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N.J., Nekrasov, V.: Editing plant genomes with CRISPR/Cas9. Curr. Opinion Plant Biotechnol. **32**, 76–84 (2015). https://doi.org/10.1016/j.copbio.2014.11.007
6. Engler, C., Youles, M., Gruetzner, R., Ehnert, T.-M., Werner, S., Jones, J.D.G., Patron, N.J., Marillonet, S.: A golden gate modular cloning toolbox for plants. ACS Synth. Biol. **3**(11), 839–843 (2014). https://doi.org/10.1021/sb4001504
7. Vazquez-Vilar, M., Bernabé-Orts, J.M., Fernandez-del-Carmen, A., Ziarsolo, P., Blanca, J., Granell, A., Orzaez, D.: A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard. Plant Methods. **12**, 10 (2016). https://doi.org/10.1186/s13007-016-0101-2
8. Hu, N., Xian, Z., Li, N., Liu, Y., Huang, W., Yan, F., Su, D., Chen, J., Li, Z.: Rapid and user-friendly open-source CRISPR/Cas9 system for single- or multi-site editing of tomato genome. Hort. Res. **6**, 7 (2019). https://doi.org/10.1038/s41438-018-0082-6
9. Brooks, C., Nekrasov, V., Lippman, Z., Van Eck, J.: Efficient gene editing in tomato in the first generation using the CRISPR/ Cas9 system. Plant Physiol. **166**, 1292–1297 (2014). https://doi.org/10.1104/pp.114.247577
10. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., et al.: Multiplex genome engineering using CRISPR/Cas systems. Science. **339**, 819–823 (2013). https://doi.org/10.1126/science.1231143
11. Malnoy, M., Viola, R., Jung, M.H., Koo, O.-J., Kim, S., Kim, J.-S., Velasco, R., Kanchiswamy, C.K.: DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci. **7**, 1904 (2016). https://doi.org/10.3389/fpls.2016.01904
12. Chen, K., Wang, Y., Zhang, R., Zhang, H., Gao, C.: CRISPR/Cas9 genome editing and precision plant breeding in agriculture. Ann. Rev. Plant Biol. **70**, 667–697 (2019). https://doi.org/10.1146/annurev-arplant-050718-100049
13. Willcox, J.K., Catignani, G.L., Lazarus, S.: Tomatoes and cardiovascular health. Crit. Rev. Food Sci. Nutr. **43**, 1–18 (2023). https://doi.org/10.1080/10408690390826437
14. Scarano, A., Chieppa, M., Santino, A.: Plant polyphenols-biofortified foods as a novel tool for the prevention of human gut diseases. Antioxidants. **9**, 1225 (2020). https://doi.org/10.3390/antiox9121225
15. Hunziker, J., Nishida, K., Kondo, A., Kishimoto, S., Ariizumi, T., Ezura, H.: Multiple gene substitution by target-AID base-editing technology in tomato. Sci. Rep. **10**, 20471 (2020). https://doi.org/10.1038/s41598-020-77379-2
16. Carvalho, R.F., Campos, M.L., Pino, L.E., Crestana, S.L., Zsögön, A., Lima, J.E., Benedito, V.A., Peres, L.E.: Convergence of developmental mutants into a single tomato model system: 'Micro-Tom' as an effective toolkit for plant development research. Plant Methods. **7**, 18 (2011). https://doi.org/10.1186/1746-4811-7-18

17. Čermák, T., Baltes, N.J., Cegan, R., Zhang, Y., Voytas, D.F.: High-frequency, precise modification of the tomato genome. Genome Biol. **16**, 232 (2015). https://doi.org/10.1186/s13059-015-0796-9
18. Li, X., Wang, Y., Chen, S., Tian, H., Fu, D., Zhu, B., Luo, Y., Zhu, H.: Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. Front. Plant Sci. **9**, 559 (2018). https://doi.org/10.3389/fpls.2018.00559
19. Deng, L., Wang, H., Sun, C., Li, Q., Jiang, H., Minmin, D., Li, C.-B., Li, C.: Efficient generation of pink-fruited tomatoes using CRISPR/Cas9 system. J. Gen. Genom. **45**, 51 (2017). https://doi.org/10.1016/j.jgg.2017.10.00
20. Li, J., Scarano, A., Mora Gonzalez, N., D'Orso, F., Yue, Y., Nemeth, K., Saalbach, G., de Oliveira Martins, C., Moran, R., Santino, A., Martin, C.: Biofortified tomatoes provide a new route to vitamin D sufficiency. Nature Plants. **8**(6), 611–616 (2022). https://doi.org/10.1038/s41477-022-01154-6
21. Van Vu, T., Sivankalyani, V., Kim, E.-J., Doan, D.T.H., Tran, M.T., Kim, J., Sung, Y.W., Park, M., Kang, Y.J., Kim, J.-Y.: Highly efficient homology-directed repair using CRISPR/Cpf1-geminiviral replicon in tomato. Plant Biotechnol. J. **18**, 2133–2143 (2020). https://doi.org/10.1111/pbi.13373
22. García-Murillo, L., Valencia-Lozano, E., Priego-Ranero, N.A., Cabrera-Ponce, J.L., Duarte-Arké, F.P., Vizuet-de-Rueda, J.C., Rivera-Toro, D.M., Herrera-Ubaldo, H., de Folter, S., Alvarez-Venegas, R.: CRISPRa-mediated transcriptional activation of the SlPR-1 gene in edited tomato plants. Plant Sci. **329**, 111617 (2023). https://doi.org/10.1016/j.plantsci.2023.111617
23. Kim, J.Y., Kim, J.H., Jang, Y.H., Yu, J., Bae, S., Kim, M.-S., Cho, Y.-G., Jung, Y.J., Kang, K.K.: Transcriptome and metabolite profiling of tomato SGR-knockout null lines using the CRISPR/Cas9 system. Int. J. Mol. Sci. **24**, 109 (2023). https://doi.org/10.3390/ijms24010109
24. Yang, T., Ali, M., Lin, L., Li, P., He, H., Sun, C., Wu, N., Zhang, X., Huang, T., Li, C.-B., Li, C., Deng, L.: Recoloring tomato fruit by CRISPR/Cas9-mediated multiplex gene editing. Hort. Res. **10**, 214 (2023). https://doi.org/10.1093/hr/uhac214
25. D'Ambrosio, C., Stigliani, A.L., Giorio, G.: CRISPR/Cas9 editing of carotenoid genes in tomato. Transgenic Res. **27**, 367–378 (2018). https://doi.org/10.1007/s11248-018-0079-9
26. Hunziker, J., Nishida, K., Kondo, A., Ariizumi, T., Ezura, H.: Phenotypic characterization of high carotenoid tomato mutants generated by the target-AID Base-editing technology. Front. Plant Sci. **13**, 848560 (2022). https://doi.org/10.3389/fpls.2022.848560
27. Zhi, J., Liu, X., Li, D., Huang, Y., Yan, S., Cao, B., Qiu, Z.: CRISPR/Cas9-mediated SlAN2 mutants reveal various regulatory models of anthocyanin biosynthesis in tomato plant. Plant Cell Rep. **39**, 799–809 (2020). https://doi.org/10.1007/s00299-020-02531-1
28. Danilo, B., Perrot, L., Botton, E., Nogué, F., Mazier, M.: The DFR locus: a smart landing pad for targeted transgene insertion in tomato. PLoS One. **13**, e0208395 (2018). https://doi.org/10.1371/journal.pone.0208395
29. Wang, B., Li, N., Huang, S., Hu, J., Wang, Q., Tang, Y., Yang, T., Asmutola, P., Wang, J., Yu, Q.: Enhanced soluble sugar content in tomato fruit using CRISPR/Cas9-mediated SlINVINH1 and SlVPE5 gene editing. Peer J. **9**, e12478 (2021). https://doi.org/10.7717/peerj.12478
30. Li, R., Li, R., Li, X., Fu, D., Zhu, B., Tian, H., Luo, Y., Zhu, H.: Multiplexed CRISPR/Cas9-mediated metabolic engineering of γ-aminobutyric acid levels in *Solanum lycopersicum*. Plant Biotechnol. J. **16**, 415–427 (2018). https://doi.org/10.1111/pbi.12781
31. Nonaka, S., Arai, C., Takayama, M., Matsukura, C., Ezura, H.: Efficient increase of γ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. Sci. Rep. **7**, 7057 (2017). https://doi.org/10.1038/s41598-017-06400-y
32. Tiwari, J.K., Singh, A.K., Behera, T.K.: CRISPR/Cas9 genome editing in tomato improvement: advances and applications. Front. Plant Sci. **14**, 2023 (2023). https://doi.org/10.3389/fpls.2023.1121209

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 23
Breeding for Yield Quality Parameters and Abiotic Stress in Tomato Using Genome Editing



Pedro García-Caparrós

Abstract Growing tomatoes is an important aspect of agriculture around the world because of the positive effects it has on people's health and the economy. Tomato breeders and growers have always been inspired by the market's insatiable desire for high-yielding and high-quality tomatoes. Crop production, yield, and quality are all negatively affected by abiotic stress, which includes factors like drought, salinity, heat, and cold. As climate change alters weather patterns throughout the world, farmers around the world are increasingly worried about the effects of abiotic stress on their tomato crops. The CRISPR/Cas9 gene-editing tool has attracted attention as an alternative for solving the need for high-yield and superior-quality tomatoes, as well as for managing abiotic stress in tomato plants. This method of gene editing offers new possibilities for the development of stress-tolerant tomato varieties. The present book chapter provides a comprehensive review of the current knowledge on CRISPR/Cas9 and its potential implications in tomato agriculture, with a particular emphasis on enhancing yield quality and conferring resistance to abiotic stresses. The CRISPR/Cas9 technology has the potential to enhance the taste, appearance, and nutritional value of tomatoes by accurately altering the genes responsible for flavor, color, aroma, and nutrition. The previously mentioned condition could end up in the cultivation of tomatoes that exhibit heightened levels of sweetness, as well as elevated concentrations of crucial vitamins, minerals, and antioxidants. The application of CRISPR/Cas9-mediated modifications has the possibility to augment the plant's capacity to endure abiotic stress conditions through the introduction of genes implicated in different pathways that contribute to enhanced resilience to such challenging surroundings. In conclusion, the use of CRISPR/Cas9 offers an intriguing chance for improving tomato farming through the enhancement of crop quality and yield, as well as the strengthening of tomato plants against adverse abiotic conditions.

P. García-Caparrós (✉)
Agronomy Department of Superior School Engineering, University of Almeria, Almeria, Spain

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_23

1 Introduction

The fruit of the tomato plant (*Solanum lycopersicum* Mill.), which is a multicarpellar berry, has emerged as the model species for fleshy fruits as a consequence of specific advantages for use in agronomic research along with basic research [1].

The tomato plant is a diploid organism that comprises 12 chromosomes and has a genome approximately 950 Mb in size. Because of the outstanding nutritional value of the plant and the plethora of beneficial industrial products capable of being derived from it, it is one of the most significant horticultural crops grown worldwide [2]. It is forecasted that the global tomato production, which was 41.52 million tons in 2020, will expand to 51.93 million tons in 2026 based on the universal scenario of tomato production [3].

Despite the fact that tomatoes have the potential to provide significant nutrition and income opportunities, it is crucial to address all possibilities associated with the application of genetic engineering to assure the feeding of the worldwide population as well as tomato growers' benefits. Consequently, CRISPR/Cas9 is a robust genome editing tool that enables researchers to make targeted and precise changes to DNA sequences in a relatively short period of time when compared to conventional breeding techniques.

The CRISPR/Cas9 system is a canonical genome editing system that consists of a shortened synthetic guide RNA (gRNA) sequence of 20 nucleotides that generates a complex with the Cas9 nuclease and guides it to a specific DNA sequence, in which it inserts a double-stranded break (DSB) [4]. Targeted genome editing (GE) technologies, particularly clustered regularly interspaced short palindromic repeats (CRISPR)/(CRISPR-associated protein 9) (Cas9), have the ability to yield elite cultivars with higher resistance to biotic and abiotic stress [5].

CRISPR/Cas9 is also exceptional due to its versatility and adaptability, which enables more precise editing of an organism's own genes, making it a more natural and effective method for achieving desired characteristics [6]. Moreover, the simplicity and low cost of this technique compared to cumbersome and less precise conventional engineering techniques based on the introduction of foreign genes into an organism's genome aiming to achieve desired traits led us to compile in this book chapter the most recent studies based on the application of this system (CRISPR-Cas9) in tomato for the enhancement of yield quality and abiotic stress resistance.

2 CRISPR/Cas9 Gene Editing: Unlocking the Potential to Boost Tomato Yield and Quality

There are several external and internal factors to consider when assessing the standard quality of tomatoes. The output, quality, marketability, plant health, and timing of tomato production are all influenced by the plant's ability to set and mature fruit. Tomatoes' yield and quality, as well as their marketing success, are directly tied to

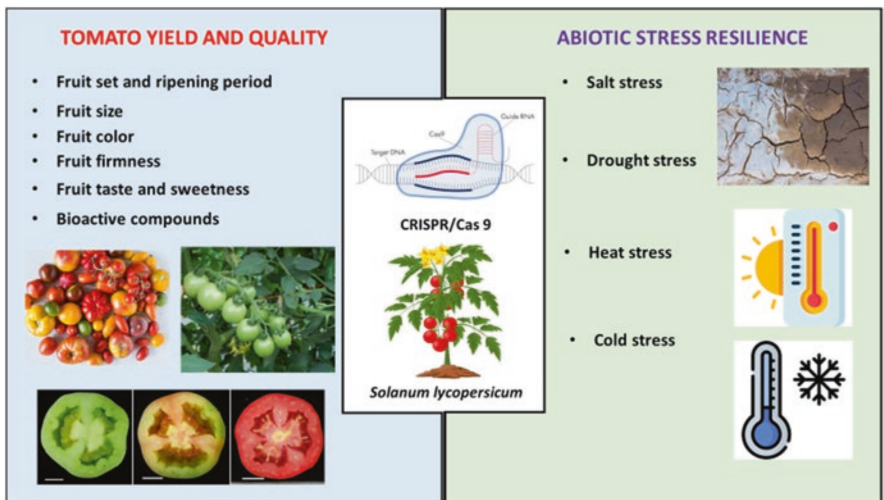


Fig. 23.1 Schematic overview about the research topic of the book chapter

how well these phases are handled [7]. The fruit size, color, and texture or firmness of fruit (external factors) have also an important impact in consumer preferences, as consumers prefer fruits those are visually appealing as well as having uniform color, size, and texture. Nonetheless, internal fruit quality parameters, such as taste, sweetness, nutraceutical properties, and the presence of bioactive compounds (lycopenes and carotenoids), are gaining importance among consumers [8]. A schematic overview of the different points explained appears in Fig. 23.1.

2.1 Fruit Set and Ripening Period

In tomato plants, the fruit set process is essential to ensure an adequate yield. Nevertheless, this physiological stage is widely affected by various factors including genetics, environment, and plant hormones [9]. A high fruit set results in a larger number of marketable tomatoes, which increases tomato growers' production and profits. In the tomato industry, the maturing period also influences the timing of harvesting and logistics.

For instance, Guo et al. [10] worked with CRISPR-Cas9-mediated SlCMT4 mutants aiming to improve the fruit set in tomato. The gene SlCMT4 (*Solanum lycopersicum* CAFFEIC ACID O-METHYLTRANSFERASE 4) is involved in the biosynthesis of lignin and also participate in the fruit set process. The loss of function of mutated plants resulted in defective stamens and pistils, small fruit size with reduced setting rate, demonstrating the crucial role of this gene in reproductive organs in tomato.

Tomato ripening is indeed a carefully orchestrated process of growth that aligns with seed maturation, and its modification through genetic engineering is of great importance because it affects fruit quality attributes such as taste and texture, nutritional value, and shelf life [11]. Using CRISPR/Cas9, Gao et al. [12] overexpressed the SlNAM1 locus in tomato plants. This gene governs the ethylene generation and fruit maturing processes in tomato plants. The overexpression of SlNAM1 did result in an earlier onset of fruit maturation, as well as quicker softening and color changes, in comparison with control plants.

SlNAC9 is a NAC transcription factor implicated in several physiological processes as well as in pigment metabolism in tomato fruit. The loss of function in SlNAC9 tomato mutants generated by genetic engineering (CRISPR/Cas9) resulted in exhibited delayed fruit ripening and reduced accumulation of carotenoids, which are important pigments that contribute to the color and nutritional quality of tomato fruit [13]. Similarly, Gao et al. [14] investigated the role of the tomato SlNAC4 (NAC transcription factor) gene in fruit maturation. Using the clustered regularly interspaced short palindromic repeats genomic targeting system (CRISPR/Cas9), overexpressed tomato mutant plants displayed differences in fruit texture, deferring the onset of softness, in contrast to the wild type or the control treatment.

The RIN gene, which encodes a MADS-box transcription factor, is activated late in tomato fruit development and boosts the transcription of genes that produce the ripening hormone ethylene [15]. Due to the potential impact of RIN gene mutations on tomato fruit ripening, Ito et al. [16] used CRISPR/Cas9 to generate RIN gene knockout mutants in tomato, and reported that the mutant fruits ripened more slowly and generated less ethylene than the control treatment. Similarly, using CRISPR/Cas9 technology, Jung et al. [17] created mutant tomato plants with LeMADS-RIN knocked out. The mutant fruits matured later than wild-type fruits and produced less ethylene. The mutant fruits also had decreased gene expression associated with ethylene synthesis.

Both FUL1 and FUL2 are considered to be homologous evolutionarily conserved in tomatoes. Both of these proteins are members of the MADS-box transcription factor family. They perform a crucial role in the management of the ripening of the fruit as well as the development of the flowers in tomato plants. The creation of double knockout mutants for these two homologous proteins using CRISPR/Cas9 slowed down the ripening of the fruit by inhibiting ethylene synthesis as well as decreasing the accumulation of carotenoid [18].

Lin et al. [19] evaluated the role of SlMIR164A (a microRNA gene involved in regulating fruit ripening and quality by influencing the expression of two target genes, SlNAM2 and SlNAM3) in tomatoes. Researchers discovered that by silencing this particular microRNA gene in tomato plants, they were capable of accelerating fruit ripening and enhance fruit nutritional quality by boosting the content of soluble sugars and acids.

2.2 Fruit Size

Fruit size is an important quality parameter for tomatoes, as it can impact factors such as yield, appearance, and taste [20]. It is also an important trait for tomato growers since determines the profitability of their crops. The packing and presentation of tomatoes are also impacted by their size. Breeding programs in tomato are focused on this aspect and in the literature, there are many references based on the improvement of this parameter [21, 22]. The methodology of CRISPR/Cas9 has been widely used in the improvement of this parameter in tomato fruit as it showed in the different references explained. For instance, Cui et al. [23] investigated the role of the tomato CONSTANS-LIKE 1 (SlCOL1) protein in regulating fruit yield in tomato plants. CONSTANS-LIKE proteins are transcription factors involved in the regulation of the flowering time and reproductive development of plants. The loss of function or the overexpression of the gene studied showed a high positive correlation with the fruit development and size.

The GLOBE gene is a major gene responsible of the shape of the fruit in tomato plants. The GLOBE gene is located on chromosome 2 in the tomato genome and encodes a transcription factor that regulates cell division and expansion during fruit development [24]. The loss of function of Solyc12g006860 generated via CRISPR/Cas9 showed that other fruit shape attributes were negatively affected while fruit size showed a positive answer [25].

BZR1-like transcription factor, BZR1.7 is a determinant trans-acting factor of the SUN gene promoter responsible of the tomato fruit elongation. Yu et al. [26] investigated the role of this TF in tomato plants generating overexpressing lines via CRISPR/Cas9 and they noted that in edited plants with overexpression of BZR1.7, tomato fruits were longer compared to control plants.

2.3 Fruit Color

The color of tomato fruit is an important quality attribute that affects consumer preference and marketability. Tomatoes are available in a range of colors including red, orange, yellow, green, and purple, and each color has unique flavor and nutritional characteristics [27]. For instance, Yang et al. [28] targeted three fruit color-related genes (PSY1, MYB12, and SGR1) to develop tomato genotypes via CRISPR/Cas9 with different fruit colors ranging from light green, brown and also pink-brown. The findings reported by these researchers is of interest to the tomato industry since they provide a strategy to obtain transgene-free plants with different colored fruits in less than 1 year.

2.4 Fruit Firmness

Fruit firmness is an important parameter for determining the quality and shelf life of tomato fruits. The firmness of the fruit is dependent on the chemical composition of the cell wall. In tomato fruits there are three types of polysacharide involved: cellulose, hemicellulose being the most abundant xyloglucan; and pectins [29]. Consumers prefer tomatoes with a firm and crisp texture because they perceive them to be more appealing and fresher. Tomatoes with a firm texture provide a pleasant and satisfying eating experience. Conversely, tomatoes with low firmness may be perceived as overripe, watery, or inferior.

In order to comprehend and exert some level of control over fruit tomato softening, it is imperative that the transcription factors (TFs) that regulate the process be identified and characterized. In this sense, by using CRISPR/Cas9 to silence the NOR-like1 (NAC Transcription factor) (Solyc07g063420) gene in tomato plants, Gao et al. [30] were able to create fruit with decreased ethylene generation, increased firmness, and decreased lycopene and other ripening-related compound levels. The result was a two-week delay in ripening compared to the control treatment.

In their study, Nie et al. [31] generated two mutants with over and down expression of CR-SlNAC4 (transcription factor involved in the softening of tomato) via CRISPR/Cas9. In both mutants, there was a strong effect of tomato fruit softening compared to the control fruit. The study also showed that SlNAC4 regulates the expression of several genes involved in fruit ripening and softening, including pectinases and cell wall-modifying enzymes.

Wang et al. [32] noted that SlXTH5, a member of the xyloglucan endotransglucosylase/hydrolase (XTH) gene family, plays a critical role in tomato fruit softening since xyloglucan is the most dominant hemicellulose in tomato fruits. Knockout mutants of SlXTH5 generated via CRISPR/Cas9 reduced fruit firmness and color index compared to control fruits.

SlBES1 transcription factor plays a crucial role in regulating tomato fruit softening. In this sense, Liu et al. [33] reported that knockout mutants of SlBES1 had firmer fruits with reduced pectin degradation compared to wild-type plants. Moreover, the increase in firmness in tomato fruits also improved the shelf life in the postharvest.

2.5 Fruit Taste and Sweetness

Taste is an important feature of tomato fruits and is linked to a complex interplay of factors such as sugar content, acidity, aroma, texture, and other chemical compounds. The taste of tomatoes is crucial in the consumer preference and purchase decisions, and therefore should be of high relevance in tomato breeding programs. Nevertheless, the improvement of this feature has been replaced by other factors such as fruit ripening, fruit size, etc. [34].

FLORAL4 gene is responsible for the production of several important floral aroma volatiles such as 2-phenylethanol, phenylacetaldehyde and 1-nitro-2-phenylethane in tomato fruit. The study conducted by Tikunov et al. [35] reported that generating mutants of FLORAL4 gene via CRISPR/Cas9 the floral aroma and overall flavor of commercial tomato fruit was severely affected.

Brix is a measure of fruit's sweetness and in the case of tomato is dependent of several factors such as cultivar, growing conditions or the stage of ripeness at which the tomato is harvested [36]. This important trait in tomato fruit quality has been improved via CRISPR/Cas9 in different experiments. For instance, Nguyen et al. [37] investigated the effect of targeted mutations via dual-gRNAs CRISPR/Cas9 system in uORF regions of the SlbZIP1, a gene involved in the sucrose-induced repression of translation (SIRT) mechanism. Tomato plants with disrupted bZIP1 uORFs had significantly higher levels of sugars and amino acids in their fruits compared to control plants. Moreover, these changes in metabolic pathways were associated with changes in the expression of several other genes involved in sugar and amino acid metabolism. Regarding the enhancement of the sweetness in tomato fruit, there are other references about CRISPR/Cas9 system design such as modification of SlINVINH1, a cell-wall invertase inhibitor resulting in tomatoes with a high sugar content without a reduction in fruit weight or plant growth [38] or the loss of function in SlVPE5, a vacuolar processing enzyme involved in sugar accumulation, in which tomato mutant lines increased soluble sugar content in fruit [39].

The brachytic phenotype (BR) in tomato is characterized by a reduction in plant height and increased compactness, which can be beneficial for certain agricultural applications. Lee et al. [40] found that tomato plants carrying the mutant FPF1 allele (Solyc01g066980) had a significant reduction in plant height although they produced fruits with better quality parameters such as higher size, increase in total soluble solids (°Brix) favoring them the commerciality of the tomato fruit.

2.6 Bioactive Compounds in Fruits

Lycopene is a natural pigment responsible for the characteristic red color of tomatoes and is also a power antioxidant with many benefits for human health, therefore tomato breeding programs are currently also focused on the enhancement of the synthesis of this pigment [41]. For instance, in one study, Li et al. [42] used CRISPR/Cas9 gene editing technology to simultaneously target five genes involved in the carotenoid metabolic pathway in tomato plants. By introducing mutations in these genes, the researchers were able to increase the levels of lycopene in tomato fruit about fivefold.

3 Empowering Tomato Crop Resilience: CRISPR/Cas9 for Abiotic Stress Adaptation

Abiotic stresses are complex in their nature and controlled by networks of different factors (e.g., genetic and environmental) that impede crop plant breeding strategies, therefore the use of the most novel genes editing technologies emphasizes the importance of this task [43].

The incidence of crop losses resulting from adverse environmental conditions has steadily increased over recent decades, attributable to both climate change and human activities. The intricate nature of abiotic stresses is governed by a multitude of factors, including genetic and environmental influences, which pose significant challenges to crop plant breeding strategies. As a result, the utilization of cutting-edge gene editing technologies is of the utmost significance. The implementation of CRISPR/Cas9 technologies is expected to facilitate plant genetic transformation, thereby improving resistance to various abiotic stresses [4].

In this section, we shall focus on the most recent publications of genome edited tomato generated via CRISPR/Cas9 with improved resistance to the main following abiotic stresses: salinity, drought, heat and chilling conditions. A schematic overview of the different points explained appears in Fig. 23.1.

3.1 Salt Stress

Salinity is considered to be a significant abiotic stressor for plants, causing considerable harm [44]. According to a recent publication by Hassani et al. [45], the estimated area of salt-affected soils in Europe is 24 Mha, which accounts for approximately 2.05% of the total worldwide salt-affected area of 1171.8 Mha. In light of these numbers, it is clear that developing via genetic engineering salt-tolerant varieties of most crops, and notably tomatoes, which are only moderately robust to salt stress, is a priority. The development of these salt tolerant cultivars allows the cultivation of tomato in soils moderately saline and also reduces the damages caused by salt stress in this crop. In this sense, we will be focusing on the most recent research that were carried out in tomato using the CRISPR/Cas9 system. For instance, Ding et al. [46] identified an uncharacterized gene named SlABIG1 from tomato, which they identified to be essential for salt tolerance and to be a component of the HD-ZIP II subfamily. The CRISPR/Cas9-generated knockout mutant exhibited higher salt tolerance, enhanced photosynthetic efficiency, reduced ROS generation, as well as improved Na^+ exclusion efficiency. On the other hand, the study conducted by Tran et al. [47] attempted to boost tomato plants' resistance to salt. Here, they used CRISPR/Cas9 to generate SlHyPRP1 (hydrid proline-rich protein 1 gene) knockout mutants to investigate the effect of salt tolerance. The data showed that the modified plants were more resistant to salt, with higher rates of survival and enhanced growth throughout the face of salt stress.

3.2 Drought Stress

The impact of drought stress on crop growth, development, and productivity is a noteworthy environmental factor. It occurs when there is an inadequate supply of water to meet the demands of crops, leading to soil moisture deficit and plant water stress. Comprehending the impact of drought stress in crops and employing genetic engineering to develop more resilient cultivars could aid in alleviating its effects and ensuring sustainable crop production amidst the altering climate patterns [48]. The susceptibility of tomatoes to water deficit is attributed to their high-water requirements and shallow root systems. Consequently, the development of modified genetically cultivars with improved drought resistance is imperative at present [49]. The development of these drought resistant cultivars is positive for the growers of tomato since they must invest in supplementary irrigation systems or water resources to combat water scarcity. Numerous recent examples in the literature demonstrate the generation of cultivars with increased tolerance to drought stress through the utilization of CRISPR/Cas9 technology. For instance, the drought tolerance of tomato plants cv. Alisa Craig was evaluated by Li et al. [50] using CRISPR/Cas9-mediated mutation of the SlNPR1 gene. This gene mutation reduces plants' resistance to water stress in different aspects such as increased stomatal conductance, generation of lipid peroxides and the higher susceptibility to oxidative damage linked to the lower levels of antioxidant enzymes. Similarly, Using CRISPR/Cas9, Chen et al. [51] created SlARF4 knockout mutants to investigate the physiological responses to drought stress in tomato plants in comparison to wild-type plants. The results indicate an improvement in the growth and water status of mutant tomato plants. In addition, arf4 mutants exhibited increased photosynthetic efficiency, decreased oxidative damage, increased antioxidant enzyme activity, and the upregulation of numerous stress-responsive genes.

Tomato's sensitivity to drought was examined by Liu et al. [52] who hypothesized that SlLBD40, a lateral organ boundaries domain transcription factor, was responsible for it. Using CRISPR/Cas9, they established mutant lines of tomato with a loss of function of the SlLBD40 gene, and those plants showed enhanced resistance to drought compared to the wild type. The mutants were more capable to deal with drought conditions, as seen through their delayed withering, reduced water loss rate, and higher survival rates.

Wang et al. [53] created two independent T1 mutant lines with the loss of function of the gene SlMAPK3 using CRISPR/Cas9 in order to compare their drought tolerance to that of the wild type. This study emphasizes the importance of MAPKs (mitogen-activated protein kinases) as crucial signaling components in plant responses to multiple stresses, including drought stress. The biochemical analysis revealed that the knock-out mutants displayed earlier wilting, increased H_2O_2 production, and decreased scavenging of reactive oxygen species, resulting in increased membrane damage.

3.3 Heat and Cold Conditions

Environmental factors such as heat and cold stress can have a significant impact on crop growth, development, and yield [54]. The ongoing climatic changes have resulted in frequent episodes of extreme heat or cold waves which dramatically affect the yields of crops, sometimes leading to their death. As a result, genetic engineering solutions are increasingly being recognized as appropriate way of dealing with this issue. Regarding CRISPR/Cas9 in tomato to counteract these abiotic stresses it can be highlighted in the recent studies. In the case of heat stress, Wang et al. [55] generated overexpressed mutants for the gene SlGRXS1 by activating the transcription factor SlWRKY3, which confers thermotolerance to tomato. Mutant tomato plants exhibited more ROS scavenging and less oxidative damage relative to their wild type counterparts. This research has confirmed that SlGRXS1 and SlWRKY3 act together to enhance the stress response network in tomato plants when they are subjected to high temperatures.

Due to its osmoprotectant properties, the capacity to maintain the cellular water status, the reduction in the synthesis of reactive oxygen species (ROS), and the regulation of gene expression, sucrose can play a crucial role in heat-stressed plants [56]. Zhang et al. [57] opted to investigate Sucrose phosphate synthase (SPS), a key rate-limiting enzyme in the sucrose synthesis pathway in crops. The researchers reported that the gain or loss of function of a novel gene (SlSPS) conferred increased or decreased thermotolerance to tomato plants, making them more or less susceptible to heat stress.

To suppress SlMAPK3 expression in tomato plants, Yu et al. [58] adopted CRISPR/Cas9 gene editing technology. The wild-type and mutant tomato plants were then subjected to heat stress. The thermotolerance of the edited plants compared to WT was confirmed by their survival in temperatures ranging from 25 to 45°. Under heat stress circumstances, the mutant plants survived longer and developed more successfully while minimizing the synthesis of potential reactive oxygen species (ROS).

The purpose of the study by Huang et al. [59] was to identify the function of the BAG9 gene in tomato heat tolerance. Tomato mutants (bag9) were able to withstand higher temperatures after overexpressing this gene using CRISPR/Cas9. Increased antioxidant enzyme activity and heat sock protein synthesis also resulted from this shift in higher gene expression in tomato plants.

As far as chilling conditions is concerned, Li et al. [60] generated CRISPR/Cas9 mutant tomato plants for the SlCBF1 gene aiming to develop a greater awareness of the tomato's chilling tolerance. The authors emphasize the important role of the CBF (C-repeat binding factor) genes in regulating plant responses to chilling stress, focusing in particular on the SlCBF1 gene, which is a known key regulator gene of chilling tolerance in tomatoes. The loss of function resulted in diminished physiological and biochemical responses of tomato plants to chilling stress, including altered levels of stress-related hormones such as jasmonic acid and abscisic acid.

Non-expresser of pathogenesis-related genes 1 (NPR1) is known for its role in crop resistance to diseases, but its function in cold-adapted tomato plants was investigated by Shu et al. [61]. It was found that CRISPR/Cas9-obtained tomato knockout mutants exhibited more cold stress tolerance than their wild-type counterparts, as evidenced by lower oxidative damage, higher antioxidant enzyme activity, and greater development of the plants.

4 Conclusion and Future Perspectives

This manuscript presents intriguing future prospects for the application of CRISPR/Cas9 technology to enhance tomato quality and abiotic stress resistance. Further investigation of the molecular mechanisms and pathways involved in tomato quality and abiotic stress response could yield important insights. Using functional genomics and systems biology approaches, such as transcriptomics, proteomics, and metabolomics, could reveal the complex molecular networks and regulatory mechanisms governing these traits, leading to a greater understanding of how CRISPR-mediated modifications affect tomato quality and stress tolerance. Future research might explore the concurrent editing of multiple genes aiming to improve tomato traits in a more comprehensive way. Combining genetic modifications for enhanced flavor, increased nutritional value, and enhanced stress tolerance, for instance, could result in the creation of tomatoes with superior overall quality and resilience.

References

1. Azzi, L., Deluche, C., Gévaudant, F., Frangne, N., Delmas, F., Hernould, M., Chevalier, C.: Fruit growth-related genes in tomato. J. Exp. Bot. **66**, 1075–1086 (2015)
2. Salava, H., Thula, S., Mohan, V., Kumar, R., Maghuly, F.: Application of genome editing in tomato breeding: mechanisms, advances, and prospects. Int. J. Mol. Sci. **22**, 682 (2021)
3. Chandrasekaran, M., Boopathi, T., Paramasivan, M.: A status-quo review on CRISPR-Cas9 gene editing applications in tomato. Int. J. Biol. Macromol. **190**, 120–129 (2021)
4. Kouhen, M., García-Caparrós, P., Twyman, R.M., Abdelly, C., Mahmoudi, H., Schillberg, S., Debez, A.: Improving environmental stress resilience in crops by genome editing: insights from extremophile plants. Crit. Rev. Biotech. **43**, 1–16 (2022)
5. Abdelrahman, M., Al-Sadi, A.M., Pour-Aboughadareh, A., Burritt, D.J., Tran, L.S.P.: Genome editing using CRISPR/Cas9–targeted mutagenesis: an opportunity for yield improvements of crop plants grown under environmental stresses. Plant Physiol. Biochem. **131**, 31–36 (2018)
6. Osakabe, Y., Watanabe, T., Sugano, S.S., Ueta, R., Ishihara, R., Shinozaki, Z., Osakabe, K.: Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. Sci. Rep. **6**, 26685 (2016)
7. Saltveit, M.E.: Fruit ripening and fruit quality. In: Tomatoes, pp. 145–170. Cabi Publishing, Wallingford (2005)
8. Wang, T., Zhang, H., Zhu, H.: CRISPR technology is revolutionizing the improvement of tomato and other fruit crops. Hortic. Res. **6**, 1 (2019)

9. Shinozaki, Y., Beauvoit, B.P., Takahara, M., Hao, S., Ezura, K., Andrieu, M.H., Nishida, K., Mori, K., Suzuki, Y., Kuhara, S., Enomoto, H., Kusano, M., Fukushima, A., Mori, T., Kojima, M., Kobayashi, M., Sakakibara, H., Saito, K., Ohtani, Y., Benard, C., Prodhomme, D., Gibon, Y., Ezura, H., Ariizumi, T.: Fruit setting rewires central metabolism via gibberellin cascades. Proc. Natl. Acad. Sci. U.S.A. **117**, 23970–23981 (2020)
10. Guo, X., Zhao, J., Chen, Z., Qiao, J., Zhang, Y., Shen, H., Hu, Z.: CRISPR/Cas9-targeted mutagenesis of SlCMT4 causes changes in plant architecture and reproductive organs in tomato. Hortic. Res. **9**, uhac081 (2022)
11. Klee, H.J., Giovannoni, J.J.: Genetics and control of tomato fruit ripening and quality attributes. Annu. Rev. Gen. **45**, 41–59 (2011)
12. Gao, Y., Fan, Z.Q., Zhang, Q., Li, H.L., Liu, G.S., Jing, Y., Zhang, Y.P., Zhu, B.Z., Zhu, H.L., Chen, J.Y., Grierson, D., Luo, Y.B., Zhao, X.D., Fu, D.Q.: A tomato NAC transcription factor, SlNAM1, positively regulates ethylene biosynthesis and the onset of tomato fruit ripening. Plant J. **108**, 1317–1331 (2021)
13. Feng, Y., Kou, X., Yuan, S., Wu, C., Zhao, X., Xue, Z., Li, Q., Huang, Z., Sun, Y.: CRISPR/Cas9-mediated SNAC9 mutants reveal the positive regulation of tomato ripening by SNAC9 and the mechanism of carotenoid metabolism regulation. Hortic. Res. **10**, uhad019 (2023)
14. Gao, Y., Zhang, Y.P., Fan, Z.Q., Jing, Y., Chen, J.Y., Grierson, D., Yang, R., Fu, D.Q.: Mutagenesis of SlNAC4 by CRISPR/Cas9 alters gene expression and softening of ripening tomato fruit. Veg. Res. **1**, 1–12 (2021)
15. Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W., Giovannoni, J.: A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science. **296**, 343–346 (2002)
16. Ito, Y., Nishizawa-Yokoi, A., Endo, M., Mikami, M., Toki, S.: CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. Biochem. Biophys. Res. Commun. **467**, 76–82 (2015)
17. Jung, Y.J., Lee, G.J., Bae, S., Kang, K.K.: Reduced ethylene production in tomato fruits upon CRSPR/Cas9-mediated LeMADS-RIN mutagenesis. Hortic. Sci. Tech. **36**, 396–405 (2018)
18. Wang, S., Lu, G., Hou, Z., Luo, Z., Wang, T., Li, H., Zhang, J., Ye, Z.: Members of the tomato FRUITFULL MADS-box family regulate style abscission and fruit ripening. J. Exp. Bot. **65**, 3005–3014 (2014)
19. Lin, D., Zhu, X., Qi, B., Gao, Z., Tian, P., Li, Z., Lin, Z., Zhang, Y., Huang, T.: SlMIR164A regulates fruit ripening and quality by controlling SlNAM2 and SlNAM3 in tomato. Plant Biotech. J. **20**, 1456–1469 (2022)
20. Coyago-Cruz, E., Corell, M., Moriana, A., Mapelli-Brahm, P., Hernanz, D., Stinco, C.M., Beltran-Singuichano, E., Meléndez-Martínez, A.J.: Study of commercial quality parameters, sugars, phenolics, carotenoids and plastids in different tomato varieties. Food Chem. **277**, 480–489 (2019)
21. Chaudhary, J., Alisha, A., Bhatt, V., Chandanshive, S., Kumar, N., Mir, Z., Kumar, A., Yadav, S.K., Shivaraj, S.M., Sonah, H., Deshmukh, R.: Mutation breeding in tomato: advances, applicability and challenges. Plan. Theory. **8**, 128 (2019)
22. Dube, J., Ddamulira, G., Maphosa, M.: Tomato breeding in Sub-Saharan Africa-challenges and opportunities: a review. Afr. Crop Sci. J. **28**, 131–140 (2020)
23. Cui, L., Zheng, F., Wang, J., Zhang, C., Zhang, D., Gao, S., Zhang, C., Ye, J., Zhang, Y., Ouyang, B., Wang, T., Hong, Z., Ye, Z., Zhang, J.: The tomato CONSTANS-LIKE protein SlCOL1 regulates fruit yield by repressing SFT gene expression. BMC Plant Biol. **22**, 429 (2022)
24. Hundleby, P., Harwood, W.: Regulatory constraints and differences of genome-edited crops around the globe. In: Genome Editing: Current Technology Advances and Applications for Crop Improvement, pp. 319–341. Springer, Cham (2022)
25. Sierra-Orozco, E., Shekasteband, R., Illa-Berenguer, E., Snouffer, A., van der Knaap, E., Lee, T.G., Hutton, S.F.: Identification and characterization of GLOBE, a major gene controlling fruit shape and impacting fruit size and marketability in tomato. Hortic. Res. **8** (2021)

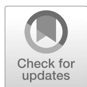
26. Yu, T., Ai, G., Xie, Q., Wang, W., Song, J., Wang, J., Tao, Y., Zhang, X., Hong, Z., Lu, Y., Ye, J., Zhang, Y., Zhang, J., Ye, Z.: Regulation of tomato fruit elongation by transcription factor BZR1.7 through promotion of SUN gene expression. Hortic. Res. **9**, uhac121 (2022)
27. Gonzali, S., Perata, P.: Fruit colour and novel mechanisms of genetic regulation of pigment production in tomato fruits. Horticulturae. **7**, 259 (2021)
28. Yang, T., Ali, M., Lin, L., Li, P., He, H., Zhu, Q., Sun, C., Wu, N., Zhang, X., Huang, T., Li, C.-B., Li, C., Deng, L.: Recoloring tomato fruit by CRISPR/Cas9-mediated multiplex gene editing. Hortic. Res. **10**, uhac214 (2023)
29. Quinet, M., Angosto, T., Yuste-Lisbona, F.J., Blanchard-Gros, R., Bigot, S., Martinez, J.P., Lutts, S.: Tomato fruit development and metabolism. Front. Plant Sci. **10**, 1554 (2019)
30. Gao, Y., Wei, W., Zhao, X., Tan, X., Fan, Z., Zhang, Y., Jing, Y., Meng, L., Zhu, B., Zhu, H., Chen, J., Jiang, C.-Z., Grierson, D., Luo, Y., Fu, D.Q.: A NAC transcription factor, NOR-like1, is a new positive regulator of tomato fruit ripening. Hortic. Res. **5**, 75 (2018)
31. Nie, H., Shi, Y., Geng, X., Xing, G.: CRISPR/Cas9-mediated targeted mutagenesis of tomato polygalacturonase gene (SlPG) delays fruit softening. Front. Plant Sci. **13**, 729128 (2022)
32. Wang, D., Lu, Q., Wang, X., Ling, H., Huang, N.: Elucidating the role of SlXTH5 in tomato fruit softening. Hortic. Plant J. (2022). https://doi.org/10.1016/j.hpj.2022.12.005
33. Liu, H., Liu, L., Liang, D., Zhang, M., Jia, C., Qi, M., Liu, Y., Shao, Z., Meng, F., Hu, S., Yin, Y., Li, C., Wang, Q.: SlBES1 promotes tomato fruit softening through transcriptional inhibition of PMEU1. Iscience. **24**, 102926 (2021)
34. Klee, H.J., Tieman, D.M.: The genetics of fruit flavour preferences. Nat. Rev. Gen. **19**, 347–356 (2018)
35. Tikunov, Y.M., Roohanitaziani, R., Meijer-Dekens, F., Molthoff, J., Paulo, J., Finkers, R., Capel, I., Carvajal-Moreno, F., Maliepaard, C., de Vries, M.N., Labrie, C.W., Verkerke, W., van Heusden, A.W., van Eeuwijk, F., Visser, R.G.F., Bovy, A.G.: The genetic and functional analysis of flavor in commercial tomato: the FLORAL4 gene underlies a QTL for FLORAL aroma volatiles in tomato fruit. Plant J. **103**, 1189–1204 (2020)
36. Jaywant, S.A., Singh, H., Arif, K.M.: Sensors and instruments for Brix measurement: a review. Sensors. **22**, 2290 (2022)
37. Nguyen, N.H., Bui, T.P., Le, N.T., Nguyen, C.X., Le, M.T.T., Dao, N.T., Phan, Q., Le, T.V., To, H.M.T., Pam, N.B., Chu, H.H., Do, P.T.: Disrupting Sc-uORFs of a transcription factor bZIP1 using CRISPR/Cas9 enhances sugar and amino acid contents in tomato (*Solanum lycopersicum*). Planta. **257**, 1–11 (2023)
38. Kawaguchi, K., Takei-Hoshi, R., Yoshikawa, I., Nishida, K., Kobayashi, M., Kusano, M., Lu, Y., Ariizumi, T., Ezura, H., Otagaki, S., Matsumoto, S., Shiratake, K.: Functional disruption of cell wall invertase inhibitor by genome editing increases sugar content of tomato fruit without decrease fruit weight. Sci. Rep. **11**, 1–12 (2021)
39. Wang, B., Li, N., Huang, S., Hu, J., Wang, Q., Tang, Y., Yang, T., Asmutola, P., Wang, J., Yu, Q.: Enhanced soluble sugar content in tomato fruit using CRISPR/Cas9-mediated SlINVINH1 and SlVPE5 gene editing. PeerJ. **9**, e12478 (2021)
40. Lee, M.B., Shekasteband, R., Hutton, S.F., Lee, T.G.: A mutant allele of the flowering promoting factor 1 gene at the tomato BRACHYTIC locus reduces plant height with high quality fruit. Plant Direct. **6**, e422 (2022)
41. Imran, M., Ghorat, F., Ul-Haq, I., Ur-Rehman, H., Aslam, F., Heydari, M., Shariati, M.A., Okuskhanova, E., Yessimbekov, Z., Thiruvengadam, M., Hashempur, M.H., Rebezov, M.: Lycopene as a natural antioxidant used to prevent human health disorders. Antioxidants. **9**, 706 (2020)
42. Li, X., Wang, Y., Chen, S., Tian, H., Fu, D., Zhu, B., Luo, Y., Zhu, H.: Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. Front. Plant Sci. **9**, 559 (2018)
43. Zhang, H., Zhu, J., Gong, Z., Zhu, J.K.: Abiotic stress responses in plants. Nat. Rev. Gen. **23**, 104–119 (2022)

44. Hamed, K.B., Castagna, A., Ranieri, A., Garcia-Caparros, P., Santin, M., Hernandez, J.A., Espin, G.B.: Halophyte based Mediterranean agriculture in the contexts of food insecurity and global climate change. Environ. Exp. Bot. **191**, 104601 (2021)
45. Hassani, A., Azapagic, A., Shokri, N.: Predicting long-term dynamics of soil salinity and sodicity on a global scale. Proc. Natl. Acad. Sci. U.S.A. **117**, 33017–33027 (2020)
46. Ding, F., Qiang, X., Jia, Z., Li, L., Hu, J., Yin, M., Xia, S., Chen, B., Qi, J., Li, Q., Gao, Y., Zhang, S., Sun, S., Ma, C., Yang, L., Li, Y.: Knockout of a novel salt responsive gene SlABIG1 enhance salinity tolerance in tomato. Environ. Exp. Bot. **200**, 104903 (2022)
47. Tran, M.T., Doan, D.T.H., Kim, J., Song, Y.J., Sung, Y.W., Das, S., Kim, E.-J., Son, G.H., Kim, S.H., Vu, T.V., Kim, J.Y.: CRISPR/Cas9-based precise excision of SlHyPRP1 domain (s) to obtain salt stress-tolerant tomato. Plant Cell Rep. **40**, 999–1011 (2021)
48. Ozturk, M., Turkyilmaz Unal, B., García-Caparrós, P., Khursheed, A., Gul, A., Hasanuzzaman, M.: Osmoregulation and its actions during the drought stress in plants. Physiol. Plant. **172**, 1321–1335 (2021)
49. Krishna, R., Karkute, S.G., Ansari, W.A., Jaiswal, D.K., Verma, J.P., Singh, M.: Transgenic tomatoes for abiotic stress tolerance: status and way ahead. 3 Biotech. **9**, 1–14 (2019)
50. Li, R., Liu, C., Zhao, R., Wang, L., Chen, L., Yu, W., Zhang, S., Sheng, J., Shen, L.: CRISPR/Cas9-mediated SlNPR1 mutagenesis reduces tomato plant drought tolerance. BMC Plant Biol. **19**, 1–13 (2019)
51. Chen, M., Zhu, X., Liu, X., Wu, C., Yu, C., Hu, G., Chen, L., Chen, R., Bouzayen, M., Zouine, M., Hao, Y.: Knockout of auxin response factor SlARF4 improves tomato resistance to water deficit. Int. J. Mol. Sci. **22**, 3347 (2021)
52. Liu, L., Zhang, J., Xu, J., Li, Y., Guo, L., Wang, Z., Zhang, X., Zhao, B., Guo, Y.-D., Zhang, N.: CRISPR/Cas9 targeted mutagenesis of SlLBD40, a lateral organ boundaries domain transcription factor, enhances drought tolerance in tomato. Plant Sci. **301**, 110683 (2020)
53. Wang, L., Chen, L., Li, R., Zhao, R., Yang, M., Sheng, J., Shen, L.: Reduced drought tolerance by CRISPR/Cas9-mediated SlMAPK3 mutagenesis in tomato plants. J. Agric. Food Chem. **65**, 8674–8682 (2017)
54. Rivero, R.M., Mittler, R., Blumwald, E., Zandalinas, S.I.: Developing climate-resilient crops: improving plant tolerance to stress combination. Plant J. **109**, 373–389 (2022)
55. Wang, Y., Gai, W., Yuan, L., Shang, L., Li, F., Gong, Z., Ge, P., Wang, Y., Tao, J., Zhang, X., Dong, H., Zhang, Y.: Heat-inducible SlWRKY3 confers thermotolerance by activating the SlGRXS1 gene cluster in tomato. Hortic. Plant J. (2022). https://doi.org/10.1016/j.hpj.2022.12.006
56. Cohen, I., Zandalinas, S.I., Huck, C., Fritschi, F.B., Mittler, R.: Meta-analysis of drought and heat stress combination impact on crop yield and yield components. Physiol. Plant. **171**, 66–76 (2021)
57. Zhang, Y., Zeng, D., Liu, Y., Zhu, W.: SlSPS, a sucrose phosphate synthase gene, mediates plant growth and thermotolerance in tomato. Horticulturae. **8**, 491 (2022)
58. Yu, W., Wang, L., Zhao, R., Sheng, J., Zhang, S., Li, R., Shen, L.: Knockout of SlMAPK3 enhances tolerance to heat stress involving ROS homeostasis in tomato plants. BMC Plant Biol. **19**, 1–13 (2019)
59. Huang, H., Liu, C., Yang, C., Kanwar, M.K., Shao, S., Qi, Z., Zhou, J.: BAG9 confers thermotolerance by regulating cellular redox homeostasis and the stability of heat shock proteins in *Solanum lycopersicum*. Antioxidants. **11**, 1467 (2022)
60. Li, R., Zhang, L., Wang, L., Chen, L., Zhao, R., Sheng, J., Shen, L.: Reduction of tomato-plant chilling tolerance by CRISPR-Cas9-mediated SlCBF1 mutagenesis. J. Agric. Food Chem. **66**, 9042–9051 (2018)
61. Shu, P., Li, Y., Xiang, L., Sheng, J., Shen, L.: SlNPR1 modulates chilling stress resistance in tomato plant by alleviating oxidative damage and affecting the synthesis of ferulic acid. Sci. Hortic. **307**, 111486 (2023)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 24
Genome Editing-Based Strategies Used to Enhance Crop Resistance to Parasitic Weeds



Kubilay Yıldırım, Musa Kavas, Melekşen Akın, and İlkay Sevgen Küçük

Abstract In contrast to most autotrophic plants, which produce carbohydrates from carbon dioxide using photosynthesis, parasitic weed plants rely on host plants to form vascular connections through which they withdraw the required nutritive resources and water. Many important crop plants are infested by these heterotrophic plants leading to tremendous yield losses and rendering agricultural lands uncultivable. The parasitic weeds are physically attached to the host plants and therefore their control is challenging due to the lack of selective methods for killing the weeds without damaging the host crop. Fortunately, many host plants have pre-haustorium resistance, host initiation responses and post-attachment tolerance to these parasitic weeds. However, parasitic weeds have high fecundity, dispersal efficiency, and persistent seed storage in the soil all of which enable them to adapt to new hosts and break down the crop resistance. Recent discoveries in genome editing and gene silencing-based technologies open new opportunities to enhance crop resistance to parasitic weeds. Some genome editing-based studies targeting the seed germination of parasitic weeds created almost complete resistance in crop species. In this chapter, we give an overview of the host-parasitic interaction and host defence responses that can be targeted by genome editing or gene silencing technologies.

Keywords Parasitic weeds · Host · Defence response · Genome editing · CRISPR · RNAi

K. Yıldırım (✉) · İ. S. Küçük
Department of Molecular Biology and Genetics, Ondokuz Mayıs University, Samsun, Türkiye
e-mail: kubilay.yildirim@omu.edu.tr

M. Kavas
Department of Agricultural Biotechnology, Ondokuz Mayıs University, Samsun, Türkiye

M. Akın
Department of Horticulture, Iğdır University, Igdır, Türkiye

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_24

1 Introduction

Plants are autotrophic organisms using light as energy to convert inorganic carbon into carbohydrates by photosynthesis. However, some plants have evolved specialized organs (haustorium) to attach and form vascular connections with autotrophic plants to absorb their water and nutrients. This heterotrophic lifestyle is used by parasitic plants/weeds and has a profound negative impact on many agriculturally important crops, forests and whole dynamics of ecological systems [1]. Parasitic plants could be grouped as facultative or obligatory according to their dependency on the host. Facultative parasitic plants (hemiparasitic) have their own chlorophyll and can complete their life cycle independently of a host. However, if there is an available host plant to obtain nutrients and water with less investment in the assimilation system, they become parasitic. Obligate parasitic plants (holoparasites) lack chlorophyll and they depend completely on their hosts for seed germination and survival. Parasitic plants can also be separated as root feeders or shoot feeders based on the invaded host tissue. Depending on their vascular connections with their host, they could be xylem feeders, phloem feeders, or both [2].

Parasitic plants in lower diversified agricultural systems can cause tremendous yield losses rendering agricultural lands uncultivable [3]. Traditional control methods such as hand weeding and herbicide treatment are too expensive and labour-intensive to regulate parasitic plant infestations in crops. These methods also are ineffective due to the tight physiological connection between the host and the parasitic weed and the re-emergence of parasitic plants after damaging of the host. Parasitic plants generally produce plentiful small seeds contaminating the soil or the crop seeds before parasitism is established. The seeds of parasitic plants remain viable in the dormant state for many years and germinate after receiving the host signals [4].

Reducing the impact and spread of parasitic weeds on crops and agricultural production requires an understanding of the molecular machinery behind the interactions between the parasite and the host plants. Pre and post-attachment as well as haustorium initiation resistance mechanisms in specific cultivars, mutants, or species have been identified and many host metabolites required for the germination of parasitic weed seeds have been identified. The availability of whole-genome sequences and transcriptomes of several parasitic plants facilitated the investigation of genes responsible for host–parasite interactions, and the identification of the genes involved in resistance or susceptibility responses of crops [5–7]. All this knowledge can be used to enhance resistance in crop species to these weeds by deploying molecular breeding and advanced genome editing strategies. In this chapter, we provide a comprehensive overview of new genome editing or gene silencing-based approaches applied to crops to enhance parasitic weed resistance and their prospective applications on the molecular mechanisms involved in host-parasitic weed interaction.

2 Genome Editing-Based Strategies Used to Enhance Parasitic Weeds Resistance in Crops

Many agriculturally important plants are attacked by specific parasitic plants, which induces a host defence response to inhibit the attachment of parasitic weeds or reduce the infestation. Based on whether the resistance mechanism functions before or after parasitic plants attach to their hosts, resistance responses can be classified as pre-haustorium resistance, haustorium initiation resistance or post-attachment resistance [8]. In addition to the classical transgenic approach, newly discovered biotechnological strategies (RNAi, VİGS and CRISPR) have been implemented to develop a high level of crop resistance to parasitic weeds in recent years [9–11]. In the review, we grouped these studies according to their target resistance mechanisms.

2.1 Genome Editing for Pre-HAUSTORIUM Resistance in Crops

The discovery of some terpenoid lactones in crops such as strigolactones (SLs) and sesquiterpene lactones (STLs), [11, 12] is a milestone in understanding the interaction between parasitic weeds and their hosts. Secondary metabolites synthesized by host roots in trace amounts have several important physiological processes in host plants from shoot branching to arbuscular mycorrhizal symbiosis. Terpenoid lactones were then realized to be also the germination stimulants for several obligate root parasitic plants [13, 14]. The seeds of these parasitic plants do not germinate unless they receive terpenoid lactones as a chemical signal from their host roots. Therefore, the parasite-host interaction has evolved in a sophisticated way to detect the presence of STLs or SLs by parasitic weeds and coordinate their germination and development with the host's lifecycle [15, 16]. Receiving the signal molecule from the host for seed germination and growth towards the host organs are critical steps in the parasitic plant life cycle. Resistant host plants take a preventive pre-attachment strategy by making themselves invisible to parasitic plants by decreasing or completely stopping the production of germination stimulant molecules [17]. Therefore, reducing the number of stimulants exuded by host plants is considered to be a key factor for the host resistance achieved by inhibition of parasitic weed seed germination. Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9)-mediated mutagenesis, virus-induced gene silencing (VIGS) and RNA interference (RNAi) mediated gene silencing strategies have been used to disrupt strigolactones (SLs) biosynthesis in host plants [18–23]. In this way, the germination of seeds of parasitic plants was suppressed and almost complete resistance to parasitic weeds was achieved in genome-edited host plants (Table 24.1).

Table 24.1 Crops and parasitic plants modified by genome editing mediated strategies to enhance host resistance to parasitic weeds

Parasite	Mode of resistance	Plant species	Reference
P. aegyptiaca	RNAi-mediated silencing of the parasite gene M6PR2	Tomato	[28]
P. ramosa	RNAi-mediated silencing of host gene CCD7	Tomato	[18]
P. ramosa	RNAi-mediated silencing of host gene CCD8	Tomato	[19]
P. aegyptiaca	VIGS-mediated knockdown of the CCD7 and CCD8 genes of parasitic weed	Tobacco	[20]
S. hermonthica	VIGS-mediated silencing of ACS, M6PR, and Prx1 genes of the parasitic weed	Tomato	[29]
P. ramosa	RNAi-mediated silencing of the host gene CCD8	Tomato	[13]
S. hermonthica	CRISPR/Cas9-mediated knockout of host CCD7	Rice	[21]
P. aegyptiaca	CRISPR/Cas9-mediated knockout of host CCD8	Tomato	[22]
S. hermonthica	CRISPR/Cas9-mediated knockout of host MAX1	Tomato	[23]
O. crenata			
P. aegyptiaca			

SLs were first isolated in cotton root exudates as a germination stimulant of *Striga lutea* [24]. Subsequent research revealed that these compounds also function as endogenous hormones to inhibit shoot branching or tillering. SL biosynthesis begins with the convertion of all-trans-β-carotene to 9-cis-β-carotene with an enzyme called β-carotene isomerase (DWARF27 or D27). Then, carotenoid cleavage dioxygenase 7 (CCD7) cleaves 9-cis-β-carotene into the volatile β-ionone and 9-cis-β-apo-10′-carotenal. This former intermediate is catalyzed by CCD8 to yield carlactone which is the precursor for all SLs (Fig. 24.1). In *Arabidopsis thaliana*, carlactone is converted into carlactonoic acid by the cytochrome P450 monooxygenase (MORE AXILLARY GROWTH 1-MAX1) (Fig. 24.1a). In rice, MAX1 homologs convert carlactone into 4-deoxyorobanchol and orobanchol [25].

RNA interference (RNAi) uses an antisense siRNA strand to associate with the RNA-induced silencing complex (RISC) to target homologous RNA molecules for degradation and gene silencing in plants [26]. RNAi was previously used to silence several key genes encoding critical enzymes functional in SL biosynthesis. Gene silencing of CCD7 and CCD8 transcripts in tomatoes using antisense siRNA resulted in decreased levels of SL in the host, leading to reduced germination of the root parasitic weed [18, 19, 27].

Kohlen et al. (2012) showed that silencing of the host CCD8 gene in tomato lines by hpRNA technique reduces infestation of *P. ramosa* by 90% in the transgenic plants [19]. In another study, Aly et al. (2014) used a tobacco rattle virus–VIGS system for the transient knockdown of CCD7 or CCD8 in *P. aegyptiaca*. The result of the study demonstrated significant inhibition of parasite-tubercle development and the infestation of *Nicotiana benthamiana* plants [20]. A similar approach was used for the control of root parasitic weeds based on the simultaneous trans-specific

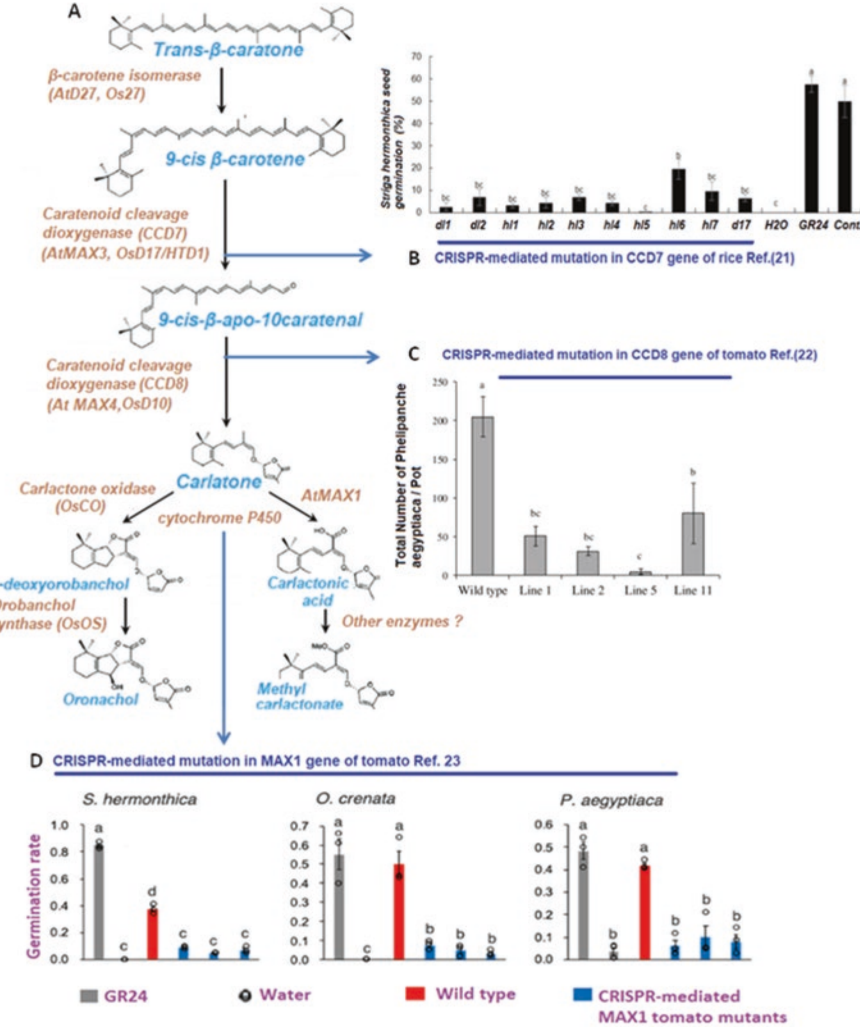


Fig. 24.1 Strigolactones (SLs) released from the host roots are the main stimulants for seed germination of parasitic weeds. Therefore the genes encoding the enzymes functional in SL biosynthesis (**a**) were the targets for CRISPR-mediated gene knockout studies. CRISPR-mediated disruption of the CC7, CCD8 and MAX1 genes in rice (**b**) and tomato (**c**, **d**) to reduce SL content in the root exudates. All the SL-deficient mutant plants exhibited reduced or poor germination in the seeds of parasitic plants such as *S. hermonthica, O. crenata* and *P. aegyptiaca*

gene silencing of parasite genes [29]. In this study, multiple DNA fragments (ACS, M6PR, and Prx1) of *P. aegyptiaca* genes were targeted by RNAi. The results of the experiment showed the movement of mobile exogenous siRNA from the host to the parasite, which lead to the decreased expression of parasitic genes essential for the parasite tubercles growing on the host plants.

CRISPR/Cas9 is the newest genome editing approach used to silence or modify the genes of plant species to enhance resistance to parasitic plants. This efficient and simple genome editing tool requires a small-guided RNA (sgRNA) complementary to a target gene sequence and Cas9 enzyme that recognize sgRNA for precise cutting of DNA and leading to dsDNA breaks [30]. During DNA repair by non-homologous end joining, insertion or deletion may occur at the break sites, silencing the protein's function [31]. CRISPR/Cas9 has been recently applied to knock out the CCD7 gene in rice (*Oryza sativa*) to reduce SL content in the roots [21]. CCD7 mutants exhibited increased tillering, combined with reduced height and extremely poor levels of SL production compared to the wild-type control. Striga seed germination was almost completely inhibited by the root exudates of some CCD7 mutants compared to that of control and the standard SL analogue GR24 (Fig. 24.1b). In another study, CRISPR/Cas9-mediated mutagenesis of the CCD8 gene was used to enhance host resistance to the parasitic weed *P. aegyptiaca* [22]. In this study, Cas9/single guide RNA constructs were targeted to the second exon of CCD8 in tomato plants. Several mutant tomato lines with heritable insertions or deletions in CCD8 gene were recorded to be SL-deficient. Compared to control tomato plants, the CCD8 mutant lines had morphological changes such as dwarfing, excessive shoot branching and adventitious root formation. In addition, some SL-deficient CCD8 mutants exhibited an almost complete reduction in seed germination of *P. aegyptiaca* and its infestation compared to non-mutated tomato plants (Fig. 24.1c) [22]. Wakabayashi et al. (2019) knocked out the cytochrome P450 (MAX1) gene, SlCYP722C, coding for an orobanchol synthase enzyme in tomato, by using a CRISPR system (Fig. 24.1d) [23]. Indels in the gene that resulted in biallelic frameshift mutations were identified in the T1 transgenic plants and T2 progeny lines. Orobanchol production was completely inhibited in the root exudates of MAX1 mutant tomato plants. Unlike the CCD8 mutant tomato lines created by Bari et al. [22], MAX1 mutants did not show prominent phenotypes such as increased shoot branching and reduced stem length. Production of the fruits and seeds was normal in the T1 MAX1 mutant tomato lines normally, and no serious yield loss occurred in mutant T2 progeny. Most importantly, root exudates of MAX1 mutant tomato plants reduced the induction of germination of seeds of root parasitic weeds, *Striga hermonthica*, *Orobanche crenata*, and *Phelipanche aegyptiaca*, compared to WT without changing the plant architecture.

Secretion of toxic compounds inhibiting the seed germination of parasitic weeds is another strategy for host resistance against parasitic weeds. Many phytotoxins or natural amino acids were found to interfere with the early growth stages of the parasitic weeds. These metabolites have negative effects on seed germination or germ tube elongation [32]. Serghini et al. (2001) found that the resistant sunflower genotypes release defensive secondary metabolites called 7-hydroxylated coumarins from their root to create a toxic environment for *O. cernua* [33]. In another study, transgenic tobacco overexpressing an antibacterial peptide sarcotoxin IA enhanced resistance to *Phelipanche spp*. by its toxic effects on this parasitic weed [34].

2.1.1 Genome Editing for HAUSTORIUM Initiation Resistance in Crops

Once a germination signal is released from the host and detected by the parasitic plants, a haustorium contact is established between the host and the parasite. Therefore, instead of reducing the germination of parasitic seeds, inhibition of haustorium formation via several Haustorium Induction Factors (HIFs) could be also another strategy for crop resistance. HIFs are released from the parasitic plants to enable haustoria penetration into host organs following haustorium attachment [35]. A quinone molecule, 2,6-dimethoxy-1,4-benzoquinone (DMBQ), released from sorghum root extract was the first defined HIF molecule in the parasitic plants. DMBQ was recorded to induce both obligate and facultative parasitic haustoria development within hours [36]. Interestingly, two genes (TvQR1 and TvQR2) encoding a type of quinone oxidoreductases in *Triphysaria versicolor* (facultative parasitic plants) were identified to be responsible for the induction of DMBQ. TvQR1 was estimated to generate the first step in the signal-transduction pathway for haustorium development while TvQR2 was thought to be responsible for the removal of the signal with a detoxification system [37]. RNA interference (RNAi) technology was used to silence TvQR1and TvQR2 transcripts in *Triphysaria* roots for the evaluation of their functional role in haustoria formation. In the study, RNAi vectors designed to target TvQR1 and TvQR2 were transformed into *Triphysaria* roots via *Agrobacterium rhizogenes.* The competence of transgenic *Triphysaria* roots was accomplished by *Arabidopsis* root contact test. The results of haustoria formation in response to host contact indicated a significant decrease in haustorium development in roots silenced for QR1 but not in roots silenced for QR2. This experiment implicates QR1 as the first identified gene necessary for the redox bioactivation of haustorial-inducing factors [38].

2.1.2 Genome Editing to Enhance Post-attachment Resistance in Crops

Even after the seeds of parasitic weeds germinate and attach to the host roots, hormone-mediated defence response in host plants can be triggered to cope with this parasite attack. Defence-related plant hormones, especially jasmonic acid (JA) and salicylic acid (SA), are known to contribute to crop resistance to parasitic weeds by direct inhibition of their contact with the host or enhancing the host plant vascular body. For instance, treatment of SA on red clover roots reduced the houstaria formation of *O. minor* by lignification in the host endodermis cell layers [39]. Induction of SA and pathogenesis-related gene transcripts were also reported to enhance the resistance response of sunflowers to *O. cumana* [40]. JA is known to be involved in cell wall damage–induced lignin biosynthesis and, therefore, it directly contributed to the host resistance by a hypersensitive-like response in plants [35]. Several studies have concentrated on the loss of function analysis of these hormones in crop species. For instance, Brading et al. (2000) created a transgenic tomato expressing salicylate hydroxylase. This enzyme converted SA immediately to

inactive catechol and created SA-deficient tomato [41]. In another study, the radiation-based mutation was created on the tomato CORONATINE-INSENSITIVE1 gene which reduced the expression of JA-responsive genes [42]. Runyon et al. (2010) used both mutant tomato genotypes to test their resistance to parasitic weeds. The results indicated that parasitic plants grown on the SA and JA mutant tomatoes were more aggressive and had more biomass than those grown on their wild-type counterparts [43].

In another study, RNAi was used to knock down (kd) the expression JA-inducible WRKY transcription factor in rice [44]. Remarkably, WRKY45-kd rice genotypes exhibited severe susceptibility to *S. hermonthica*. The size and number of the *S. hermonthica* seedlings that attached and developed in mutant rice genotypes were almost threefold higher compared with wild-type rice. Therefore, a reduction in endogenous JA levels resulted in enhanced susceptibility to *S. hermonthica.* External application of JA was found to completely recover the resistance ability of mutant rice to this parasitic plant [5, 45, 46].

Hypersensitive response (HR) is a common mechanism which leads to localized cell death and necrosis at the infectious site to defend against pathogens and prevent the spread of infection in the plant body [47]. Some studies indicated that hosts have evolved the ability to detect parasitic plant–specific signals to initiate signal transduction cascades that lead to an HR and prevent the haustorium penetration process of parasitic plants [48]. For instance, a cowpea cultivar resistant to *S. gesnerioides* was found to trigger a downstream signalling cascade to activate the avirulence (Avr) proteins, which is a positive regulator of the HR [49]. A similar case was also reported for the interaction between sunflowers and *O. cumana*. Sunflower recognizes an avirulence protein (AVROR7) from *O. cumana* via the kinase domain of the HAOR7 protein, which then activates signalling cascades for the induction of HR [50].

3 Prospective Applications of Genome Editing-Based Systems for the Control of Parasitic Plants in Crops

Genome editing-based strategies used to silence host or parasite genes may serve as an important strategy to obtain more effective and durable crop resistance to parasitic weeds. Unlike other types of natural resistance, genome editing-based strategies could be easily applied to susceptible crop cultivars. Moreover, parasite species share homology in the target gene sequence and, therefore, an established strategy could be effective against other parasitic weed species. For instance, Aly et al. (2009) revealed that M6PR gene has high sequence similarity between *P. aegyptiaca, P. ramosa* and *O. crenata* species, suggesting that a single RNAi or CRISPR-based protocol can be used to manipulate sensitivity to several species at the same time. In addition, multiple candidate parasitic genes can be cloned in the same construct and pyramided in susceptible hosts for gene editing, thus

significantly reducing the risk of the development of new virulent parasitic weeds [28]. A limited number of studies described in-vitro transformation and regeneration systems for *P. aegyptiaca* [51]. and *P. ramosa* [52]. However, the establishment of genome editing-based protocols needs more effort to acquire high-quality genomic data, reverse-genetics information and reliable parasite transformation systems to target key processes in the host–parasite interaction. Nevertheless, current molecular knowledge could still be a target for CRISPR-based genome editing studies. For instance, RNAi or CRISPR-based silencing of parasite genes functional in host cell-wall degradation and penetration (pectin methylesterase, polygalacturonase, rhamnogalacturonase or peroxidases) may reduce host penetrability during haustorium formation and initial parasitic stages. Another promising strategy could be the reduction of the parasite's seed productivity by silencing the genes involved in flower and seed formation pathways. A general conclusion emerging from research in the last 20 years is that the intimate physical and physiological connection of parasites with their hosts can be used as a key target point, where its greatest potential lies in developing parasite resistance utilizing molecule or macromolecule exchange. Since host-released stimulants such as hormones, seconder metabolites and signals are the most critical key factor in germination and infestation of parasitic weeds, genes involved in these stimulant biosynthesis communications, signalling, and perception should be further studied and identified for the best targets for genome editing. A more thorough understanding of molecular interaction between host and parasite will enable manipulation of their in-vivo interactions and activity to control root parasitic weed germination without damaging the crop plant.

References

1. Hu, L., Wang, J., Yang, C., Islam, F., Bouwmeester, H.J., et al.: The effect of virulence and resistance mechanisms on the interactions between parasitic plants and their hosts. Int. J. Mol. Sci. **21**, 9013 (2020)
2. Fernández-Aparicio, M., Delavault, P., Timko, M.P.: Management of infection by parasitic weeds: a review. Plants (Basel). **9**(9), 1184 (2020)
3. Delavault, P.: Are root parasitic plants like any other plant pathogens? New Phytol. **226**, 641–643 (2020)
4. Mutuku, J.M., Cui, S., Yoshida, S., Shirasu, K.: Orobanchaceae parasite–host interactions. New Phytol. **230**, 46–59 (2021)
5. Yıldırım, K., Kavas, M., Kaya, R., Seçgin, Z., Can, C., Sevgen, I., Saraç, G., Tahan, V.: Genome-based identification of beet curly top Iran virus infecting sugar beet in Turkey and investigation of its pathogenicity by agroinfection. J. Virol. Methods. **300**, 114380 (2021)
6. Fishman, M.R., Shirasu, K.: How to resist parasitic plants: pre- and post-attachment strategies. Curr. Opin. Plant Biol. **62**, 102004 (2021)
7. Panstruga, R., Moscou, M.J.: What is the molecular basis of nonhost resistance? Mol. Plant-Microbe Interact. **33**, 1253–1264 (2020)
8. Aksoy, E., Yildirim, K., Kavas, M., Kayihan, C., Yerlikaya, B.A., Calik, I., Sevgen, I., Demirel, U.: General guidelines for CRISPR/Cas-based genome editing in plants. Mol. Biol. Rep. **49**, 12151–12164 (2022)

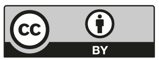
9. Yıldırım, K., Kavas, M., Küçük, İ.S., Seçgin, Z., Saraç, Ç.G.: Development of highly efficient resistance to Beet Curly Top Iran Virus (Becurtovirus) in Sugar Beet (B. vulgaris) via CRISPR/Cas9 system. Int. J. Mol. Sci. **24**(7), 6515 (2023)
10. Miladinovic, D., Antunes, D., Yildirim, K., Bakhsh, A., Cvejić, S., Kondić-Špika, A., Jeromela, A.M., Opsahl-Sorteberg, H.G., Zambounis, A., Hilioti, Z.: Targeted plant improvement through genome editing: from laboratory to field. Plant Cell Rep. **40**, 935–951 (2021)
11. Xie, X., Yoneyama, K., Yoneyama, K.: The strigolactone story. Annu. Rev. Phytopathol. **48**, 93–117 (2010)
12. Chadwick, M., Trewin, H., Gawthrop, F., Wagstaff, C.: Sesquiterpenoids lactones: benefits to plants and people. Int. J. Mol. Sci. **14**(6), 12780–12805 (2013)
13. Cheng, X., Floková, K., Bouwmeester, H., Ruyter-Spira, C.: The role of endogenous strigolactones and their interaction with ABA during the infection process of the parasitic weed Phelipanche ramosa in tomato plants. Front. Plant Sci. **8**, 392 (2017)
14. Raupp, F.M., Spring, O.: New sesquiterpene lactones from sunflower root exudate as germination stimulants for Orobanche cumana. J. Agri. Food Chem. **61**, 10481–10487 (2013)
15. López-Ráez, J.A., Matusova, R., Cardoso, C., Jamil, M., Charnikhova, T., et al.: Strigolactones: ecological significance and use as a target for parasitic plant control. Pest Manag. Sci. **65**, 471–477 (2009)
16. Spring, O.: Sesquiterpene lactones in sunflower oil. LWT. **142**, 111047 (2021)
17. Ashapkin, V.V., Kutueva, L.I., Aleksandrushkina, N.I., Vanyushin, B.F., Teofanova, D.R., Zagorchev, L.I.: Genomic and epigenomic mechanisms of the interaction between parasitic and host plants. Int. J. Mol. Sci. **24**, 2647 (2023)
18. Vogel, J.T., Walter, M.H., Giavalisco, P., Lytovchenko, A., Kohlen, W., Charnikhova, T., Simkin, A.J., Goulet, C., Strack, D., Bouwmeester, H.J., et al.: SlCCD7 controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. Plant J. **61**, 300–311 (2010)
19. Kohlen, W., Charnikhova, T., Lammers, M., Pollina, T., Tóth, P., Haider, I., Pozo, M.J., Maagd, R.A., Ruyter-Spira, C., Bouwmeester, H.J., et al.: The tomato CAROTENOID CLEAVAGE DIOXYGENASE 8 (SlCCD8) regulates rhizosphere signaling, plant architecture and affects reproductive development through strigolactone biosynthesis. New Phytol. **196**, 535–547 (2012)
20. Aly, R., Dubey, N.K., Yahyaa, M., Abu-Nassar, J., Ibdah, M.: Gene silencing of CCD7 and CCD8 in Phelipanche aegyptiaca by tobacco rattle virus system retarded the parasite development on the host. Plant Signal. Behav. **9**(8), e29376 (2014)
21. Butt, H., Jamil, M., Wang, J.Y., Al-Babili, S., Mahfouz, M.: Engineering plant architecture via CRISPR/Cas9-mediated alteration of strigolactone biosynthesis. BMC Plant Biol. **18**, 174 (2018)
22. Bari, V.K., Nassar, J.A., Kheredin, S.M., et al.: CRISPR/Cas9-mediated mutagenesis of CAROTENOID CLEAVAGE DIOXYGENASE 8 in tomato provides resistance against the parasitic weed Phelipanche aegyptiaca. Sci. Rep. **9**, e11438 (2019)
23. Wakabayashi, T., Hamana, M., Mori, A., et al.: Direct conversion of carlactonoic acid to orobanchol by cytochrome P450 CYP722C in strigolactone biosynthesis. Sci. Adv. **5**, eaax9067 (2019)
24. Lee, H.A., Lee, H.Y., Seo, E., Lee, J., Kim, S.B., et al.: Current understandings of plant nonhost resistance. Mol. Plant-Microbe Interact. **30**, 5–15 (2017)
25. Mashiguchi, K., Seto, Y., Yamaguchi, S.: Strigolactone biosynthesis, transport and perception. Plant J. **105**(2), 335–350 (2021)
26. de Framond, A., Rich, P.J., McMillan, J., Ejeta, G.: Effects on Striga parasitism of transgenic maize armed with RNAi constructs targeting essential S. asiatica genes. In: Gebisa, E., Jonathan, G. (eds.) Integrating New Technologies for Striga Control, pp. 185–196. World Scientific, Singapore (2007)
27. Aly, R., Matzrafi, M., Bari, V.K.: Using biotechnological approaches to develop crop resistance to root parasitic weeds. Planta. **253**(5), 97 (2021)

28. Aly, R., Cholakh, H., Joel, D.M., Leibman, D., Steinitz, B., Zelcer, A., Naglis, A., Yarden, O., Gal-On, A.: Gene silencing of mannose 6-phosphate reductase in the parasitic weed Orobanche aegyptiaca through the production of homologous dsRNA sequences in the host plant. Plant Biotechnol. J. **7**, 487–498 (2009)
29. Dubey, N.K., Eizenberg, H., Leibman, D., Wolf, D., Edelstein, M., Abu-Nassar, J., Marzouk, S., Gal-On, A., Aly, R.: Enhanced host-parasite resistance based on down-regulation of Phelipanche aegyptiaca target genes is likely by mobile small RNA. Front. Plant Sci. **8**, 1574 (2017)
30. Seçgin, Z., Kavas, M., Yildirim, K.: Optimization of agrobacterium-mediated transformation and regeneration for CRISPR/Cas9 genome editing of commercial tomato cultivars. Turk. J. Agric. For. **45**, 704–716 (2021)
31. Secgin, Z., Uluisik, S., Yıldırım, K., Abdulla, M.F., Mostafa, K., Kavas, M.: Genome-wide identification of the Aconitase gene family in tomato (Solanum lycopersicum) and CRISPR-based functional characterization of SlACO2 on male-sterility. Int. J. Mol. Sci. **23**(22), 13963 (2022)
32. Vurro, M., Boari, A., Evidente, A., Andolfi, A., Zermane, N.: Natural metabolites for parasitic weed management. Pest Manag. Sci. **65**(5), 566–571 (2009)
33. Serghini, K., de Luque, A.P., Castejón-Muñoz, M., García-Torres, L., Jorrín, J.V.: Sunflower (Helianthus annuus L.) response to broomrape (Orobanche cernua Loefl.) parasitism: induced synthesis and excretion of 7-hydroxylated simple coumarins. J. Exp. Bot. **52**, 2227–2234 (2001)
34. Hamamouch, N., Westwood, J.H., Banner, I., Cramer, C.L., Gepstein, S., Aly, R.: A peptide from insects protects transgenic tobacco from a parasitic weed. Transgenic Res. **14**(3), 227–236 (2005)
35. Albanova, I.A., Zagorchev, L.I., Teofanova, D.R., Odjakova, M.K., Kutueva, L.I., Ashapkin, V.V.: Host resistance to parasitic plants—current knowledge and future perspectives. Plan. Theory. **12**, 1447 (2023)
36. Goyet, V., Wada, S., Cui, S., Wakatake, T., Shirasu, K., et al.: Haustorium inducing factors for parasitic Orobanchaceae. Front. Plant Sci. **10**, 1056 (2019)
37. Matvienko, M., Wojtowicz, A., Wrobel, R., Jamison, D., Goldwasser, Y., Yoder, J.I.: Quinone oxidoreductase message levels are differentially regulated in parasitic and non-parasitic plants exposed to allelopathic quinones. Plant J. **25**(4), 375–387 (2001)
38. Bandaranayake, P.C., Filappova, T., Tomilov, A., Tomilova, N.B., Jamison-McClung, D., Ngo, Q., Inoue, K., Yoder, J.I.: A single-electron reducing quinone oxidoreductase is necessary to induce haustorium development in the root parasitic plant Triphysaria. Plant Cell. **22**(4), 1404–1419 (2010)
39. Kusumoto, D., Goldwasser, Y., Xie, X., Yoneyama, K., Takeuchi, Y., Yoneyama, K.: Resistance of red clover (Trifolium pratense) to the root parasitic plant Orobanche minor is activated by salicylate but not by jasmonate. Ann. Bot. **100**, 537–544 (2007)
40. Letousey, P., De Zelicourt, A., Vieira Dos Santos, C., Thoiron, S., Monteau, F., Simier, P., Thalouarn, P., Delavault, P.: Molecular analysis of resistance mechanisms to Orobanche cumana in sunflower. Plant Pathol. **56**, 536–546 (2007)
41. Brading, P.A., Hammond-Kosack, K.E., Parr, A., Jones, J.D.G.: Salicylic acid is not required for Cf-2- and Cf-9-dependent resistance of tomato to Cladosporium fulvum. Plant J. **23**, 305–318 (2000)
42. Li, L., Zhao, Y.F., McCaig, B.C., Wingerd, B.A., Wang, J.H., Whalon, M.E., Pichersky, E., Howe, G.A.: The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell. **16**, 126–143 (2004)
43. Runyon, J.B., Mescher, M.C., Felton, G.W., De Moraes, C.M.: Parasitism by Cuscuta pentagona sequen- tially induces JA and SA defence pathways in tomato. Plant Cell Environ. **33**, 290–303 (2010)
44. Shimono, M., Sugano, S., Nakayama, A., et al.: Rice WRKY45 plays a crucial role in Benzothiadiazole-inducible blast resistance. Plant Cell. **19**(6), 2064–2076 (2007)

45. Mutuku, J.M., Cui, S., Hori, C., Takeda, Y., Tobimatsu, Y., et al.: The structural integrity of lignin is crucial for resistance against Striga hermonthica parasitism in rice. Plant Physiol. **179**, 1796–1809 (2019)
46. Mutuku, J.M., Yoshida, S., Shimizu, T., Ichihashi, Y., Wakatake, T., et al.: The WRKY45-dependent signaling pathway is required for resistance against Striga hermonthica parasitism. Plant Physiol. **168**, 1152–1163 (2015)
47. Yildirim, K., Boylu, B., Atici, E., TKahraman Akkaya, M.S.: In Turkish wheat cultivars the resistance allele of LR34 is ineffective against leaf rust. J. Plant Dis. Prot. **119**, 135–141 (2012)
48. Saucet, S.B., Shirasu, K.: Molecular parasitic plant–host interactions. PLoS Pathog. **12**, e1005978 (2016)
49. Su, C., Liu, H., Wafula, E.K., Honaas, L., de Pamphilis, C.W., Timko, M.P.: SHR4z, a novel decoy effector from the haustorium of the parasitic weed Striga gesnerioides, suppresses host plant immunity. New Phytol. **226**(3), 891–908 (2020)
50. Duriez, P., Vautrin, S., Auriac, M.C., Bazerque, J., Boniface, M.C., et al.: A receptor-like kinase enhances sunflower resistance to Orobanche cumana. Nat. Plant. **5**, 1211–1215 (2019)
51. Fernandez-Aparicio, M., Rubiales, D., Bandaranayake, P.C., Yoder, J.I., Westwood, J.H.: Transformation and regeneration of the holoparasitic plant Phelipanche aegyptiaca. Plant Methods. **7**, 36 (2011)
52. Libiakova, D., Ruyter-Spira, C., Bouwmeester, H.J., Matusova, R.: Agrobacterium rhizogenes transformed calli of the holoparasitic plant Phelipanche ramosa maintain parasitic competence. Plant Cell Tissue Organ Cult. **135**, 321–329 (2018)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.



Part III
Policies and Regulations

Chapter 25
Genome Editing in Biotech Regulations Worldwide

Thorben Sprink and Ralf Wilhelm

Abstract Since the introduction of genome editing techniques in breeding and the first commercial products on the market, various governments or jurisdictions have attempted to clarify the legal classification of genome editing in relation to their genetic engineering regulations. Only a few countries, including Europe, fully apply their strict genetic engineering laws to genome-edited organisms or products derived from them. Most countries with liberal regulations base classification on the absence of foreign DNA in the final product (including the USA and Canada, which de facto have no specific GMO laws). Countries such as Australia and Japan have introduced subcategories when sequence templates have been used in the genome editing process. Several countries, including Europe, are in the process of revising their GMO legislation. The international legislative landscape is thus dynamic. The heterogeneity of regulatory regimes poses a challenge for international trade. This chapter summarises the status as of June 2023 and provides a brief introduction to the main legal concepts.

1 Introduction

The commercial use of genome editing technologies is closely linked to national legislation in the field of genetic engineering and biotechnology, as well as to consumer acceptance. The first genome editing techniques in plant breeding were developed before the beginning of this century, but it was not until the use of CRISPR-Cas systems that they became widely established in the range of applications. Since the technique represents an active intervention in the genome to insert a modification at a specific genomic site, the question also arose as to how the techniques should be classified under genetic engineering law. The rapid development of genome editing techniques poses a challenge to national regulations and

T. Sprink · R. Wilhelm (✉)
Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants,
Institute for Biosafety in Plant Biotechnology, Quedlinburg, Germany
e-mail: ralf.wilhelm@julius-kuehn.de

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_25

Table 25.1 Genome-edited products/organisms released to national markets (until May 2023)

Product	Target gene(s)	Method	Phenotype	Market release	Company
Soybean [2, 3]	FAD2-1A, FAD2-1B	TALEN	High oleic acid	2019[a]	Calyxt (USA)
Tomato [4]	GAD3	CRISPR-Cas9	High GABA	2021	Sanatech Seeds (Japan)
Red sea bream [5]	Myostatin	CRISPR-Cas9	More muscle mass	2021	Regional Fish Institute (Japan)
Tiger puffer fish [5]	Leptin receptor	CRISPR-Cas9	Increased appetite	2021	Regional Fish Institute (Japan)
Mustard greens [6]	Myrosinase	CRISPR-Cas9	Reduced pungency	2023	Pairwise (USA)

[a]The product pipeline and business model of Calyxt has changed. The product was marketed by Calyxt until 2021

international treaties worldwide, as most existing laws and regulations do not provide explicit reference to the techniques and their applications as such. To date few genome-edited products have entered the market in the North America (USA, Canada) and Japan (Table 25.1).

Current legislations or guidelines may distinguish between the genome editing techniques, SDN-1, SDN-2, SDN-3, respectively [1] and whether foreign DNA and/or sequence templates have been used during generation of the genome-edited organism.

2 Countries/Regions with Strict Regulations

Few countries/regions i.e. European Union (EU), New Zealand, South Africa, Venezuela, Peru and Costa Rica (Fig. 25.1) consider genome edited organisms (plant, animals, respectively; microorganisms maybe unclear) as GMO sensu stricto [7–14]. For these countries genome edited organisms bear broader challenges for law enforcement and compliance especially in relation to international trade. Though mutations can be detected with established molecular biotic methods the identification of the technique or the natural event that caused the mutation is bound to a priori information about the uniqueness of the event and the modified genome sequence(s) [15, 16]. Such data are rarely readily available and hence the detection of unintended residues from (prohibited) genome-edited plants in internationally traded commodities becomes erratic. This problem of law enforcement cannot be solved by labelling regulations and it is also challenging liability and redress. Nevertheless, labelling of "GMO" and the provision of a specific detection method prior to market release is mandatory e.g. in the EU regulatory framework. The EU as well as New Zealand are currently reviewing their respective GMO-regulations. The European Commission has published a legislative proposal in July 2023 that

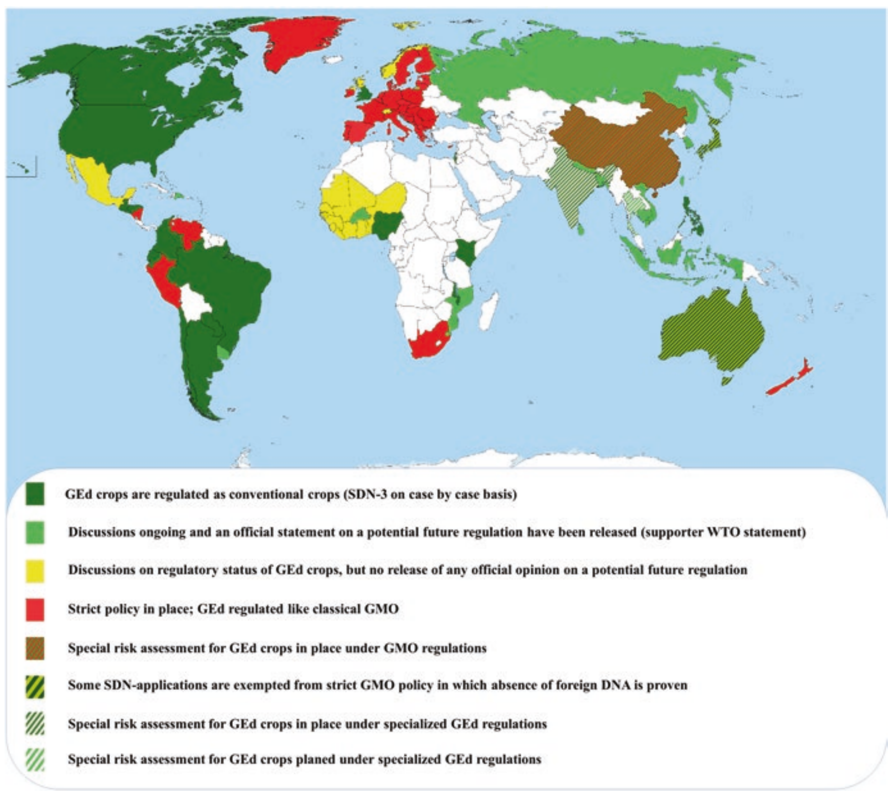


Fig. 25.1 Regulations on genome edited food around the world (updated [7]). The regulatory status of genome-edited (GEd) crops on a global schema

suggests regulatory relaxations for genome-edited and cisgenetic plants and starts the legislation process in the EU [17]. De facto or purposely interrelated, the EU initiated two further policy actions. There is the "Sustainable food system framework initiative" [18] as a framing regulation and the "Revision of the plant and forest reproductive material legislation" [19]. The latter is less acknowledged in the public discussions, but of considerable relevance for the breeding sector as well.

Several countries are developing a liberal handling of organisms derived with new genomic techniques (NGTs) based on the Cartagena Protocol [20] that defines a living modified organism as "any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology", emphasizing the novelty of a (re)combined sequence. Nevertheless, different countries tend to deal with the novelty aspect considerably differently. This raises challenges for law enforcement, compliance and liability that result when the specific genome editing method would need to be identified from a DNA-sequence of a sample from unknown products or commodities.

3 Countries/Regions with Liberal Regulations for Specific SDN Applications

In Japan three genome-edited organisms have already been introduced into the market (Table 25.1) and crosses using these already registered organisms do not need to be evaluated again and are free from obligations. Japan [21] refers to the Cartagena protocol and exempt SDN-1, SDN-2 and Oligonucleotide-Directed Mutagenesis (ODM) as long as the absence of foreign DNA integration is proven in the latter cases. A guidance on how to proof the absence of foreign DNA with legal sufficiency is pending.

Australia [22] published legal guidelines that essentially exempt organisms derived by SDN-1 mechanisms from restrict regulations of GMO (i.e. if an external sequence template was not used and if the organisms are free from foreign sequences).

4 Countries with Liberal Regulations (for Organisms Free of Foreign DNA)

Several countries followed Argentina deregulating organisms derived by genome editing if they do not contain introduced foreign DNA-sequences. Argentina has updated the regulation several times and it now includes animals and microorganisms [23]. Nevertheless, a pre-assessment or notification may be necessary to assess compliance. This does not mean that sequence information is published as such. Since 2015 more than 35 Prior Consultation Instances has been raised, 66% of those from local developers. Similar regulations are established in Chile in 2017 [24], Brazil and Colombia in 2018 [24], Paraguay, Honduras, Guatemala and El Salvador in 2019 [24].

Likewise, Nigeria [25], Kenia [26] and Malawi [27] published guidelines based on notification and a case-by-case assessment which comes to a decision within few weeks. Essentially, the regulations refer to the absence of “novel combination of DNA” in the genomes whereas small InDels and substitutions are not seen as such.

There are no specific legislations for bioengineering in Canada and USA and at least one product is currently on the market (see Table 25.1). With regards to seeds, Canada follows a product based approach to regulation as recently confirmed by the Canadian Food Inspection Agency (CFIA): “*It is the scientific opinion of the CFIA that genome editing technologies do not present any unique or specifically identifiable environmental or human health safety concerns as compared to other technologies of plant development. For this reason, genome-edited plants are regulated using a product-based approach, like any other product of plant breeding. Namely, it is the traits that a plant exhibits and whether these traits would have a significant negative impact on environmental safety that are used to determine whether a plant would be subject to Part V of the Seeds Regulations*.” [28] Nevertheless, following

the Division 28 of Part B of the Food and Drug Regulations about novel foods Health Canada states "*Foods derived from plants that have been genetically modified such that they contain foreign DNA in the final plant product require pre-market notification and assessment as novel foods*." [29] "Novelty" essentially relates to terms like "history of safe use" and "familiarity" with the composition of the final food product. Residues of the CRISPR/Cas-System in the genome would trigger additional safety assessments like any other transgenic organism.

The legislation and regulation of genome-edited crops in the USA is more complex while a specific regulation for bioengineering does not exist but a "Coordinated Framework for regulation of Biotechnology" [30]. Based on the Plant Protection Act (PPA) the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture (USDA/APHIS), based on the Food, Drug and Cosmetics Act (FDCA), the Food and Drug Agency (FDA) and based on the FDCA and the Federal Insecticide, Fungicide, and Rodenticide Act [FIFRA of the Environmental Protection Agency (EPA)] regulate products of biotechnology applications. For several years there are governmental activities to streamline the application. Since 2021 USDA APHIS implemented the Revised Biotechnology Regulations (previously SECURE rule) to provide clear and efficient regulatory pathways for applicants, when the plant products are unlikely to pose a plant pest risk. Products derived by means of genome editing under in most conditions are free from restrictions based on the PPA when changes in the plant product's genome are either deletion(s), targeted substitutions of a single base pair or solely introductions from sequences derived from the plant's natural gene pool or edits from sequences which are known to correspond in the plants natural gene pool.

The Philippines established a procedure to regulate genome-edited plants based on the key criteria are whether they possess novel combinations of genetic material not achievable by conventional breeding [31].

5 Other Regulatory Frameworks

China has released guidelines for the safety evaluation of genome-edited plants for agricultural use that do not harbour exogenous DNA-sequences (SDN-1, SDN 2). It provides a tiered assessment based on the risk profile of the target trait. The first category (low risk) refers to plants/traits that do not increase the risk to environmental and food safety, the second to increased environmental risks, the third to increased food safety risks, and the fourth to increases in both environmental and food risks. Different requirements apply for cultivation and or import aside from some general items describing the plant and trait: (1) molecular characterization, editing method applied, data on the edited sequence, presence of residual vector sequences, and off-target analysis; (2) stability of the edit and the trait over at least three generations. At present the guidelines do not specify how to classify a product according to the four categories, what may indicate a case-by-case decision procedure. These requirements are in line with the ones requested in the guidelines for safety

evaluation of GMOs. However, genome-edited crops are still managed under GMO regulation, but may require much less complicated food and environmental safety evaluations compared to classical GMOs and may reduce time for regulation from six down to one to two years. However, these guidelines also differentiate between local and foreign developers as foreign firms are not allowed to invest in Chinas biotech sector [32].

A risk based concept was also implemented by India and an appropriate tiered based risk assessment is foreseen to categorizing genome editing in three categories. In a first category, products should be addressed with single or few base pair edits or In/Dels. The assessment confirms targeted edits as well as absence of any biological relevant off target genomic changes, and, if necessary, a phenotypic equivalence to a comparator will be checked on a case by case basis. The second category addressed targeted base pair edits in which the assessment is compiled by phenotypic equivalence and trait efficacy through appropriate contained and/or confined field trials. The third category addresses products harbouring targeted edit(s) with synthetic/foreign DNA. The assessment is the same as for traditional GMOs [33].

Thailand also drafted a risk assessment of genome editing products in which a liberal assessment of SDN1 has been foreseen. However, this draft has not passed official release, yet [34].

6 Other Countries – Ongoing Consultations

In Europe the policy of the EU is considerably important for non-EU countries – which are trading partners. Nevertheless, England, Norway and Switzerland have discussed somewhat differing regulations for genome-edited plants.

The Norwegian Biotechnology Advisory Board initiated a reconsideration of the regulatory framework for GMOs. It sketched a tiered scheme for risk assessment of GMOs including genome-edited organisms combining biological, environmental as well as social criteria [35].

The Swiss Genetic Technology Act (Art. 37a) [36] mandates the Federal Council to develop a draft decree for a risk-based approval procedure for transgene-free GMOs by mid-2024. The current genetic engineering act considers genome-edited organisms as GMO and the year-long moratorium for cultivation of GMO in Switzerland will apply. Nevertheless, field trials are supported.

In 2020 UK left the EU and England – independent of Scotland, Wales and Northern Ireland – moved towards specific regulations for genome-edited organisms. On 23rd March 2023 the “Genetic Technology (Precision Breeding) Act 2023” [37] came into force. It allows for genetic changes that could also have been produced naturally or through “traditional” breeding. It rules that genome-edited (precision bred) plants and animals can be released or marketed in England based on notification and risk assessment provisions.

It is expected that South Korea as well as Taiwan intend to publish clarifications on the status of genome-edited organisms in 2023. Several African countries are reconsidering the regulatory status of GE plants, but it remains open when decisions will be taken.

7 Compliance, Law Enforcement, Detection and Identification

As mentioned above, the legal framework is closely linked to the issue of enforcement and compliance as well as labelling demands, which in turn is linked to the issue of detection, i.e., identification of the genome editing process associated with the DNA sequence of an organism. Unlike organisms obtained by classical transgenesis, organisms edited by ODM, SDN-1, or SDN-2 do not contain sequence elements belonging to elements associated with tranformations (e.g. S35-promotor, Nos-terminator) that simplify broader screening. Moreover, a targeted mutation often does not differ from a random mutation. Hence, a precondition for identification is the information about the uniqueness of a sequence caused by genome editing. The unique sequence length cannot be freely set as recombination events (crossover at meiosis during seed propagation) as well as random mutations may alter the sequence "naturally" [38]. Even large modifications e.g. introgressions, occur in conventional breeding programs [39]. Therefore, there are calls for an international sequence database [40] for unique sequences that identify a genome-edited organism to support screening and detection. The crucial dependence on this information and detection challenges are nicely depicted by the debate about the detection of Cibus herbicide tolerant canola. There is no doubt that any SNP can be sensitively detected in a sample with mixed background. Since at various time Cibus provided contradictory information about the origin of the SNP in its modified canola variety, the sequence itself does not reveal the actual process of modification [41–44]. For genome-edited organisms that are not considered GMOs in various legislations there is neither a legal obligation to pass detailed sequence information to public international repositories nor an obligation to provide a detection method, as is requested for market release of GMOs in the EU. Within a territory with a uniform legal basis (e.g. in the EU), the establishment of a register may be considered possible, but considering international trade with mixed commodities, law enforcement is challenging. In jurisdictions with differing regulations for SDN-1 and SDN-2 the handling of genome-edited organisms will become even more problematic. In addition, laws that refer to equivalence of genome-edited sequences with sequences that may be generated through conventional breeding will need to specify borderlines. As large introgressions (>>1000 bp) occur naturally and deliberately in conventional breeding, the legally fixed sequence length may affect trade with conventional breeding products as well.

A positive or negative certification regime may be considered that is based on documentation. Such certification systems have been established e.g. in the organic food sector or for certified regional products. To scrutinize detailed compliance, audits based on extended documentation at each step of the supply or value chain would be necessary. Employing blockchain-based tracking systems is recently discussed for agro-food value chains [e.g. 45]. Nevertheless, monitoring the actual physical compliance would need appropriate identification methods (see above).

8 Conclusions

The majority of countries currently do not explicitly regulate genome edited organisms – for various reasons. The legal practice may stay unclear for some countries for the next years. Several major players in international trade (of agricultural goods) already clarified their legal classification of genome-edited organisms in specific legislations, guidance documents, decrees or else related to the handling of biotechnology and bioengineering (GMO). The countries are applying separate regulatory requirements for organisms that do not harbour foreign gene sequences while strict GMO regulations apply in a few countries and the EU. This challenges international trade and national law enforcement and compliance (detection and identification of genome-edited organisms) due to the international heterogeneity of regulations. Hence, there are frequent calls for international harmonisation of legislation on genome-edited organisms to end divergent national regulations.

This situation is likely continuing for at least a few more years, affecting international trade between some countries and preventing or delaying the application and use of genome editing and its products in regions with restrictive regulations. Therefore, the prospects and progress of genome editing in breeding will vary from region to region and also for international and regional players.

Acknowledgements The paper is a continuing update of the initial work performed in the course of the projects "CHIC" funded by the European Union's Horizon 2020 research and innovation programme, grant agreement No. 760891 and "ELSA-GEA" funded by the German Federal Ministry of Education and Research (BMBF) grant agreement No. (01GP1613B).

References

1. Podevin, N., Davies, H.V., et al.: Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. Trends Biotechnol. **31**, 375–383 (2013). https://doi.org/10.1016/j.tibtech.2013.03.004
2. Haun, W., Coffman, A., et al.: Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol. J. **12**, 934–940 (2014). https://doi.org/10.1111/pbi.12201

3. U.S. Food and Drug Administration: Biotechnology Notification File No. 000164: High oleic acid soybean, FAD2KO (2019)
4. Waltz, E.: GABA-enriched tomato is first CRISPR-edited food to enter market. Nat. Biotechnol. **40**, 9–11 (2022). https://doi.org/10.1038/d41587-021-00026-2
5. Japan embraces CRISPR-edited fish: Nat. Biotechnol. **40**, 10 (2022). https://doi.org/10.1038/s41587-021-01197-8
6. Karlson, D., Mojica, J.P., et al.: Targeted mutagenesis of the multicopy Myrosinase gene family in Allotetraploid Brassica juncea reduces pungency in fresh leaves across environments. Plan. Theory. **11**, 2494 (2022). https://doi.org/10.3390/plants11192494
7. Sprink, T., Wilhelm, R., Hartung, F.: Genome editing around the globe: an update on policies and perceptions. Plant Physiol. **190**, 1579–1587 (2022). https://doi.org/10.1093/plphys/kiac359
8. European Parliament and the Council (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/2020/EEC. Official Journal of the European Communities L106/1–38
9. CURIA: Judgement of the Court (Grand Chamber) 25 July 2018 in Case C-528/16. https://curia.europa.eu/juris/document/document.jsf?mode=DOC&pageIndex=0&docid=204387&part=1&doclang=EN&text=&dir=&occ=first&cid=1044840. Accessed 25 May 2023
10. Kershen, D.L.: Sustainability council of New Zealand trust v. the environmental protection authority: genome editing technologies and the law. GM Crops Food. **6**, 216–222 (2015). https://doi.org/10.1080/21645698.2015.1122859
11. Republic of South Africa: South Africa's Regulatory Approach for New Breeding Techniques (2021). https://gain.fas.usda.gov/Download.aspx?p=1347&q=51ee2f85-c423-4b66-9bae-2813c7bc3f6c. Accessed 24 May 2023
12. US Department of Agriculture, Foreign Agricultural Service: Agricultural Biotechnology Annual. Report VE2022-0031 (2022). https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agricultural%20Biotechnology%20Annual_Caracas_Venezuela_VE2022-0031. Accessed 25 May 2023
13. Kuiken, T., Kuzma, J.: Genome Editing in Latin America: Regional Regulatory Overview. Inter-American Development Bank Environment, Rural Development and Disaster Risk Management Division Peru (2022)
14. US Department of Agriculture, Foreign Agricultural Service: Agricultural Biotechnology. Annual. Report CS2022-0019 (2022). https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agricultural%20Biotechnology%20Annual_San%20Jose_Costa%20Rica_CS2022-0019. Accessed 25 May 2023
15. Grohmann, L., Keilwagen, J., et al.: Detection and identification of genome editing in plants: challenges and opportunities. Front. Plant Sci. **10**, 236 (2019). https://doi.org/10.3389/fpls.2019.00236
16. European Network of GMO Laboratories (ENGL): Detection of food and feed plant products obtained by new mutagenesis techniques (JRC116289), 26 March 2019
17. European Commission: Farm to Fork Strategy: public consultation on new genomic techniques (2022). https://food.ec.europa.eu/system/files/2022-04/sc_modif-genet_pub-cons-factsheet.pdf. Accessed 13 May 2023
18. European Commission: Sustainable EU Food System – New Initiative (2021). https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/13174-Sustainable-EU-food-system-new-initiative_en. Accessed 25 May 2023
19. European Commission: Reproductive Material Legislation (2021). https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/13083-Plant-and-forest-reproductive-material-revised-rules-_en. Accessed 25 May 2023
20. Cartagena Protocol on Biosafety to the Convention on Biological Diversity. The Secretariat of the Convention on Biological Diversity, Montreal ISBN: 92-807-1924-6 (2000)
21. Kondo, K., Taguchi, C.: Japanese regulatory framework and approach for genome-edited foods based on latest scientific findings. Food Safety. **10**, 113–128 (2022)

22. Mallapaty, S.: Australian gene-editing rules adopt 'middle ground'. Nature. **23** (2019). https://doi.org/10.1038/d41586-019-01282-8
23. Goberna, M.F., Whelan, A.I., et al.: Genomic editing: the evolution in regulatory management accompanying scientific progress. Front. Bioeng. Biotechnol. **10**, 835378 (2022). https://doi.org/10.3389/fbioe.2022.835378
24. Menz, J., Modrzejewski, D., et al.: Genome edited crops touch the market: a view on the global development and regulatory environment. Front. Plant Sci. **11**, 586027 (2020)
25. Nigerian National Biosafety Management Agency (NBMA): National Genome editing Guidelines (2020). https://nbma.gov.ng/wp-content/uploads/2022/03/NATIONAL-GENE-EDITING-GUIDELINE.pdf. Accessed 24 May 2023
26. National Biosafety Authority of Kenia (NBAK): Guidelines for Determining the Regulatory Process of Genome Edited Organisms and Products in Kenya (2022). https://www.biosafetykenya.go.ke/images/GENOME-EDITING-GUIDELINES-FINAL-VERSION-25th-Feb-2022-03.pdf. Accessed 24 May 2023
27. ISAAA AfriCenter: Malawi's Genome Editing Guidelines key in promoting supportive environment for new breeding technologies (2022). https://africenter.isaaa.org/malawis-genome-editing-guidelines-key-promoting-supportive-environment-new-breeding-technologies. Accessed 23 May 2023
28. Canadian Food Inspection Agency: Directive 2009–09: Plants with novel traits regulated under Part V of the Seeds Regulations: Guidelines for determining when to notify the CFIA (2023). https://inspection.canada.ca/plant-varieties/plants-with-novel-traits/applicants/directive-2009-09/eng/1304466419931/1304466812439#a2. Accessed 24 May 2023
29. Health Canada: Guidelines for the Safety Assessment of Novel Foods (2022). https://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/guidance-documents/guidelines-safety-assessment-novel-foods-derived-plants-microorganisms/guidelines-safety-assessment-novel-foods-2006.html. Accessed 24 May 2022
30. Hoffman, N.E.: Revisions to USDA biotechnology regulations: the SECURE rule. Proc. Natl. Acad. Sci. U.S.A. **118**, e2004841118 (2021). https://doi.org/10.1073/pnas.2004841118
31. Republic of the Philippines, Office of the Secretary. Memorandum Circular No. 08, Series of 2022: Rules and procedures to evaluate and determine when products of plant breeding innovations (PBIs) are covered under the DOST-DA-DENR-DOH-DILG Joint Departments Cercular No.1 Series of 2021 (JDC1, s2021) based on the NCBP Resolution No. 1. Series of 2020
32. US Department of Agriculture, Foreign Agricultural Service: MARA Issues First Ever Gene-Editing Guidelines. Report Number: CH2022-0015 (2022). https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=MARA%20Issues%20First%20Ever%20Gene-Editing%20Guidelines_Beijing_China%20-%20People%27s%20Republic%20of_01-26-2022.pdf. Accessed 25 May 2023
33. Government of India, Office of Science and technology, Department of Biotechnology: Office Memorandum Sub.: Guidelines for the Safety Assessment of Genome Edited Plants. File No. PID-15011/1/2022-PPB-DBT (2022) https://dbtindia.gov.in/sites/default/files/Final_%2011052022_Annexure-I%2C%20Genome_Edited_Plants_2022_Hyperlink.pdf. Accessed 25 May 2023
34. US Department of Agriculture, Foreign Agricultural Service: Agricultural Biotechnology Annual. Report Number: TH2022-0071 (2022). https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agricultural%20Biotechnology%20Annual_Bangkok_Thailand_TH2022-0071.pdf. Accessed 25 May 2023
35. Norwegian Biotechnology Advisory Board: (A Forward-Looking Regulatory Framework for GMO (2018). https://www.bioteknologiradet.no/filarkiv/2019/03/2019-04-16-Genteknologiloven-sammendrag-ENGELSK.pdf. Accessed 24 May 2023
36. Swiss Federal Act on Non-Human Gene Technology (Gene Technology Act, GTA) of 21 March 2003 (Status as of 1 January 2022)
37. UK Genetic Technology (Precision Breeding) Act 2023, CHAPTER 6. https://www.legislation.gov.uk/ukpga/2023/6/enacted/data.pdf. Accessed 25 May 2023

38. Gürtler, P., Pallarz, S., et al.: Detection of commercialized plant products derived from new genomic techniques (NGT) – practical examples and current perspectives. Food Control. **152**, 109869 (2023). https://doi.org/10.1016/j.foodcont.2023.109869
39. Keilwagen, J., Lehnert, H., et al.: Detecting major introgressions in wheat and their putative origins using coverage analysis. Sci. Rep. UK. **12**, 1908 (2022). https://doi.org/10.1038/s41598-022-05865-w
40. Eckerstorfer, M.F., Dolezel, M., et al.: An EU perspective on biosafety considerations for plants developed by genome editing and other new genetic modification techniques (nGMs). Front. Bioeng. Biotechnol. **7**, 31 (2019). https://doi.org/10.3389/fbioe.2019.00031
41. Health Canada: Novel Food Information – Cibus Canola Event 5715 (2016). https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/novel-food-information-cibus-canola-event-5715-imidazolinone-sulfonylurea-herbicide-tolerant.html. Accessed 25 May 2023
42. Canadian Food Inspection Agency: DD 2013-100: Determination of the Safety of Cibus Canada Inc. (Incorporated)'s Canola (Brassica napus L. (Linnaeus)) Event 5715 (2020). https://www.inspection.gc.ca/plant-varieties/plants-with-novel-traits/approved-under-review/decision-documents/dd-2013-100/eng/1427383332253/1427383674669. Accessed 25 May 2023
43. Chhalliyil, P., Ilves, H., et al.: A real-time quantitative PCR method specific for detection and quantification of the first commercialized genome-edited plant. Foods. **9**, 1245 (2020). https://doi.org/10.3390/foods9091245
44. Weidner, C., Edelmann, S., et al.: Assessment of the real-time PCR method claiming to be specific for detection and quantification of the first commercialised genome-edited plant. Food Anal. Methods. **15**, 2107–2125 (2022). https://doi.org/10.1007/s12161-022-02237-y
45. Tyagi, K.: A global blockchain-based agro-food value chain to facilitate trade and sustainable blocks of healthy lives and food for all. Humanit. Soc. Sci. Commun. **10**, 196 (2023). https://doi.org/10.1057/s41599-023-01658-2

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 26
Interpreting Precision Breeding: Key Legal Concepts Under International Law and Current Domestic Regulatory Approaches in the Global South

André Rosado

Abstract In various countries, especially in the Global South, there is legal uncertainty about which products or organisms derived from precision breeding, also called new breeding technologies, are subject to biosafety regulation. It is not clear whether which precision breeding products are classified as Living Modified Organisms (LMOs), or Genetically Modified Organisms (GMOs), and therefore be subject to regulatory oversight under biosafety laws.

This section, under Chap. 3 of Policies and Regulations, provides an overview of key definitions under international and national legislation to clarify the regulatory status of precision breeding products. This is done by assessing provisions under international biosafety law and national legislation in selected countries in the Global South. The outcome of this section is to provide a baseline for further discussion about the regulatory status of precision breeding globally.

First, the background is presented, covering the development of international and national legal frameworks governing biosafety of LMOs and GMOs. Second, the legal definition of LMO and related terms under international law is discussed. Third, the GMO definition of national law in selected jurisdictions is presented. Fourth, a snapshot of the emergence of regulations governing precision breeding in the Global South is analyzed. Finally, key future perspectives to the regulatory status of precision breeding products are suggested.

A. Rosado (✉)
Faculty of Science, KU Leuven, Leuven, Belgium

Centro de Investigaciones de Zonas Aridas, Universidad Nacional Agraria La Molina, Lima, Peru
e-mail: ayrtonandre.rosadohuaynasi@student.kuleuven.be

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_26

1 International and National Legal Frameworks for Biosafety

Biotechnology products, such as LMOs and GMOs, are subject to regulation. Rules to regulate the safe use of LMOs and GMOs are established under international and national biosafety law.

For instance, the Convention on Biological Diversity (CBD) requires in Art. 8(g) that Parties "*to establish or maintain means to regulate, manage or control the risks associated with the use and release of living modified organisms resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account the risks to human health*".

In addition to the obligation pertaining to national biosafety systems, the Cartagena Protocol on Biosafety (CPB), a subsequent protocol to the CBD, governs the transboundary movements of LMOs [1].

According to Art. 1 CPB, the objective to the CPB is: "*to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements*".

The CPB was adopted in 2000 and entered into force in 2003 [2]. Currently, 173 countries are Parties to the CPB. This means that such countries have either ratified, accepted, approved or accessed the CPB [3]. It is relevant to note that most Parties to the CPB are located in the Global South. In fact, both the text of the CPB and its adoption by jurisdictions in the Global South were promoted by influential actors in the Global North such as the European Union. In fact, the CPB stipulations were contested by other Global North prominent actors in the international environmental system, such as the United States of America [4].

According to Art. 2 CPB, CPB's Parties are required to, *inter alia*, adopt national legal measures to implement their obligations under the CPB. Since the ratification of the CBD and the CPB, an increasing number of Parties have adopted domestic measures to govern the use of the LMO and to implement the CPB.

With the adoption of the CPB in 2000, some Parties to the CPB rapidly started to adopt national legislation to regulate the use of LMOs within their territories [5]. In the Global South, some countries have adopted a national biosafety legislation to govern LMOs. For example, in 2007, Malaysia adopted the Biosafety Act No. 678 to, *inter alia*, regulate the use of LMOs with the objectives of protecting human, plant and animal health, the environment and biological diversity. Similarly, in 2015, Dominican Republic adopted the Law No. 219-15 regarding the safety of biotechnology to ensure safe use of LMOs and prevent adverse effects that they could cause to biodiversity, human health and the environment.

Other countries in the Global South, which are also Parties to the CPB, have designed biosafety laws to regulate the safe use of GMOs. These countries regulate

GMOs instead of LMOs. For instance, in 2005, Mexico enacted the Biosafety Law of Genetically Modified Organisms with the purpose of regulating various activities of GMOs to prevent, avoid or reduce the possible risks that these activities might entail to human health, environment and biological diversity. Similarly, in 2009, Kenya adopted the Biosafety Act No. 2 to facilitate responsible research into, and minimize the risks that may be posed by the GMOs to human health and the environment. Therefore, regardless the ratification of the CPB, some Parties have opted to use the term GMO, instead of LMOs, to design national measures to implement the CPB.

In the Global South, biotechnology products have been subject to regulatory oversight even before the adoption of the CPB. For instance, in 1999, Peru enacted the Law No. 27104 to prevent risks associated with the use of biotechnology and to protect the human health, the environment and biodiversity by regulating, managing and controlling the risks associated with LMOs. Similarly, in 1997, South Africa adopted the Genetically Modified Organism Act No. 15 to promote the responsible development, production, and use of GMOs to limit possible harmful consequences to the environment.

However, not all countries in the Global South have a biosafety legal framework to govern LMOs or GMOs. In fact, in the Global South, the purpose to adopt biosafety policies, legislation and regulations is diverse. For instance, most countries have adopted biosafety legal frameworks as an end product of the ratification and entry into force of the CPB [5, 6]. Other countries, such as Argentina and Brazil are major exporters of agrobiotechnology commodities, and this may be a contributing element to their advanced level of trade-related biosafety frameworks [7, 8]. Some other countries have opted to design biosafety policies to restrict the use of biotechnology products or have yet to promulgate any biosafety legal instrument [5]. Clearly, the economic importance of the agricultural sector and the role of biotechnology in each country are fundamental triggers for the rapid adoption of biosafety policies and laws [7]. Additionally, political will would be a key factor in the success of adoption biosafety policies and legislation [5].

Overall, some countries are designing bills of legal instruments to regulate LMOs, or GMOs, and implement the CBD and the CPB at a national level. For example, most Small Island States in the Caribbean, and the Pacific, are drafting biosafety legal measures [5]. Similarly, most Least Developed countries in Africa lack biosafety legislation [9].

2 What Is a Living Modified Organism (LMO)?

The term LMO has a universal legal definition that has been described under international environmental law. The concept of LMO was initially introduced in international law by the CBD in 1992 [10]. Two articles under the CBD presented the notion of LMO.

Firstly, Art. 8(g) CBD, concerning *in situ* conservation, indicates that each contracting party shall, as far as possible and as appropriate, "*establish or maintain means to regulate, manage or control the risks associated with the use and release of Living Modified Organisms resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account the risks to human health*".

Second, the concept of LMO is also presented in Art. 19(3) CBD referring to handling of biotechnology and distribution of its benefits. Art. 19(3) CBD notes that "*the Parties shall consider the need for and modalities of a protocol setting out appropriate procedures, including, in particular, advance informed agreement, in the field of the safe transfer, handling and use of any Living Modified Organism resulting from biotechnology that may have adverse effect on the conservation and sustainable use of biological diversity*".

The CBD only mentions the notion of LMO with the aim to develop appropriate measures to control their possible risks to biodiversity and human health. However, the definition of LMO is subsequently clarified in the CPB in 2003. According to Art. 3(g) CPB, a LMO refers to "*any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology*".

Some Parties to the CPB have included the legal definition of LMO into their national biosafety legislation [11, 12].

Activity 26.1: Adoption of the LMO Definition Under National Laws

- Which other Parties to the CPB have adopted the LMO definition in their national biosafety law?
- Are there non-Parties to the CPB that also follow the LMO definition?

3 Key Features of the LMO Definition

Based on Art. 3(g) CPB, the LMO definition contains three key features. A product is considered to be a LMO when it owns all these three characteristics:

- Be a living organism,
- Possess a novel combination of genetic material, and,
- Be developed by the use of modern biotechnology.

Each feature of the LMO definition includes relevant legal terms that have also been defined under international law. Overall, the LMO definition contains four legal terms that are described by international law. These are: "living organism", "genetic material", "biotechnology" and "modern biotechnology".

3.1 What Is a Living Organism?

According to the LMO definition of the CPB, LMO are living organisms. Art. 3(h) CPB defines the concept of living organism as "*any biological entity capable of transferring or replicating genetic material, including sterile organisms, viruses and viroids*". According to this definition, a living organism possesses two joint characteristics.

Firstly, a living organism is any biological entity, including sterile organisms, virus and viroids. Living organisms are alive beings which are also referred to as biological entities, living beings, living systems or natural entities [13]. They occur in various forms [14]. For example, animals (including human beings), plants, bacteria, protozoa and fungi are living organisms [15].

Furthermore, entities such as sterile organisms, viruses and viroids are also included as living organisms according to Art. 3(h) CPB. It is noteworthy that some viruses and viroids are not alive entities [10]. However, the definition of living organisms, as stipulated by Art. 3(h) CPB, is broad as it includes non-living things, such as viruses and viroids.

Secondly, a living organism must be capable of transferring or replicating genetic material. Biological entities possess various capacities, including the ability to transfer or replicate their genetic material [13]. Similarly, viruses and viroids also possess this ability [10].

3.2 What Is a Novel Combination of Genetic Material?

Based on the LMO definition of the CPB, a LMO has to possess a novel combination of genetic material. However, there is no definition of the concept of novel combination of genetic material under international law. Rather, the term genetic material is in:

Art. 2 CBD, genetic material refers to "*any material of plant, animal, microbial or other origin containing functional units of heredity*". Similarly, Art. 2 International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) defines genetic material as: "*any material of plant origin, including reproductive and vegetative propagating material, containing functional units of heredity*".

Overall, the definition of genetic material by the CBD is broad, as it encompasses any genetic material of various types of living organisms, including plants, animals or microbes. In contrast, the definition of the ITPGRFA exclusively refers to genetic material of plant origin. However, both definitions agree that genetic material must contain functional units of heredity, which are composed of nucleic acids, such deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) carrying genetic information or genes [10, 16].

3.3 What Is Modern Biotechnology?

Before explaining the concept of modern biotechnology, it is relevant to describe what biotechnology means. Art. 2 CBD defines biotechnology as "*any technological application that uses biological systems, living organisms, or derivates thereof, to make or modify products or processes for specific use*".

The term biotechnology was first introduced by natural sciences in 1919 to refer the scientific methodologies that allow products to be developed from raw materials with the support of living organisms [17]. For instance, biotechnology is used for the selective breeding of plants and animals or the use of microorganisms in the production of beer, bread and wine [18].

Modern biotechnology is one type of biotechnology. According to Art. 3(i) CPB, modern biotechnology refers to the application of "*in vitro nucleic acid techniques, including recombinant DNA (rDNA) and direct injection of nucleic acid into cells or organelles, or fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection*". Both, *in vitro* nucleic acid techniques and cell fusion are primarily used for agriculture, textile, food and feed production, including the development of LMOs [19].

Additionally, the definition of modern biotechnology makes reference that these techniques are not same as methods used in traditional breeding and selection. However, the meaning of traditional breeding and selection is not defined under international law. Conventional, traditional, or natural could refer to traditional breeding and selection techniques, such as natural selection, cross-breeding, protoplast fusion, and chemical- or radiation-induced mutation [20]. These techniques modify the genetic material within the crossable gene pool of a species, but they do not introduce genetic information from other organisms [20].

4 What Is a Genetically Modified Organism (GMO)?

There is no international legal definition of GMO. Instead, the term GMO is introduced and defined only under domestic and regional law in certain jurisdictions. As such, some countries have designed their own definition of GMO, which is incorporated into their national biosafety laws. For instance, in the Global South, biosafety legislations in selected countries in Africa, Asia and Latin America and the Caribbean use the term GMO instead of LMOs (Table 26.1). It is relevant to note that, regardless the ratification to the CPB, some Parties to the CPB have decided to use the term GMO instead of LMO.

As shown in Table 26.1, the definition of GMO varies between jurisdictions. Therefore, it is not possible to describe main characteristics of the GMO definition that can be universally valid. This is because there is no standard definition of the term GMO. In fact, the GMO definition contains different or similar features among countries with a biosafety law to regulate GMOs. Interestingly, these features of the

Table 26.1 GMO definition under national biosafety laws in certain jurisdictions

Region/ Country	Main biosafety legal instrument	Year of enactment	GMO definition
Africa			
Kenya	Biosafety Act No. 2 An Act of Parliament to regulate activities in Genetically Modified Organisms to establish the National Biosafety Authority, and for connected purposes	2009	"*Any organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology techniques*".
Nigeria	National Biosafety Management Act	2015	"*Any organism living or non-living that possesses a novel combination of genetic material obtained through the use of modern biotechnology*".
South Africa	Genetically Modified Organism Act No. 15	1997	"*An organism the genes or genetic material of which has been modified in a way that does not occur naturally through mating or natural recombination or both, and 'genetic modification' shall have a corresponding meaning*".
Asia			
The Philippines	Joint Department Circular No.1 of the Rules and Regulations for the Research and Development, Handling and Use, Transboundary Movement, Release into the Environment, and Management of Genetically-Modified Plant and Plant Products Derived from the Use of Modern Biotechnology	2021	"*Also refers to Living Modified Organism under the Cartagena Protocol on Biosafety and refers to any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology*".
Viet Nam	Biodiversity Law No. 20	2008	"*An organism whose genetic structure has been modified by the gene transfer technology*".
Latin America and the Caribbean			
Brazil	Law No. 11,105	2005	"*An organism whose genetic material, DNA/RNA has been altered by any genetic engineering technique*".

(continued)

Table 26.1 (continued)

Region/ Country	Main biosafety legal instrument	Year of enactment	GMO definition
El Salvador	Decree No. 78 for the Special Regulation for the safe use of Genetically Modified Organisms	2008	"*Any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology. For the purpose of this regulation, the term Genetically Modified Organism is understood as synonym of the term Living Modified Organism used by the Cartagena Protocol on Biosafety*".
Mexico	Biosafety Law of Genetically Modified Organisms	2005	"*Any organism living, with the exemption of humans, that possesses a novel combination of genetic material obtained through the specific use of modern biotechnological techniques defined in this Law*".
Panama	Biosafety Law No. 48	2002	"*Any living organism in which the genetic material has been modified in a way that does not occur naturally or in a different way than natural*"

GMO definition can be similar or different in comparison to the LMO definition of the CPB. Overall, the GMO definition can be equal, similar or different to the LMO definition.

First, for some countries with a biosafety law to regulate GMOs, the definition of GMOs is equal to the LMO definition of the CPB. This phenomenon occurs when revising biosafety laws in The Philippines and El Salvador. In fact, under such legislation, there is a clear indication that GMO is a synonym of the term LMO under the CPB.

Second, for other countries that have adopted a biosafety law of GMOs, the term GMO is very similar to the LMO definition of the CPB. the GMO concept under biosafety legislation of GMOs in Mexico, Nigeria and Kenya contains almost all the features of the LMO definition. For example, according to these laws, a GMO has to possess a novel combination of genetic material and have to be developed through the use of modern biotechnology.

Third, biosafety legislation in some other countries have design a GMO definition that is very different to the concept of LMO. This occurs in countries such as Brazil, Panama, South Africa and Vietnam. Here, there is no indication of modern biotechnology; instead, other scientific terms are mentioned such as genetic engineering, gene transfer technology or in a way that does not occur naturally. Similarly, these legal instruments only mention that the genetic material of the GMO has to be altered or modified. There is no reference that GMO has to possess a novel combination of genetic material.

Activity 26.2: Adoption of the GMO Definition Under National Laws

- Which other Parties to the CPB have designed a GMO definition?
- Are there non-Parties to the CPB that follow a GMO definition?
- Which are the similarities or differences between the GMO definition of such countries and the LMO definition?

5 Regulatory Approaches Governing Precision Breeding

Under international law, there is no binding specific provision to regulate precision breeding or new breeding techniques. Some international organizations including the Secretariat to the CBD, Organization Economic Cooperation and Development (OECD), Food and Agriculture Organization of the United Nations (FAO) have made efforts to support countries when defining what precision breeding refers to. For instance, these organizations have designed guidelines to explain some precision breeding or other related techniques such as genome editing, synthetic biology and/or gene drive systems [21–23]. However, the definitions proposed by these organizations are not legally binding as those concepts have been developed as guidance only.

However, under national law, rules to regulate some techniques that could be placed under the umbrella term precision breeding exist. Recently, few countries, including in the Global South, with a biosafety legislation of LMOs or GMOs have adopted a new regulation to clarify the regulatory status of precision breeding [24] (Fig. 26.1). Interestingly, most of these countries are located in Latin America and the Caribbean region.

Overall, 13 countries have designed guidelines or regulations that have been adopted in certain countries in the Global South (Table 26.2). The development of these guidelines and regulations results is a consequence that some precision breeding techniques can generate products that can be classified as LMOs or GMOs under biosafety legislation and they can result in organisms that can also occur in nature or through conventional breeding.

Regulations in most of these countries acknowledge the fact that some precision breeding techniques could develop LMOs or GMOs, while others may not. Therefore, the purpose of these regulations is to develop an administrative procedure, and an application form for applicants to query to the National Competent Authority (NCA) whether or not their products are considered as LMOs or GMOs. The NCAs in these countries are:

- In Africa: National Biosafety Authority in Kenya and National Biosafety Management Agency in Nigeria;
- In Asia: Department of Biotechnology under the Ministry of Science and Technology in India and Department of Agriculture in The Philippines; and,

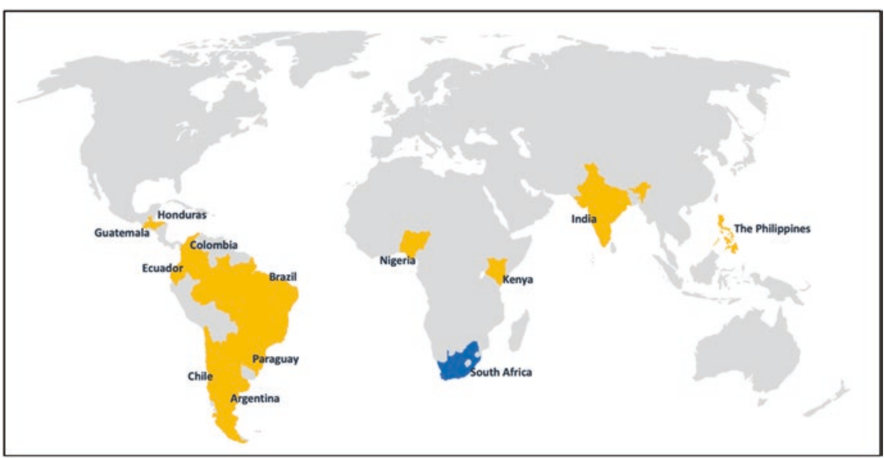


Fig. 26.1 Overview of countries in the Global South with a regulation or guidelines to regulate some technologies of precision breeding. Countries in yellow have designed a similar approach to regulate precision breeding products. Whereas countries in blue opted to adopt other type of approach

- In Latin America and the Caribbean: Ministry of Agriculture, Livestock and Fisheries in Argentina, National Biosafety Technical Commission in Brazil, Service for Agriculture and Livestock in Chile, Colombian Institute of Agriculture and Livestock in Colombia, Ministry of Environment in Ecuador, Ministry of Agriculture and Livestock in Paraguay, Ministry of Agriculture, Livestock and Food in Guatemala and National Service of Agri-Food Health and Quality in Honduras.

Overall, according to [7], the applications forms that are developed by the NCAs request the following information including:

- *data about the parental organism, such as its molecular biology and phenotype,*
- *the breeding methodology used to obtain and select the crop including the new trait or introduced characteristic, and an indication of the modified DNA sequences,*
- *evidence of stably inherited genetic changes in the final product, including technologies used to discard a stably inserted new combination of, or foreign, genetic material in the final organism.*

The NCAs in these countries analyze the information provided by the applicant and evaluate whether the products have a novel combination of genetic material or not. If the final organism has a novel combination of genetic material, it is considered as LMO or GMOs, and falls under the biosafety regulatory oversight [7, 12, 25].

Therefore, a common definition in most of these countries is what constitutes a novel combination of genetic material. This is because for all countries in Fig. 26.1

Table 26.2 Regulations governing precision breeding in selected countries

Region/ Country	Main regulation governing precision breeding	Year of enactment	Meaning of novel combination, or foreign, of genetic material
Africa			
Kenya	Guidelines for determining the regulatory process of genome editing techniques in Kenya	2022	*Refers to a novel combination of genetic material from sexually non- compatible species through the use of modern biotechnology techniques.*
Nigeria	National Biosafety Guidelines on Gene Editing	2020	*a combination of DNA sequences which is possible only through modern biotechnology and is not possible to find in nature or obtained through conventional breeding techniques.*
South Africa	Public Note: South Africa's Regulatory Approach for New Breeding Techniques	2021	–
Asia			
India	Guidelines for the safety assessment of genome edited plants	2022	–
The Philippines	Memorandum Circular No.08 Series of 2022. Rules and procedure to evaluate and determine when products of plan breeding innovations (PBIs) are covered under the DOST-DA-DENR-DOH-DILG Joint Department Circular No. 1 Series of 2021	2022	*A resultant genetic combination in a living organism that is not possible through conventional breeding.*
Latin America and the Caribbean			
Argentina	Resolution 173/2015	2015	*A stable and joint insertion into the genome of one or more genes or DNA sequences that are part of a defined genetic construct*
Brazil	Normative Resolution 16	2016	–
Chile	Consultation Form	2017	*To a stable insertion of one or more genes or DNA sequences encoding proteins, RNAi, double-stranded RNA, signal peptides or regulatory sequences*

(continued)

Table 26.2 (continued)

Region/ Country	Main regulation governing precision breeding	Year of enactment	Meaning of novel combination, or foreign, of genetic material
Colombia	Resolution 00029299	2018	*A gene, set of genes or DNA sequences that are part of a defined genetic construction and that have been introduced in the genome of an organism on a stable way, by the use of modern biotechnology, overcoming natural physiological barriers of reproduction.*
Ecuador	Regulation of the Organic Code for the Environment No. 752	2019	–
Guatemala	Technical Regulation 65.06.01:18	2018	*A stable insertion in the genome, of one or more genes or DNA sequences that codify: DNA double helix DNA, RNA, proteins or regulatory sequences, that cannot be obtained by conventional breeding or are not found in nature.*
Honduras	Technical Regulation 65.06.01:18	2018	*A stable insertion in the genome, of one or more genes or DNA sequences that codify: DNA double helix DNA, RNA, proteins or regulatory sequences, that cannot be obtained by conventional breeding or are not found in nature.*
Paraguay	Resolution 565	2019	–

(in yellow), excluding South Africa, precision breeding products that lack a novel combination, or foreign, of genetic material are not defined as LMOs, or GMOs, according to their national biosafety legislation. The presence of novel combination, foreign or exogenous, of genetic material in the final product is the main consideration to classify a product as a LMO or GMO. Clearly, the characteristics of this definition may slightly or significantly vary between countries as indicated in Table 26.2.

To the contrary, the NCA of South Africa, the Executive Council for Genetically Modified Organisms in South Africa, had adopted a communication indicating that all products derived from new breeding techniques will be subject to regulation. Therefore, all products of new breeding techniques will be treated similarly to GMOs, and they will fall under subject to the Genetically Modified Organism Act No. 15 of 1997.

Some other countries in the Global South are currently discussing the need to adopt regulations for precision breeding or are designing drafts of guidelines to regulate precision breeding products. For example, this occurs in various countries

in Latin America and the Caribbean [7]. Similarly, few countries in South East Asia are discussing whether or not regulate precision breeding products [26]. Additionally, ongoing discussions are taking place in some West African countries [27].

Activity 26.3: Approval of Regulations Concerning Precision Breeding

- Which other Parties to the CPB have adopted regulations concerning precision breeding?
- Are there non-Parties to the CPB that have design precision breeding regulations?
- What is the trigger to regulate precision breeding countries in these countries?

6 Future Regulatory Perspectives on Precision Breeding

Some countries in the Global South, especially countries in Latin America and the Caribbean region, have designed regulations to assess the regulatory status of some precision breeding techniques. In fact, these countries have an extensive experience governing LMOs and/or GMOs since the 90s. Additionally, these countries are major producers of agricultural and biotechnological commodities [5]. As such, it was expected that these countries would rapidly adopt such new measures on precision breeding, as they have an interest to insert such these products into national and international markets. Clearly, the rapid adoption of procedures relevant to precision breeding is strongly linked to the role of agriculture and biotechnology in the countries and with national economic, social and political perspectives [7]. As such, it is likely that other countries with a similar attitude would likely be willing to adopt similar or slightly similar regulations on precision breeding.

However, countries in the Global South have diverse positions in terms of biosafety law and the role of agrobiotechnology in their economies. Countries that rely in other types of agriculture, e.g. organic, or have developed bans to the use of biotechnology products for agriculture would continue excluding the governance of precision breeding products. This is simply because precision breeding products are one alternative that can be utilized by certain countries but not necessarily the only one that can be used across all countries in the Global South.

It is fundamental to highlight the right of sovereignty of countries over their territory. For the Global South, clearly, there is no one-size-fits-all approach to promote the use of precision breeding in all countries. Nor all countries should follow a singular and common approach to govern precision breeding products. This would occur only when such approaches, if relevant and needed, fit the conditions and national interest of the countries about precision breeding.

Specially in the Global South, countries are complex actors and their socio-political and economic context and priorities would play a key role when deciding to what extent the adoption of regulation of precision breeding is relevant or not. For instance, countries that currently lack a biosafety regulatory system to assess the use

of LMOs or GMOs such as Small Island States or most Least Developed Countries in Africa would probably, in the future, also lack precision breeding regulations as such matter is not a key priority for these countries. Clearly, this phenomenon could obviously change over time.

7 Summary

In the Global South, 13 nations have adopted regulations or guidelines to govern some precision breeding techniques. Most of these countries have designed a similar approach to assess the regulatory status of precision breeding products. The main criterion for such countries is whether or not the final product contains a novel combination of genetic material. To this aim, countries have designed a concept to legally define what novel combination of genetic material refers to. Such definition may vary or may be similar among nations.

However, overall, most jurisdictions in the Global South require legal clarity about whether products or organisms derived from precision breeding would be subject to biosafety regulation. For such countries, it is still not clear whether precision breeding products would be classified as LMO or GMOs, and therefore be subject to regulatory oversight under biosafety laws. Further studies are required to explore the interest or current developments of such countries to design regulations or guidelines to regulate some precision breeding techniques.

This section provided an overview of key definitions under international and national legislation to clarify the regulatory status of precision breeding products. This was done by assessing provisions under the CPB and national biosafety laws in selected countries in the Global South. This hopefully would aim to provide a baseline and a global perspective for further discussion about the regulatory status of precision breeding in other nations.

8 Further Reading

For a detailed overview of the LMO definition see [10] and revise the CPB. With regards to the GMO definition, read the national biosafety legislation and regulations of GMOs of the country of interest. Such legal instruments are available in the national gazettes or official journals of each country.

Acknowledgements The author would like to thank (in alphabetic order) to Dennis Eriksson, Jale Tosun, Noelia Valderrama-Bhraunx and Pieter Van der Meer for their helpful comments and suggestions.

References

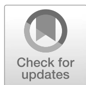
1. Secretariat to the Convention on Biological Diversity: Cartagena Protocol on Biosafety to the Convention on Biological Diversity (2000)
2. Secretariat to the Convention on Biological Diversity: The Cartagena Protocol on Biosafety. https://bch.cbd.int/protocol/ (2023a)
3. United Nations Treaty Collection: Status of Cartagena Protocol on Biosafety to the Convention on Biological Diversity. https://treaties.un.org/Pages/ViewDetails.aspx?src=TREATY&mtdsg_no=XXVII-8-a&chapter=27&clang=_en (2022)
4. Schulze, K., Tosun, J.: Rival regulatory regimes in international environmental politics: the case of biosafety. Public Adm. **94**, 54–72 (2016)
5. Rosado, A., Craig, W.: Biosafety regulatory systems overseeing the use of genetically modified organisms in the Latin America and the Caribbean region. AgBioforum. **20**, 120–132 (2017)
6. McLean, M., Foley, M., Pehu, E.: The status and impact of biosafety regulation in developing economies since the ratification of the Cartagena Protocol. http://www-wds.world- bank.org/external/default/WDSContentServer/WDSP/IB/2012/06/12/000356161_20120612021559/Rendered/PDF/ 698300WP0P11210egulations0final0web.pdf (2012)
7. Rosado, A., Eriksson, D.: Biosafety Legislation and the regulatory status of the products of precision breeding in the Latin America and the Caribbean Region. Plants, People, Planet. **4**, 1–18 (2022)
8. Smith, P., Katovich, E.: Are GMO policies "trade related"? Empirical analysis of Latin America. Appl. Econ. Perspect. Policy. **39**, 286–312 (2017)
9. African Biosafety Network of Expertise: African Biosafety Status Map. https://www.nepad.org/programme/african-biosafety-network-of-expertise-abne (2021)
10. Mackenzie, R., Burhenne-Guilmin, F., La Vina, A., Weksman, J.: An Explanatory Guide to the Cartagena Protocol on Biosafety. https://portals.iucn.org/library/efiles/documents/eplp-046.pdf (2003)
11. Frison, C., Manga, S., Cordonier, M.: Implementing sustainable development through national biosafety frameworks. In: Cordonier, M.-C., Perron-Welch, F., Frison, C. (eds.) Legal Aspects of Implementing the Cartagena Protocol on Biosafety. Cambridge University Press (2013)
12. Whelan, A., Lema, M.: Regulatory framework for gene editing and other new breeding techniques (NBTs) in Argentina. GM Crops Food. **3**, 253–265 (2015)
13. Wash, D.: Organisms, Agency and Evolution. Cambridge University Press (2015)
14. Deplazes, A., Huppenbauer, M.: Synthetic organisms and living machines: positioning the products of synthetic biology at the borderline between living and non-living matter. Syst. Synth. Biol. **3**, 55–63 (2009)
15. Kadhila, N.: Biology Module 1. Cambridge University Press (2009)
16. Suza, W., Lee, D.: Genetic, Agriculture and Biotechnology. Iowa State University Digital Press (2021)
17. Organization of Economic Cooperation and Development: Policy Brief: Modern Biotechnology and the OECD. https://www.oecd.org/science/emerging-tech/1890904.pdf (1999)
18. Organization of Economic Cooperation and Development: Glossary of Statistical Terms: Biotechnology. https://stats.oecd.org/glossary/detail.asp?ID=219 (2001)
19. Sensi, A., Ghosh, K., Takeushi, M., Sonnino, A.: Building biosafety capacities: FAO's experience and outlook. https://www.fao.org/3/i1033e/i1033e.pdf (2009)
20. Kinderlerer, J.: The Cartagena protocol on biosafety. Collection of Biosafety Reviews. https://conacyt.mx/cibiogem/images/cibiogem/comunicacion/publicaciones/CBR-V4/Kinderlerer.pdf (2008)
21. Secretariat to the Convention on Biological Diversity: Synthetic Biology. https://www.cbd.int/synbio/ (2023b)
22. Organization of Economic and Cooperation Development: Genome editing: applications in agriculture https://www.oecd.org/environment/genome-editing-agriculture/ (2023)

23. Food and Agriculture Organization of the United Nations: Genome editing and agrifood systems. https://www.fao.org/3/cc3579en/cc3579en.pdf (2022)
24. Schmidt, S., Belisle, M., Frommer, W.: The evolving landscape around genome editing in agriculture. EMBO Rep. **21**, 1–4 (2020)
25. Lema, M.: Regulatory aspects of gene editing in Argentina. Transgenic Res. **28**, 147–150 (2019)
26. Buchholzer, M., Frommer, W.: An increasing number of countries regulate genome editing in crops. New Phytol. **237**, 12–15 (2022)
27. Sprink, T., Wilhelm, R., Hartung, F.: Genome editing around the globe: an update on policies and options. Plant Physiol. **190**, 1579–1587 (2022)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 27
CRISPR Processes Patents in Green Biotechnology: Collaborative Licensing Models



Agnès Ricroch

Abstract A strong patent system is necessary to enable innovation by incentivising investments in research and development (R&D) and promoting the dissemination of knowledge, including through licensing, as this is considered vital for the development and commercialisation of new products. The new genomic techniques (NGTs) defined by the European Union (EU) in 2021 include the CRISPR system which can selectively modify DNA sequences in a genome. More than 11,000 CRISPR-related patent applications have already been filed worldwide mainly in USA and China. The proliferation of patents on CRISPR for green biotechnology applications and the dispute between two of the technology's inventors (UC Berkeley and the Broad Institute of MIT, USA) could be barriers to innovation. With regard to intellectual property (IP) and the patent protection, the conditions and opportunities of alternative licensing models to overcome the difficulties created by the complex patent landscape of CRISPR technology are examined. Patent pools and clearing houses are the two models attracting most interest that leads to a one-stop licensing point allowing cross-licensing and facilitating freedom to operate. The conditions for success and acceptability of collaborative licensing platforms are discussed.

1 The Protection of New Genomic Techniques (NGTs)

Plant biotechnology research for new traits and thus the development of new varieties is risky and costly. Plant innovation needs a return on investment to encourage private research and public-private partnerships [1]. Intellectual Property (IP) is a tool used to: (i) disseminate knowledge and innovation to speed-up innovation cycles, (ii) encourage collaboration and open innovation, (iii) build a sustainably growing knowledge and innovation pool, (iv) enable fair access and benefit sharing, and (v) prevent creation misappropriation and, when owned or licensed, allows for

A. Ricroch (✉)
The Jean-Monnet Faculty. Idest Laboratory, Paris-Saclay University, Sceaux, France
e-mail: agnes.ricroch@universite-paris-saclay.fr

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_27

so-called freedom-to-operate (FTO). An FTO analysis (or clearance/infringement search) clarifies if a product or its potential commercialization infringes on other existing IP rights. It is an expensive undertaking. It begins by searching for issued or pending patents and thereafter, involves analysing the claimed scope of protection to get a legal opinion as to whether the product, process, or service potentially infringes on any patents owned by others. It establishes a list of potential patent holders, with whom the future (prospective) licensee must then contact and successfully negotiate a license for each patent. The negotiation process involves costs for both the licensee and the patent holders. On 29 April 2021, the European Commission (EC) published a study on the status of new genomic techniques (NGTs) that defined NGTs as "techniques capable of modifying the genetic material of an organism that have emerged or been developed since 2001", *i.e.* after the existing EU legislation on genetically modified organism (GMO) was adopted in 2001 (Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC). NGTs include (i) CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), TALEN (Transcription Activator-Like Effector Nucleases), zinc-finger nucleases, meganuclease techniques, and prime editing; (ii) mutagenesis techniques, such as oligonucleotide directed mutagenesis; and (iii) epigenome-editing techniques, such as RNA-dependent DNA methylation.

With regard to IP and the patent protection of biotechnological inventions (under Directive 98/44/EC54), this EU study acknowledged the benefits of patents and licensing in promoting innovation and the development of NGTs and their products. The licensing landscape is developing rapidly with licensing agreements, some exclusive, some non-exclusive, on a diverse range of CRISPR technologies and application fields, from agriculture to therapeutics. More than 11,000 CRISPR-related patent applications have already been filed worldwide, with the majority being filed in USA and China [2] leading to some concerns about the complex patent landscape for NGTs with multiple players holding patents, and uncertainty surrounding the IP situation. *IPStudies*, a company based in Switzerland, has compiled a list of more than 100 variants of CRISPR enzymes beyond the best-known discovery of Cas9 in 2012, with some commercial players attempting to claim them exhaustively to ensure as much IP exclusivity as possible (dCas, dCas9, Cas12a, Cas13a…).

Prime editing, using a modified Cas9 protein that makes a single-stranded cut substantially expands the scope and capabilities of genome editing, a versatile and precise method that directly writes new genetic information into a specified DNA site (see Chap. 3). Prime editing shows higher or similar efficiency and fewer by-products than homology-directed repair, has complementary strengths and weaknesses compared to base editing, and induces much lower off-target editing than Cas9 nuclease at known Cas9 off-target sites. CRISPR-Cas-based techniques are still evolving and the list of NGTs is expected to expand further in the coming years (*e.g.* base editing). New CRISPR-associated enzymes have been engineered, such as base editors that are better able to make specific edits.

However, optimized tissue culture and transformation protocols should be in place to make genome editing a routine tool for plant breeding. More than 370,000 higher plant species exist *in natura*. But scientists can only make transformation (transgenesis and genome editing) successful in a few dozens of these.

1.1 Patents Characteristics

The protection conferred by a patent to biological material possessing specific characteristics that result from an invention [1]. (i) Patents usually have a term of protection for 20 years (starting from the filing of the application). As with all IP rights (IPRs), patent protection is also territorial, meaning that it provides protection only on a given territory. The scope of a patent on an invention is determined by the so-called patent claims. (ii) The protection conferred by a patent to biological material possessing specific characteristics that result from an invention, shall extend to any biological material which has derived from the original biological material through propagation or multiplication and possessing those same properties. Noting that in the case of a self-replicating biological material, the right of exhaustion does not apply at the first sale. (iii) A patented biotechnological invention incorporated into a variety remains protected in this variety but by no means is the variety itself patented, as this would be contrary to EU legislation prohibiting the patenting of varieties. Thus, the genome of this variety, when it no longer contains the patented biotechnological invention, is completely free of patent rights.

Under the European Patent Convention which has been amended by the decisions of the Administrative Council since the publication of the 17th edition (November 2020), patents are granted only for inventions that are new, involve an inventive step and are industrially applicable. An invention meets these requirements if it: (i) was not known to the public in any form, (ii) is not obvious to a person skilled in the art, and (iii) can be manufactured or used industrially. They are valid in individual countries, for a specified period. New plant varieties in the EU are completely excluded from patentability. Plant variety rights are regulated under Council Regulation (EC) No 2100/94 of 27 July 1994 on Community plant variety rights. In Europe, transgenic or edited plants carrying a patented event (transgenic or edited trait) fall within the scope of the patent because this element is not limited to a single variety and at the same time a transgenic or edited variety can also be individually protected by a Plant Variety Protection (PVP) certificate.

1.2 The CRISPR Patent Dispute and Legal Uncertainty

Biotechnology in agriculture is a lucrative industry and its growth is going to continue. Universities hold most of the key patents using the CRISPR technology needed to deploy this technology [3]. Thus, the role of the public sector in

fundamental research and surrogate companies such as the University of California, Berkeley, the Broad Institute of MIT, and Harvard, USA, are very relevant in holding fundamental/key patents of CRISPR technology. Key patents have no economically viable substitutes; a party needs a license to utilize the Intellectual Property Right (IPR) [4].

There has been extensive interest in IP issues surrounding CRISPR, including a patent dispute between two of the technology's inventors, Jennifer Doudna (the University of California, Berkeley, USA) and Emmanuelle Charpentier (currently in Max-Planck Institute, Germany) on one side, and Feng Zhang (the Broad Institute of MIT and Harvard, USA) on the other over who first invented the gene-editing system and who should benefit from key patents [2]. This CRISPR-Cas9 dispute, which began in 2016, is still ongoing.

Companies now also have the option of avoiding these key patents (UC Berkeley, the Broad Institute and Harvard) altogether by using different CRISPR systems. Such systems occur naturally in many bacteria and Achaea and can have various properties. Relatively few of these use CRISPR–Cas9; instead, they use alternative enzymes such as Cas12a, Cas13a or Cas14, the latter being remarkably small and easy to transport into human cells. Labs have also engineered new CRISPR-associated enzymes, such as base editors, that are better able to make specific edits.

The IP around CRISPR is becoming increasingly complex. Commercial deployments are complicated by the legal uncertainty associated with the lack of patent clarity on CRISPR. Indeed, in the context of the dispute, patent offices in the USA and the EU have issued different decisions on the validity of CRISPR patents. The United States Patent and Trademark Office (USPTO) has repeatedly ruled in favour of the Broad Institute and, on February 28, 2022, determined that it was the first inventor. The decisions are on who is entitled to receive patent protection [5]. In the EU, since the main CRISPR-Cas9 patents in the Broad Institute's portfolio were rejected due to lack of documentation, UC Berkeley is considered first by the European Patent Office (EPO) ("EP2800811" (November 10, 2020)). There is a need for greater clarity in patent rights in order to make researchers feel secure in developing further technological innovations using the CRISPR system.

Despite this legal uncertainty as to the final determination of ownership and patentability, the licensing landscape is developing rapidly with licensing agreements, some exclusive, some non-exclusive, on a diverse range of CRISPR technologies and application fields, from agriculture to therapeutics.

2 Alternative Models: Licensing Platforms and IP Clearing Houses

The current CRISPR patent landscape presents a variety of barriers to research, innovation and profit. In order to overcome the difficulties created by the overall presence of patents, academics and breeders of the private sector are debating

alternative licensing models. Patent pools and clearing houses are the two models attracting most interest.

2.1 Concerns

Patent Profusion With a steady increase of more than 200 patent families published each month IP studies shows the emergence of several new trends concerning both the enabling technology itself, and application developments. There are so many property rights in the hands of various owners—with whom parties must reach agreements to enable them to aggregate the rights they need in order to legally perform their activities—that it will prove difficult to negotiate licences for patented inventions successfully. High transaction costs may stand in the way of an agreement. If a high number of agreements with rights holders is required, transaction costs may lead parties to decide that the bargaining process is not worthwhile. Hence, a socially optimum level of consumption of the resource may not be achieved, resulting in "under-utilization" of the property which will have a blocking effect on further innovation. Moreover, the fact that licensees have to acquire many licences in order to avoid patent infringements, may lead to elevated royalty fees, caused by royalty stacking. Because the licensee will usually pass on the cost of these fees to the final consumer, the final development and manufacture of products may be obstructed [6]**.**

A growing cause for concern in the CRISPR sphere is the so-called "tragedy of the anticommons." There are two reasons why anti-commons could affect the CRISPR patent pool; a thicket of patents, and the current licensing model being implemented. (i) There is a potential "thicket" of patents in the CRISPR sphere with some fragmentation of IPRs because the technology tends to be patented and licensed on a gene-by-gene basis. (ii) The surrogate licensing model currently in use raises concerns about research bottlenecks [7]. A research bottleneck could occur because the exclusive licensing to surrogates has limited the availability of CRISPR technology as a global platform, and traditional protections against the overly broad surrogate licenses will not work.

Concentration of Players The concentration of players in the seed market has led to high seed prices, reduced seed variety choices, and great dependence on farmers. Transgenesis technology has led to an increased concentration of ownership and power in agri-food systems through patents, contracts and licence agreements. Patent rights and the way they are granted and exercised contribute to a decrease in the diversity of breeding companies and threaten innovation in plant breeding. It is argued that the position of patents, combined with technological developments, has led to substantial consolidation of breeding companies in recent decades.

Farmers and producers fear that their freedom of choice will be threatened and that no varieties specifically meeting their needs will be developed for certain crops

(orphan or underutilized crops). Genome editing holds great promise for increasing crop productivity, and there is particular interest in advancing the breeding of orphan crops, which are often burdened by undesirable characteristics resembling wild relatives. In order to diversify cropping systems, orphan or underutilized crops are better adapted to local or marginalized environments [8].

Access for Small and Medium-Sized Enterprises (SMEs) to Patents The high cost of patenting innovations and high patent licence fees can be a barrier to market entry for SMEs (together with high business concentration) due to; (i) the complexity of patenting and the resulting monopolization, (ii) the licensing of patented products and the respective transaction costs, and (iii) the lack of transparency and FTO analyses, *e.g.* due to the complex patent landscape of the CRISPR technology. These same aspects may limit access to NGTs as mentioned in the EC study on the status of new genomic techniques in 2021.

2.2 Some Solutions of Collaborative Licensing Models: The Patent Pools

Alternative collaborative licensing models such as patent pools and IP clearing houses may play a significant role in facilitating access to patents and untangling a potential patent "thicket".

A patent pool for the purpose of joint package licensing is an agreement between two or more patent owners to license one or more of their patents as a package either to one another or to third parties willing to pay the associated royalties [9]. The package is managed either directly by patentees to licensees, or indirectly through a new entity specifically established to administer the pool. The first licensing pool was established in 1856 among members of the sewing machine industry. More recently some pools are being established in biotechnology, such as the Golden Rice pool or the SARS-1 (severe acute respiratory syndrome) pool.

This package of IPRs is then licensed on a non-exclusive basis, providing licensees with affordability and FTO, while giving licensors adequate royalty returns [10]. A patent pool can provide competitive advantages by integrating complementary technologies, reducing transaction costs, clearing blocking positions, and avoiding costly infringement litigation. By promoting the dissemination of technology, patent pools can be pro-competition.

Classically, the creation of a patent pool involves four major characteristics. (1) It is built around the voluntary inclusion of key and specific IPR holders. (i) "Key" patents are those that have no economically viable substitute; a party needs a license to use the IPR. Key patents are those for which the application is general, such as a technique that applies to all genomes. (ii) "Specific" patents are those for which the application is specific (such as a modification of a genetic sequence for a given trait in a given species). (2) It relies on a model. A model is an attempt to encapsulate the details and patents necessary to enable uniformity of practice across a diverse range

of implementations. (3) It requires pool administrators to conduct an in-depth and continual search of the patent landscape. Thus, a patent pool must be open to all IPR holders, but each patent must be analysed individually to determine if it is needed before possible inclusion; independent high-level scientists and lawyers analyse both the patent landscape and the potential key patents. (4) It can be subject to anti-competition laws. Any issues regarding anti-competition laws and patent pools have largely been resolved following the decision of the Department of Justice to grant MPEG LA pro-competitive clearance in 1997. The starting point for an anti-trust analysis of any patent pool is an investigation of the validity of the patents and their relationship with each other.

In the absence of a patent pool, users (licensees) have to enter into negotiations with all relevant patent holders, which is a time-consuming and expensive process. In the presence of a patent pool, licensees turn to the patent pool for the rights as one package, which results in simplification and a significant reduction of transaction costs.

Example: MPEG LA

The platform MPEG LA, an independent licensing agent based in Denver (USA), aims to pool CRISPR-Cas9 patents into a one-stop licensing point (accessibility to a multi-user market will maximize CRISPR's life-enhancing potential) [11]. Its model offers fair, reasonable, and non-discriminatory access to essential IP from multiple patent holders under a single license as an alternative to separate licenses.

By assisting users with the implementation of their technology choices, MPEG LA offers licensing solutions that provide access to fundamental IP, freedom to operate (FTO), reduced risk of litigation, and predictability in the business planning process. In turn, this enables inventors, research institutions, and other owners to monetize and accelerate market adoption of their assets in the global market while also substantially reducing the cost of licensing.

The CRISPR-Cas9 Reference Model is authored by MPEG LA for MPEG LA's use in the formation of *a CRISPR-Cas9 Joint Licensing Platform* which aims to provide one-stop, worldwide licenses to CRISPR-Cas9 patent rights as a convenient alternative to negotiating separate licenses with individual patent owners, and pursues the broader purpose of fostering innovation in genome engineering and accelerating the development and deployment of CRISPR-based products, therapies, and services. The Reference Model is intended only to support the efficiency of a single licensing transaction that allows access to as many specific patents as possible for the benefit of the market and is consistent with applicable legal requirements. Inclusion requires that at least one claim is directed to the CRISPR-Cas9 System. MPEG LA's *CRISPR Cas-9 Joint Licensing Platform* will give technology owners the opportunity to share in mass-market royalties from their CRISPR technology while also enjoying, with other developers, broad access to other important CRISPR technologies. As a voluntary, market-based business solution to the problems relating to patent access, designed to balance and resolve competing market and public interests, an independently managed patent pool represents the greatest opportunity to unleash CRISPR's full potential for the benefit of humanity.

To ensure that no single party has control over the licensing of the package, the organisation works with all the included patent holders to create a single set of licensing terms and conditions, upon which all included patent holders must agree. The patent holders also hold most of the enforcement powers. While MPEG LA can enforce contractual provisions, it does not file patent enforcement lawsuits on its own; instead, it must notify the patentees that they may want to file an enforcement suit.

MPEG LA develops its "CRISPR-Cas9 Reference Model", which describes how to patent essentiality will be determined with respect to the CRISPR platform (the CRISPR-Cas9 System is defined). This outline of essentiality in MPEG LA's Reference Model is meant to encompass all patents key and specific to the underlying CRISPR platform. If a patent meets the established criteria, it will be eligible for inclusion in the pool. To collect these platform patents, MPEG LA is seeking "target-agnostic" patents that do not require a specific genome. The Reference Model discloses the criteria for pool inclusion. MPEG LA's initiative was to provide a worldwide non-exclusive license to multiple patents held by multiple entities in a single transaction.

2.3 Some Solutions of Collaborative Licensing Models: The Clearing House Models

An alternative mechanism supporting licensing negotiations is the clearing house model. The term clearing house is derived from banking institutions and refers to the mechanism by which cheques and bills are exchanged among member banks in order to transfer only the net balances in cash. The platform may provide information on patented technologies, bring together potential providers (licensors) and users (licensees) of patented technologies, and may provide additional services, *e.g.* negotiating licensing conditions, and collecting and distributing royalties. An IP clearing house was analysed for agricultural biotechnology [12].

They are five models of clearing houses: (1) The information clearing house, (2) The technology exchange clearing house, (3) The open access clearing house, (4) The standardized licenses clearing house and (5) The royalty collection clearing house (RCCH). The RCCH model could be useful in providing access to and use of patented inventions in NGTs and novel traits.

The RCCH comprises all the features of the previous models (1, 2 and 4), but also collects license fees from users on behalf of the patent holder in return for the use of certain technologies or services. For the user, RCCH organizations would simplify licensing negotiations and, therefore, facilitate access to and use of patented inventions. For the patent holder, increased visibility of their patent rights and the streamlining of royalty collection and monitoring may lead to a rise in licensing and thus, licensing revenue. At the same time, awareness and respect for IPRs may grow among researchers and their public and private institutions, leading to

decreased enforcement costs through fewer infringements. Hence, a reasonable price for licensees (royalties, transaction costs) and licensors (royalties, transaction costs, and enforcement costs) may be achieved. The patent holder is reimbursed by the clearing house pursuant to a set allocation formula. In addition, a RCCH may offer other services such as the monitoring of patents transferred to the clearing house or an independent dispute resolution mechanism.

However, the RCCH may have some drawbacks. (i) Patent holders may be reluctant to voluntarily participate in it. They would have to grant a licence to the clearing house which would then issue licences to all applicants without discrimination and on a non-exclusive basis in accordance with competition law. As a consequence, patent holders would lose some control over their business licensing strategy. (ii) Unless the RCCH represents a high proportion of all relevant patented inventions, it might not be a viable and effective alternative, nor could it prevent the emergence of an anti-commons effect. (iii) RCCH might be more complicated and costly to set up in comparison with the other clearing house models. Experts (high-level scientists and lawyers) will have to be hired to evaluate the often very complex patents, to match licensees with the patented inventions, to develop standardized licence agreements, and for monitoring and dispute resolution purposes. (iv) The standardized licences might not allow for measures highly appreciated in commercial licensing practices, such as the setting of milestones, due diligence, and the maintenance of long-term business relationships. (v) The exchange of relevant technical know-how is often fundamental for the smooth application and further development of a patented invention. Know-how is generally protected as a trade secret, but the clearing house will probably not be able to guarantee the preservation of secrecy when know-how is exchanged. Thus, with respect to complex technologies, direct negotiations between the licensor and the licensee on the issue of know-how may still be required, which may diminish some of the advantages of the royalty collection clearing house. This drawback might be a reason to advocate for the establishment of an RCCH that is limited to inventions that do not require the exchange of technical know-how, such as patented DNA sequences and mutations.

In the absence of a clearing house, licensees must enter into negotiations with all patent holders. In the presence of a clearing house, licensees turn to the patent pool for the acquisition of required rights.

Example: Agricultural Crop Licensing Platform (ACLP)

The ACLP, located in Brussels (Belgium), is open to all private or public sector organizations involved in plant breeding or trait research and development, and having employees and tangible assets in the 'territory' (the geographical scope of the ACLP is 38 member states of the EPO along with Russia and Ukraine). The initiative is currently driven by 10 European plant breeding companies and trait developers representing a wide range of agricultural crops and includes small, medium-sized, and large companies. The ACLP is financed by membership fees. Small members enjoy free membership during the five first years of the existence of the ACLP. The scope is all patented traits present in commercial varieties that are sold on the open market in the 'territory' ("Trait") and all agricultural crops as defined by CPVO

(The *Community Plant Variety Office* is the EU agency responsible for protecting plant variety rights (PVR) in the EU). The ACLP provides an innovative, simple legal framework for all breeders to use and enables transparent access to patented traits including genome edited traits.

This initiative provides breeding and guaranteed marketing rights for commercial traits which in turn fosters the transfer of technology. Transparent information on all commercial varieties that contain patented traits within the territory is shared via PINTO (*Patent Information and Transparency On-line*). PINTO was established with the aim of improving transparency regarding plant varieties that might fall under the scope of patents or patent applications and is updated continuously by *Euroseeds*.

The entitlement of members to a commercial variety containing a patented trait is established as soon as it is sold by a member on the open market in the Territory. Member's rights are; (i) to obtain a non-assertion agreement from the patent holder for breeding in the Territory with the patented trait(s) including using its specific markers, (ii) to obtain a commercial license from the patent holder for the production and sale of the varieties, bred under the non-assertion agreement and containing the patented trait(s), within in the Territory.

The key expected outcomes of the ACLP are as follows; (i) novel patented plant traits, including those produced by new genomic technologies, are available among ACLP members on fair conditions, (ii) the sustainable development of novel varieties be enabled by information and rights shared amongst members, under the framework of the ACLP, (iii) the facilitation of technology transfer will make it easier for the ACLP members to further innovate.

Principal rights and obligations applicable to members of the ACLP Initiative are; (i) members are entitled to any commercial variety containing a patented trait, as soon as is sold by a member on the open market in the Territory; (ii) for members to obtain from the patent holder, a non-assertion agreement for the breeding of plants containing patented trait(s), including using its specific markers, within the Territory, (iii) for members to obtain from the patent holder, a commercial license for the production and sale of varieties, bred under the non-assertion agreement and containing the patented trait(s), within the Territory.

Licensing can take the form of the *Standard License Agreement* (SLA) of the ACLP in which case only royalties need to be agreed between the patented trait holder and the interested member. In circumstances where no agreement on royalties is reached within a prescribed period of time, a process known as "Baseball" Arbitration [13] begins.

The ACLP will foster bilateral agreements between members as the standard licensing agreement will not fit all specific factual situations best. The ACLP gives never less assurance that a commercial license will be available under the *Standard License Agreement* (SLA). For breeding activities, the ACLP acts as a technology exchange clearing house: all members get a non-assert for breeding activities which is not available in all states of the territory. The standardized licenses clearing house is also a component of the ACLP.

3 Success and Acceptability of Licensing Platforms and IP Clearing Houses

3.1 The Public Sector

The public sector plays an important role in fundamental research and represents a substantial source of IP in agricultural biotech. There are quantitative and qualitative distinctions between the public- and private-sector IP portfolios (1/3 of CRISPR patents are held by private companies) [2]. It is important to distinguish between 'enabling' technologies representing the research tools needed to create NGT varieties, and 'trait' technologies which provide the genetic basis for new functionalities. The public sector contributes more and more to the discovery of new trait technologies. The extent to which private and public-sector inventions can be used to assemble a platform of enabling technologies and gene-trait technologies sufficient to develop (new edited varieties) have been examined [12]. A major challenge facing the management of public-sector IP is the high degree of fragmentation of technology ownership across numerous institutions, especially in light of the need for multiple technology components to provide FTO in edited crops. Based in part on some of the economic principles of an 'IP clearing-house,' a model of public-sector collaboration, including data sharing and patent pooling, has recently been suggested as a solution that could directly address this issue. It seems that the technologies patented by the public sector might indeed be capable of providing a platform of technologies that could be sufficient to enable the development of new NGT varieties and cultivars. Such a strategy may be particularly important in the future for sharing access to key enabling technologies and enabling innovators to develop and deploy the trait technology projects with the public sector. Public funding for research emphasizes the discovery of a wide spectrum of previously unknown gene functions whereas private research focuses on application-driven research in a narrower range of established product lines.

3.2 Ethical Licensing

Ethical licensing is one way of thinking about the role of universities and other public institutions in the regulatory process, but there is a wealth of other initiatives promoting ethical licensing. IP pooling, clearing houses and open source initiatives are examples of private ordering mechanisms that differ from solutions aimed at changing or harmonizing the legislative framework in that they are generated by the users themselves. Thus, a voluntary pool or a clearing house model could promote commercialization and include provisions for royalty-free research use by public institutions, while addressing ethical concerns about particular CRISPR applications.

A successful patent pool could do more than solve licensing issues (fragmentation and downstream litigation, the patent thicket and licensor issues such as the

surrogate licensing model). It could also mitigate ethical concerns. Point 9 in the *Nine Points* to Consider in Licensing University Technology (AUTM, Washington, DC, 2007) states: "*Through thoughtful management and licensing of intellectual property, however, drugs, therapies, and agricultural technologies developed at universities can at least help to alleviate suffering from disease or hunger in historically marginalized population groups*"**.**

License restrictions could be used, in theory, to require that R&D funds be used, in part, to ethically promote access to NGTs (access for SMEs or to research/biotech institutes in developing countries) and also to develop traits for orphan crops in developing countries.

MPEG LA and its prospective licensors could potentially solve said ethical dilemmas through ethical constraints in their licensing agreement (ethical limitations in their licensing agreements by granting licenses for only very specific applications). This is largely the case with *Monsanto*'s license from the Broad Institute covering the use of CRISPR-Cas9 for a variety of agricultural purposes in 2016. This non-exclusive license agreement will deliver a wide array of crop improvements to global agriculture. The Broad Institute's license to *Monsanto* covering the use of CRISPR-Cas9 for a variety of agricultural purposes requires *Monsanto* to allow its farming customers to save and re-sew seed from one season to the next, in contrast to some of *Monsanto's* past practices. Requiring this of Monsanto provides greater access to the outcomes of CRISPR technology to farmers, who would otherwise be required to purchase expensive new seeds each year from *Monsanto*. The Broad Institute has used ethical limitations in their licensing agreements by granting licenses for only very specific applications (discussing Broad's non-exclusive licensing with *Bayer* for specific agricultural applications such as genetic modification of plant varieties). In October 2017 *DuPont* entered into a joint non-exclusive licensing agreement with the Broad Institute for use in commercial agricultural research and product development (except gene drive and tobacco for human use) on the CRISPR-Cas9 IP held by the Broad Institute and its collaborators.

Only genetic modification (mutation, allele replacement or gene insertion) by NGTs can be patented. Prohibiting patents on native sequence, crossover processes and random mutations is important in defending the principle that all the information contained in a plant genome belongs to the scientific heritage of humanity.

3.3 Open Licensing Systems (with CRISPR IP Research Tools Available)

Companies would benefit from a CRISPR patent pool that would provide a non-exclusive license to CRISPR as a research tool, and it would appear that many of the exclusive licenses already granted would permit this field of use to be included in a pool license.

In the context of CRISPR, this non-commercial use is done through a non-profit repository and licensor of patents on CRISPR technologies for academic

organizations. *AddGene*, a company (a non-profit organization) based in the USA and UK, serves academic and non-profit institutions that provide access to CRISPR constructs and plasmids through a standardized Biological Material Transfer Agreement (BMTA). *AddGene*'s BMTAs contain patent licenses for the academic use of the underlying technology. For example, the University of California, the Broad Institute, and hundreds of other institutions have agreed to make many of their CRISPR IP research tools available for free or at a reduced cost through *AddGene* [14].

3.4 Patent Quality

The scope of patents should be clearly defined for two major reasons. (i) When the product (a trait in crops) is only vaguely defined and its essential character is difficult to determine, the utility of the pool may be diminished in upstream research, as it may be too narrow to be useful for product development. (ii) Biotech patents tend to be incomplete meaning that the innovation must be completed before the final product is incorporated and brought to market. Incompleteness and long development cycles can make it difficult to define specific patents. This can be a problem because, if a pool does not contain specific rights, the pool may no longer be a one-stop licensing point for potential licensees.

4 Conclusion

Although patent pooling (licensing platforms and IP clearing houses) is an attractive solution for the licensing of IPR in fragmented fields such as NGTs, there are many challenges that could hinder the formation of such a pool. Public perception of NGTs is critical to their adoption by the market. Understanding and awareness enable consumers to make informed choices, therefore it is necessary to provide consumers with information. With respect to IP and patent protection, alternative licensing models could overcome the difficulties caused by the complex patent landscape of CRISPR technology. Patent pools and clearing houses are a promising approach. When access and use to a certain technology are hindered by the existence of multiple patents, a patent pool could be a useful model to facilitate access and reduce potential litigation risk. The package of IPRs is licensed on a non-exclusive basis, allowing licensees to benefit from affordability and freedom to operate while providing licensors with adequate royalties. Non-exclusive licensing schemes could allow many companies to enter the market, creating a commercial ecosystem that has strengthened innovation and the economy. The ACLP, a clearing house, can be mentioned as a solution for products produced by the CRISPR technology for plant breeding in the agricultural sector in Europe. A CRISPR patent pool depends on the willingness of a sufficient number of IP owners to join the pool

to ease licensing burdens and costs. The CRISPR platform will take off in a big way when a major company and all key players (major research institutions) announce that they will join the patent pool. The platform should be a low-complexity platform that lawyers and breeders are familiar and comfortable with.

Acknowledgements The author gratefully acknowledges the help of the 4iP Council and Dr. Marcel Kuntz. The views expressed in this paper are solely those of the author.

References

1. Le Buanec, B., Ricroch, A.: Intellectual property protection of plant innovation. In: Ricroch, A., Chopra, S., Kuntz, M. (eds.) Plant Biotechnology – Experience and Future Prospects, 2nd edn. Springer (2021)
2. Martin-Laffon, J., Kuntz, M., Ricroch, A.: Worldwide CRISPR patent landscape shows strong geographical biases. Nat. Biotechnol. **37**, 601–621 (2019)
3. Ricroch, A.E., Martin-Laffon, J., Rault, B., Pallares, V.C., Kuntz, M.: Next biotechnological plants for addressing global challenges: the contribution of transgenesis and new breeding techniques. New Biotechnol. **66**, 25–35 (2021)
4. Lawrance, S., Brooks, F., Ross, J.: The competition law issues of the CRISPR patent pool. Lexology. (2018)
5. Cohen, J.: CRISPR's Nobel Prize winners defeated in key patent claim for genome editor. Science. (2022)
6. Contreras, J.L.: Policy forum, the anticommons at 20: concerns for research continue. Science. **335**(337) (2018)
7. Contreras, J.L., Sherkow, J.S.: CRISPR, surrogate licensing, and scientific discovery. Science. **698**, 698–699 (2017)
8. Talabi, A.O., Vikram, P., Thushar, S., Rahman, H., Ahmadzai, H., Nhamo, N., Shahid, M., Singh, R.K.: Orphan crops, a best fit for dietary enrichment and diversification in highly deteriorated marginal environments. Front. Plant Sci. **13**, 839704 (2022)
9. Verbeure, B., Van Zimmeren, E., Matthijs, G., Van Overwalle, G.: Patent pools and diagnostic testing. Trends Biotechnol. **24**(3), 115–120 (2006)
10. Horn, L.: Patent pools for CRISPR technology. Science. **355**(6331), 1274 (2017)
11. Neville, P.: MPEG LA's use of a patent pool to solve the CRISPR industry's licensing problems. Utah Law Rev. **2**, 535–567 (2020)
12. Graff, G.D., Zilberman, D.: An intellectual property clearinghouse for agricultural biotechnology. Nat. Biotechnol. **19**, 1179–1181 (2001)
13. Pauwelyn, J.: Baseball arbitration to resolve international law disputes: hit or miss. Florida Tax. Rev. **22**, 40 (2018)
14. Panagopoulos, A., Sideri, K.: Prospect patents and CRISPR; rivalry and ethical licensing in a semi-commons environment. J. Law Biosci. **8**, 2 (2021)

Resources

Addgene: https://www.addgene.org/crispr/
Agricultural Crop Licensing Platform (ACLP): https://aclp.eu/
Association of University Technology Managers, AUTM: https://autm.net/about-tech-transfer/principles-and-guidelines/nine-points-to-consider-when-licensing-university

Broad Institute: https://www.broadinstitute.org/
EC Study on the status of new genomic techniques under Union law and in light of the Court of Justice ruling in Case C-528/16: https://food.ec.europa.eu/system/files/2021-04/gmo_mod-bio_ngt_eu-study.pdf
European Patent Office: https://register.epo.org/application?lng=fr&number=EP13793997
Euroseeds: https://euroseeds.eu
IP Studies: https://www.ipstudies.ch/media/crispr-patent-analytics/
MPEG LA: https://www.mpegla.com

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Part IV
Public and Stakeholder Perceptions

Chapter 28
The View of the European Seed Sector on Genome Editing Tools in Plant Breeding

Petra Jorasch and Nick Vangheluwe

Abstract Reconciling sustainability with agricultural productivity relies strongly on crop improvement. The continuous development of new plant breeding methods and high investment in crop research has triggered an unprecedented acceleration of progress in crop productivity. Companies active in the seed sector are innovative to meet the challenging and ever-evolving needs of growers, farmers, consumers, and other actors of the agri-food value chain. Communication and stakeholder engagement are extremely important to identify shared values and goals in the transition to more sustainable food systems. The seed sector is characterised by a multitude of collaborations and partnerships that contribute to the development of improved, high-quality, and diverse plant varieties that address specific market needs. In this article, we describe on behalf of Euroseeds the view of the European seed sector on plant breeding innovation.

Keywords European seed sector · Plant breeding · Genome editing

1 Plant Breeding Has a Track Record to Contribute to Sustainability

Plant breeding involves processes by which new technologies and findings from plant sciences and other research domains are transformed into improved plant varieties. Plant breeding has strongly contributed to increased yields and production in arable farming, and subsequently to improved market and trade conditions, increased food availability, higher economic prosperity and additional farm income while avoiding additional land use, greenhouse gas (GHG) emissions, and loss of biodiversity [1].

Scientists have shown for instance for wheat, which is a major staple food around the world, that resistance breeding can contribute to achieving the United Nations

P. Jorasch (✉) · N. Vangheluwe
Euroseeds, Avenue des Arts, Brussels, Belgium
e-mail: PetraJorasch@euroseeds.eu; NickVangheluwe@euroseeds.eu

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_28

Sustainable Development Goals (SDGs) [2]. They were able to show that resistance breeding in wheat has increased crop production, and hence the economic profitability due to a reduction in the use of fungicides. Another study could verify that five decades of wheat breeding progress in western Europe has enhanced cultivar performance not only under optimal production conditions but also in production systems with reduced agrochemical inputs. New cultivars incrementally accumulated genetic variants conferring favourable effects on key yield parameters, disease resistance, nutrient use efficiency, photosynthetic efficiency, and grain quality [3].

Europe's seed sector is committed to delivering on the SDGs (2, 3, 8, 9, 12, 13 and 15) by improving the sustainability of food production, by maintaining and promoting Europe's high food quality and standards, by ensuring that the European agri-food sector can remain fair and competitive, and by contributing to Europe's climate, environmental and biodiversity goals. The European Union (EU) Farm to Fork (F2F) strategy includes several policy objectives that have implications for agricultural production in the EU and beyond.

Plant breeding in the EU contributes to socio-economic and environmental sustainability and can partially compensate production losses potentially resulting from the implementation of the EU F2F and Biodiversity strategies [4]. Policy makers seem to have implicitly concluded that the additional net benefits resulting from the EU F2F and Biodiversity Strategies outweigh the losses in production surplus. But studies do not support this claim without further technological and institutional changes, such as supporting the application of modern biotechnology by reducing regulatory hurdles for plant-based innovations [5]. Modern plant breeding including biotechnological tools must be enabled to provide farmers more efficiently with improved plant varieties to sustainably secure their productivity [1].

2 The Ever-Evolving Plant Breeders' Toolbox

Like evolution itself plant breeding depends upon genetic variability within crops and their relatives as a basis for developing new plant varieties with improved characteristics. For thousands of years, humans have been improving crops to suit better their needs. Conventional plant breeding methods, transgenesis or newer plant breeding methods are all important components of the plant breeders' toolbox (Fig. 28.1). By building on the mechanisms created by nature, most of the latest innovations in plant breeding methods simply reduce the complexity of breeding and achieve the relevant breeding goals in less time and with greater precision.

Fig. 28.1 Integration of new plant breeding tools into the breeding cycle. Plant breeding depends upon genetic variability within crops and their relatives as a basis for developing new plant varieties with improved characteristics. Plant breeders are continuously integrating the latest methods in plant biology and genetics into their breeding toolbox to efficiently use existing diversity but also to induce new genetic variation. Over the past years ever more precise and efficient plant breeding methods have been developed. This plant breeding innovation leap is based on an in-depth understanding of plant genomes and refinement of breeding methods, enabling more efficient, more precise, and faster progress in achieving the desired breeding goals

3 The Role of New Breeding Techniques According to the European Seed Sector

The targeted development of improved plant varieties is important to mitigate climate change effects like new plant pests or diseases. These can be devastating to crops and lead to huge pre-harvest losses. Other new plant varieties provide quality improvements, such as better taste (*e.g.*, in fruits and vegetables), processing advantages or nutritional enhancements, such as desirable proteins or lower saturated fats. In addition, New Genomic Techniques (NGTs) -such as genome editing- are technologies that could also facilitate the improvement of so-called orphan species that have benefitted little from innovations in genetic selection as well as the domestication of related wild species thus increasing the genetic diversity within crop species [6].

The results of a survey among 62 private plant breeding companies conducted by Euroseeds highlights the enormous interest of companies in using NGTs for a wide range of crop species and traits and the negative impact of the current regulatory situation in the EU on companies' decisions for investments in NGT-related research and development (R&D) activities for the EU market and beyond [7].

The Commission study on NGTs [8] confirmed that plants resulting from NGTs have the potential to contribute to a more sustainable food system as part of the

objectives of the European Green Deal and the F2F Strategy. At the same time, the study found that the current genetically modified organism (GMO) legislation, adopted in 2001, is not fit for purpose for these innovative technologies. This was also confirmed by the results of the public consultation conducted by the Commission as part of the impact assessment to draft a policy proposal. Almost 80% of the participants who responded to the consultation acknowledged that the current regulatory framework is not adequate for plants resulting from targeted mutagenesis and cisgenesis [9].

4 Communication Practices and Needs for Plant Breeding Innovation

The EU-funded Horizon 2020 project CropBooster-P assessed European stakeholders' information and communication behaviour on plant genome editing including survey results of a total of 100 respondents from the seed and plant breeding sector [10].

The most referred crop characteristics communicated about by breeders and seed and plant breeding organisations relate to yield and yield stability. This suggests that communication about economic aspects of crop production and economic sustainability of farmers (as the customers of new seeds) are of utmost importance. Breeders and seed and plant breeding organisations stressed the importance to communicate safety as well as sustainability aspects in future efforts (Fig. 28.2). This does not necessarily imply that safety communication is a consequence of a

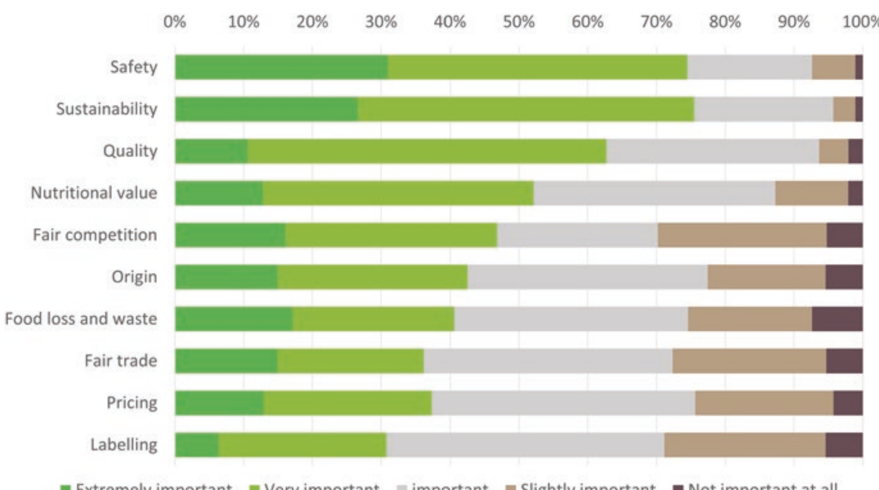


Fig. 28.2 Ranking of topics for future communication efforts by breeders and seed and plant breeding organisations. N = 94

Social media
Benefits to producers
Participatory events
Less dependent on pesticides
Specific examples
Basics of plant breeding
Benefits to consumers
Field days

Fig. 28.3 Word cloud of examples for effective communication based upon the free text answers in the survey from breeders and seed and plant breeding organisations

risk-focused approach. For breeders, safety and quality are important because seeds are at the basis of food safety and health requirements related to crops that are grown for food, feed, and bio-based production [11].

The potential benefits, examples of applications, and comparisons of genome editing and conventional breeding methods were identified as the most important topics to communicate about NGTs. This indicates that the ongoing policy discussions in Europe about a differentiated regulatory framework for plants resulting from targeted mutagenesis and cisgenesis resonate with breeders.

Finally, respondents could share in the survey examples of communication relating to plant research, crop improvement and breeding or crop production which, in their experience was effective. The answers were thematically analysed to identify recurring topics, which are depicted in the word cloud below (Fig. 28.3). Most shared experiences by the seed and plant breeding sector are explaining the basics of plant breeding and its benefits to consumers and primary producers as well as providing specific examples (*e.g.*, the need to reduce pesticide use) and engaging with interested stakeholders on field days and participatory events.

5 A Multitude of Collaborations in the Seed Sector

The seed and plant breeding sector engages with many different stakeholders as R&D activities take place along the value -and process chains from the field to the shop counter to meet the challenging and ever-evolving needs of farmers and other actors of the agri-food value chain, including consumer preferences and policy objectives. In addition, cooperation between companies, public research institutes and other actors is important to address research needs and gaps in crop improvement strategies. A multi-stakeholder perspective on the role of crop improvement in future-proofing the European food system was developed in the CropBooster-P

project, which revealed that for instance examining downstream sustainability impacts will be valuable to identify concrete targets for plant breeding innovation as a food systems solution [12].

COST actions are interdisciplinary research networks that bring together researchers and innovators from academia, small and medium-sized enterprises, public institutions, and other relevant organisations or interested parties to investigate a topic of their choice for 4 years [13]. The COST Action PlantEd has been a unique platform for multi-stakeholder engagement on the topic of plant genome editing [14]. The multi-actor approach put forward in H2020 and Horizon Europe research programmes, helps to facilitate the involvement of the seed and plant breeding sector in research projects to co-develop solutions, which could be more readily applied in.

Sustainability, safety-related aspects, and transparency appear to be the most important topics for communication about plant genome editing by the surveyed stakeholders in the CropBooster-P project [10]. These stakeholders include academia, farmers, seed and breeding sector, environmental and consumer organisations, journalists, and policy makers. The topic of sustainability is primarily addressed in a supportive manner for NGTs [10]. However, environmental organisations prefer to use rejective or neutral argumentation in communication about sustainability in the context of plant genome editing applications. Interestingly, respondents from consumer organisations address the topic impartially, presumably because no genome-edited products have been released on the European market yet [10].

The survey results suggest that focussing on sustainability in future communication activities might increase the opportunity to agree on shared values among different agri-food stakeholders. This is in line with the results of another survey where a majority of those surveyed at farm-level (70%), consumer-level (66%), and plant scientist (60%) respondents to the survey chose sustainability as the most important aim for crop improvement in Europe [11]. Sustainability could thus provide a good starting point for constructive discussions about the regulatory framework in Europe. This recommendation is supported by the outcome of two citizen juries with consumer experts and societal stakeholders. They concluded that the awareness of NGTs by society might be increased when their application serves goals with a societal dimension such as environmental sustainability, resilience, and quality [15, 16].

6 It's Time to Act

Europe's seed sector, technology developers and public researchers have been contributing to the development of improved plant breeding methods. The private seed sector is highly innovative and invests an average of 20% and up to 30% of its turnover in R&D of improved plant varieties [7]. Each year more than 4000 new varieties are registered for cultivation in the EU [17]. In addition, plant breeding has a

proven track record for supporting sustainable agricultural production and NGTs can provide additional sustainability options by increasing efficiency and reducing complexity in plant breeding. Communicating the benefits of plant breeding, and the potential of NGTs specifically, can be a starting point for a constructive public dialogue.

NGT applications are versatile and can be used in the development of a wide range of different plant varieties. While NGTs may for some purposes be used to introduce a transgene and consequently result in a transgenic organism (transgenes are DNA fragments outside the plant species' gene pool) [18], many other types of NGT-derived plants, e.g. those derived from targeted mutagenesis and cisgenesis, are similar to those that could occur in nature or be produced by conventional breeding methods [18], e.g. by induced random mutagenesis or backcross breeding. The European Food Safety Authority (EFSA) [19] concluded that certain plants obtained by targeted mutagenesis and cisgenesis do not pose any new hazards compared to plants developed by conventional breeding.

Plant varieties developed through the latest breeding methods should therefore not be subject to different or additional regulations if they could also be obtained through earlier breeding methods or result from spontaneous processes in nature [20].

Worldwide, there is a growing number of countries that implement differentiating and enabling policy approaches for NGTs. Their regulatory decisions consider the genetic characteristics of the genome edited organism, and whether the changes introduced in its genome can (or cannot) occur naturally [21]. One of the most recent examples of such enabling policies is the United Kingdom (UK) Precision Breeding Act. [22]. The bill excludes precision bred organisms from the GMO legislation and aims to encourage agricultural and scientific innovation in the UK and could unlock the potential of new technologies like genome editing to promote sustainable and efficient farming and food production. EU agriculture and some other of the EU's most innovative sectors are at risk of being deprived of scientific progress, putting them at a competitive disadvantage compared to their counterparts in other countries.

Therefore, Europe should join the increasing number of countries that pursue a differentiated and efficient regulatory approach. The future legislation must provide clarity through principal definitions and unambiguous criteria that allow to clearly determine whether plants resulting from certain NGTs fall into the same category as conventionally bred plants (and thus should be regulated alike) or constitute GMOs according to the respective regulatory framework.

The regulatory framework for GMOs in the EU (2001/18) is unworkable, specifically for SMEs which constitute 90% of the EU's seed and breeding sector [7]. The lengthy and costly procedures risk neutralizing any efficiency gains in breeding. Currently a GMO import approval takes more than 5 years between application and approval of the GMO dossier, the last and the only cultivation approval that realized into GMO cultivation in the EU dates to the end of the 1990ies. That makes it unattractive for companies to invest in those technologies. Under such conditions, NGTs will not deliver on the goals of the F2F and Biodiversity strategies.

Consequently, Europe's leading position in innovative breeding is at stake, as are the EU's sustainability goals, jobs in agriculture, their associated value chains, and international trade flows.

References and Resources

1. Noleppa, S., Cartsburg, M.: The socio-economic and environmental values of plant breeding in the EU and for selected EU member states. HFFA Res. (2021). https://hffa-research.com/wp-content/uploads/2021/05/HFFA-Research-The-socio-economic-and-environmental-values-of-plant-breeding-in-the-EU.pdf. Accessed 27 June 2023
2. Lüttringhaus, S., et al.: Resistance breeding increases winter wheat gross margins–an economic assessment for Germany. Front. Agron. **3** (2021). https://doi.org/10.3389/fagro.2021.730894
3. Voss-Fels, K.P., et al.: Breeding improves wheat productivity under contrasting agrochemical input levels. Nat. Plants. **5**, 706–714 (2019). https://doi.org/10.1038/s41477-019-0445-5
4. Bremmer, J. et al.: Impact Assessment of EC 2030 Green Deal Targets for Sustainable Crop Production. (2021). https://edepot.wur.nl/558517. Accessed 27 June 2023
5. Wesseler, J.: The EU's farm-to-fork strategy: an assessment from the perspective of agricultural economics. Appl. Econ. Perspect. Policy. (2022). https://doi.org/10.1002/aepp.13239
6. Caranta, C. et al.: Etat des connaissances sur la contribution des techniques d'édition du génome à l'amélioration des plantes pour la transition agroécologique et l'adaptation au changement climatique. (2022). https://doi.org/10.17180/0hcx-tn70
7. Jorasch, P.: Potential, challenges, and threats for the application of new breeding techniques by the private plant breeding sector in the EU. Front. Plant Sci. **11** (2020). https://doi.org/10.3389/fpls.2020.582011
8. Commission: Study on the status of new genomic techniques under Union law and in light of the Court of Justice ruling in Case C-528/16, SWD (2021) 92 final (2021). https://food.ec.europa.eu/system/files/2021-04/gmo_mod-bio_ngt_eu-study.pdf
9. Commission: Summary Report of the Public Consultation on "Legislation for plants produced by certain new genomic techniques". (2022). https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/13119-Legislation-for-plants-produced-by-certain-new-genomic-techniques/public-consultation_en. Accessed 27 June 2023
10. Will, S., et al.: Communicating about plant breeding and genome editing in plants: assessment of European stakeholders, sources, channels and content. Food Energy Secur. **12**(**1**), e415 (2023). https://doi.org/10.1002/fes3.415
11. Kaiser, N., et al.: The role of conventional plant breeding in ensuring safe levels of naturally occurring toxins in food crops. Trends Food Sci. Technol. **100**, 51–66 (2020). https://doi.org/10.1016/j.tifs.2020.03.042
12. Stetkiewicz, S., et al.: Food system actor perspectives on future-proofing European food systems through plant breeding. Sci. Rep. **13**(**1**), 5444 (2023). https://doi.org/10.1038/s41598-023-32207-1
13. COST actions. https://www.cost.eu/about/about-cost/. Accessed 27 June 2023
14. PlantEd COST action. https://plantgenomeediting.eu/about-planted/. Accessed 27 June 2023
15. Nair, A., et al.: European consumer and societal stakeholders' response to crop improvements and new plant breeding techniques. Food Energy Secur. **12**(**1**), e417 (2023). https://doi.org/10.1002/fes3.417
16. Stetkiewicz, S., et al.: Crop improvements for future-proofing European food systems: A focus-group-driven analysis of agricultural production stakeholder priorities and viewpoints. Food Energy Secur. **12**(**1**), e362 (2023). https://doi.org/10.1002/fes3.362
17. EUPVP: Common Catalogue Information System. https://ec.europa.eu/food/plant-variety-portal/. Accessed 27 June 2023

18. EFSA Panel on Genetically Modified Organisms (GMO): Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. EFSA J. **10**(2), 2561 (2012). https://doi.org/10.2903/j.efsa.2012.2561
19. Naegeli, H., et al.: Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis. EFSA J. **8**(11), 6299 (2020). https://doi.org/10.2903/j.efsa.2020.6299
20. Euroseeds: Plant Breeding Innovation Applying the latest Plant Breeding Methods for the benefit of sustainable Agriculture, Consumers and Society. (2018). https://euroseeds.eu/app/uploads/2019/07/18.1010-Euroseeds-PBI-Position-1.pdf. Accessed 27 June 2023
21. Custers, R., et al.: Genetic alterations that do or do not occur naturally; consequences for genome edited organisms in the context of regulatory oversight. Front. Bioeng. Biotechnol. **6**, 213 (2019). https://doi.org/10.3389/fbioe.2018.00213
22. UK Government: Genetic Technology (Precision Breeding) Act. (2023). https://www.legislation.gov.uk/ukpga/2023/6/contents/enacted. Accessed 27 June 2023

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 29
The Awareness of the Polish Society on New Genomic Techniques

Wiktoria Mołodziejko, Justyna Nowakowska, and Anna Linkiewicz

Abstract New genomic techniques (NGTs) are challenging from an ethical perspective as well as from the legal and regulatory point of view. Acceptance of solutions by society is a factor that can determine the success of a technology. Increasingly, there is a growing gap between the solutions offered by biotechnology and the public, which with certain ambiguity evaluates biotechnological innovations. Here we investigate the level of knowledge of Polish society on NGTs and the perception of this techniques. Our survey was carried out on a representative group of Polish citizens from August 2022 till January 2023, and was based on a 3-part self-developed questionnaire. Questions concerned: (i) demographic characteristics, (ii) the knowledge of the NGTs including the legal state of the NGT after a ruling European Court of Justice (Case C-528/16) and (iii) respondent's attitudes towards the NGTs. As result, only 15% of respondents (n = 194) gave the correct definition of NGTs, 43% the respondents never encountered the term NGTs, and 35% could not define it. The majority of respondents recognised the potential benefits of using the technology in agriculture as well as in healthcare, but they were not convinced about the potential personal use of NGTs if they were allowed in Poland.

1 Introduction

New genomic techniques (NGTs) represent a significant breakthrough and long-awaited methods for site-specific mutations in DNA and targeted genome editing, offering promising new tools for plant and animal breeding and finally for more

W. Mołodziejko
Faculty of Biology and Environmental Sciences, Cardinal Stefan Wyszynski University in Warsaw, Warsaw, Poland
e-mail: wiktoriamolodziejko1@gmail.com

J. Nowakowska · A. Linkiewicz (✉)
Laboratory of Molecular Biology and Genetics, Institute of Biological Sciences, Cardinal Stefan Wyszynski University in Warsaw, Warsaw, Poland
e-mail: j.nowakowska@uksw.edu.pl; a.linkiewicz@uksw.edu.pl

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_29

sustainable food production. The use of NGTs allows to increase the efficiency and precision of mutations compared to the current breeding methods and can lead to the rapid obtaining of desired plant varieties and animal breeds [1, 2]. However, like any new technology, it has its pros and cons that can be highlighted in the public debate and influence consumer decisions. So far, there is no international consensus on whether and how the products of certain NGTs techniques like CRISPR/Cas9 or TALENs should be regulated and whether their use would fall within the genetically modified organisms (GMOs) regulatory framework [3]. The European Union (EU) defines NGTs as 'techniques capable of changing the genetic material of an organism and that have emerged or have been developed since 2001, that is, after the existing EU legislation on GMOs was adopted' and the products of NGTs are regarded as GMOs after the controversial decision of European Court of Justice (Case C-528/16) [4, 5].

The authors are interested in how much EU citizens are aware of the possibilities brought by the use of the CRISPR/Cas9 and other NGTs methods, and whether they know how much EU law regulates the use of these methods. This paper presents the first comprehensive survey of public opinion of Polish citizens on NGTs. The survey was carried out from August 2022 till January 2023 on the basis of a self-developed questionnaire, inspired by the questions present in the public consultation study on NGTs launched by the European Commission (EC) in April 2022 [6]. This modelling will allow for a comparative analysis of public attitudes in Poland and other EU countries. The main purpose of the study was to obtain broad information on the reception of the latest biotechnological inventions that may affect people in Poland. Our results can also be used in assessing the attitudes of Poles towards biotechnology advances, including GMOs, a study that has been conducted since the late 1990s [7–10].

2 Material and Methods

2.1 The Survey

The survey was conducted in Poland between August 2022 and January 2023 using an anonymous self-designed questionnaire, planned as a 3-part study. The first part of the survey was related to the socio-demographic characteristics of the respondents such as gender, age, education, occupation and place of residence (city size and information on the region of residence in Poland) (Table 29.1).

The second part of the survey concerned general knowledge about NGTs. The questionnaire contained closed single- or multiple-choice questions, where people first explained the meaning of the term NGT (Table 29.2). In case of an answer other than "I do not know", respondents took part in the rest of the questionnaire, which included questions 2–6 (Table 29.2). This third part of the survey included detailed questions on the use of and attitudes towards NGTs and products. The design of the survey was coordinated with sociologists from Cardinal Stefan Wyszyński University in Warsaw. The survey was conducted in Polish.

Table 29.1 Demographic characteristics of the Polish population involved in the study

Characteristic	n = 194
Gender	**n (%)**
Female	95 (49)
Male	99 (51)
Age	
Mean (years ± SD)	40.45 ± 13,72
Median (range) years	39 (19–66)
Education	**n (%)**
Primary	1 (1)
Secondary	78 (40)
Tertiary	110 (56)
Professional	5 (3)
Occupation	**n (%)**
Teacher in agricultural school	5 (3)
Student (biology)	29 (15)
Animal technician	100 (51)
Dairy industry (laboratory technician)	11 (6)
Farmer	18 (9)
Profession supporting agriculture	31 (16)
Place of residence	**n (%)**
Urban >500,000 residents	20 (11)
Urban 100,000–500,000 residents	18 (9)
Urban 20,000–100,000 residents	28 (14)
Urban <20,000 residents	28 (14)
Rural	100 (52)
Regions of Poland	**n (%)**
North	35 (18)
East	64 (33)
South	9 (5)
West	15 (8)
Central part	71 (36)

Table 29.2 Questions and choice of answers included in the survey

No	Question	Answer	**n = 194** n, (% share)
1	Do you know the new genomic techniques (NGTs) e.g. CRISPR/Cas9 or TALEN and what does it mean for you?	I don't know	83 (43%)
		I've heard of it but can't define it	68 (35%)
		These are new techniques used in laboratories since 2001, enabling the change of the genetic material of the organism, e.g. through site-directed mutagenesis	30 (15%)
		Techniques that cause changes in the body's hormonal pathways, where the product has a stably introduced foreign DNA sequence	13 (7%)
	Question	**Answer**	**n = 111** n, (% share)

(continued)

Table 29.2 (continued)

No	Question	Answer	**n = 194** n, (% share)
2	In your opinion, in which sectors of the economy could NGTs be used:	I don't know	14 (8%)
		Agricultural and food industry, e.g. to produce food with a lower proportion of plant protection products	51 (28%)
		They allow for more precise and faster plant breeding and obtaining new varieties that ensure progress in yielding	53 (30%)
		Healthcare, e.g. in the fight against certain diseases such as cancer or genetic diseases	57 (32%)
		NGTs shouldn't be used	4 (2%)
3	Are NGTs products already on the EU market?	I don't know	3 (3%)
		They are present outside the EU, e.g. soybeans with an improved fatty acid profile	44 (38%)
		They are available in the EU and in Poland	46 (40%)
		They do not yet occur	22 (19%)
4	How does Polish and European law currently regulate products made with the use of NGTs methods?	I don't know	47 (42%)
		NGTs organisms are GMOs subject to the requirements of EU GMO legislation	53 (48%)
		They are completely allowed and widely used in Agri-food production in the EU	6 (5%)
		They are forbidden, they cannot be used at all	5 (5%)
5	Do you think that the use of NGTs in agriculture:	They can be used, but subject to applicable PL and EU regulations	70 (63%)
		They should be forbidden	7 (6%)
		It should be generally allowed as in the case of classical methods of plant breeding and not subject to additional legal regulations	10 (9%)
		I have no opinion	24 (22%)
6	If products using NGTs entered the polish market, would you be interested in using them?	Yes	30 (27%)
		No	15 (14%)
		They are of no use to me	14 (13%)
		I have no opinion	52 (46%)

2.2 Characteristics of the Respondents

The survey was addressed to farmers, agricultural and food workers and professionals supporting agricultural production, as well as students of environmental sciences. All contacts and emails to which the questionnaires were sent were found in the recipients' websites or came from the website of the Ministry of Agriculture and Rural Development.

The survey was sent via Gmail (Google, California, USA) to agricultural associations, the dairy industry and other food producers, biology students and teachers (N = 194). Everyone was able to contribute via an online questionnaire available at the link to Google Forms (Google, California, USA).

The authors selected this group of respondents based on their potential level of knowledge in the field of biology and biotechnology. In addition, professionals and students were selected because they represent a population group that is receptive to current trends. As consumers, they can initiate positive changes in the development of agricultural biotechnology. The authors assumed that such a group of recipients would be less likely to drop out after the first question of the test because of their education and profession.

2.3 Statistical Analyses

The data reported here have been weighted to be nationally representative, with a margin of error of 5%. Pearson's chi-square test was used to compare the frequency of responses between the different groups, with P values of <0.05 considered statistically significant. All statistical analyses were performed using Statistica 13.3 (StatSoft, Krakow, Poland).

3 Results

3.1 Demographic Characteristics

Most of the Polish respondents were male (51%), lived in rural areas (52%) in the central (36%) and eastern (33%) parts of the country and had completed tertiary education (56%). Among the professional groups surveyed, animal technicians dominated (51%). Respondents came from different parts of Poland and represented all regions (Table 29.1).

3.2 Knowledge About NGTs Among Polish Citizens

A total of 194 Polish citizens participated in the second part of the questionnaire (Table 29.2, question 1), while 111 respondents were included in the third part (Table 29.2, questions 2–6; Table 29.3). The results show that 111 respondents (57%) knew the term NGT, but only 30 (15%) of the respondents gave the correct definition of NGT. Almost half of the respondents, 83 people (43%), had never heard of NGTs (Table 29.2).

Respondents answered questions 1–4 regardless of gender, age, education, occupation and place of residence (Table 29.3). For question 2, respondents were almost evenly split between the answers: "agri-food industry, e.g. to produce food with lower levels of pesticides" (28%), "they allow more precise and faster plant breeding and obtaining new varieties that ensure yield progress" (30%), and "medicine, e.g. in the fight against certain diseases such as cancer or genetic diseases" (32%) (Table 29.2).

Regarding knowledge about the availability of NTG products on the market, 38% of the respondents indicated that they are available outside the EU, e.g. "soybeans with an improved fatty acid profile", while 40% indicated that they are "available in the EU and in Poland" (Table 29.2, question 3). Almost half of the Polish respondents (48%) were aware of EU legislation on products produced using NGTs (Table 29.3, question 4).

When asked in which sectors of the economy NGTs could possibly be used, most responses pointed to use in healthcare (32%), but a similar number of responses (30% and 28%, respectively) pointed to use in plant breeding and in the agro-food industry. In addition, 2% of respondents do not think NGTs should be used or have no opinion (8%).

The survey revealed that majority of respondents (48%) correctly recognised that NGT organisms are subject to GMO requirements under Polish and EU law. Unfortunately, a 42% of respondents had no answer to this question, while a similar number of respondents believed that NGTs are fully allowed and widely used in agricultural and food production in the EU, or that products from NGTs methods are prohibited by law and cannot be used at all. The only significant differences in responses regarding demographic characteristics were found in questions 5 and 6 (Table 29.3).

Table 29.3 Effect of gender, age, education, occupation and place of residence on responses to each of six questions by 111 respondents, based on chi-squared test (significant P-value highlighted)

Question No.	Gender	Age	Education	Occupation	Place of residence
1	0.75841	0.36179	0.27905	0.38532	0.07893
2	0.11687	0.05277	0.19460	0.24991	0.14457
3	0.49176	0.19411	0.77831	0.67989	0.23528
4	0.23055	0.18557	0.84037	0.55324	0.99551
5	0.64519	0.14161	0.40649	0.61411	**0.01294**
6	**0.04930**	0.14161	**0.01368**	0.10893	0.74694

Questions after Table 29.2

After analysing the data in question 5 regarding the relationship between place of residence and respondents' opinion on whether NGT products should be used in agriculture, it was found that the majority of respondents (64%) indicated that NGT products can be used but should be subject to European Union law. Residents of urban areas, especially cities with <20,000 inhabitants, were in favour of this solution (89%). Interestingly, it was found that 39% of people from rural areas were not interested in legal regulations for NGTs products (Table 29.3, $p = 0.01294$).

The next statistically significant result was the correlation between gender and willingness to use NGT products once they appear on the market (Table 29.3, $p = 0.04930$). The results obtained indicate a discrepancy between the responses of women and men. In the survey, about 56% of women had no opinion, while 39% of men were willing to use NGTs products (data not shown). The relationship between education and willingness to use NGTs products once they are on the market was also observed ($p = 0.01368$). Those with tertiary education were more likely to use NGTs products (37%) than those with secondary education (14%).

4 Discussion

Genome editing methods are already applied for a diverse range of plants and animals, and at the same time there is an active debate about the possible use of these techniques in the EU. The authors of this study wanted to know to what extent EU citizens are aware of the possibilities offered by the use of CRISPR/Cas9 or other NGT methods and whether they know to what extent EU law regulates the use of these methods. The aim of the survey was to determine the knowledge and attitudes of Polish citizens, who have come into contact with agriculture as a result of their education or profession, towards the practical applications of genome editing methods, in particular CRISPR/Cas9 and TALEN methods. Questions were asked about the knowledge of the applications of the aforementioned technologies, about the legal aspects of using the methods and about people's attitudes towards the NGTs.

The survey revealed a low level of awareness of NGTs among Polish citizens. Almost half of the respondents (43%) said they had never heard of the term NGTs, and another 35% had heard of it but could not define what it meant. Only 15% of respondents gave the correct definition of NGTs. When the group was asked whether NGTs products are already on the EU market, 40% of respondents said that they are now present in Poland and on EU markets. An even lower level of awareness of NGTs was reported in Japan, where only 7% of respondents who did not have genetic diseases were aware of the term "gene editing" [11]. In Costa Rica, 4% of respondents had heard or read a little (2%), some (1%) or a lot (0.6%) about CRISPR/Cas9 [12]. Education may also influence responses. Individuals with higher education were more aware of genetically modified products [13–15], in contrast to some data from the deficit model, which showed that scientific knowledge was only weakly associated with attitudes towards the technology [16]. In the

current study, the authors were not able to find this correlation, but there is a clear link between education and willingness to use NTG products.

To better understand the public's attitude towards NGT technology, we asked for their opinion on the legal aspects of using NGT products by asking the question, 'What legal aspects should be fulfilled for the technology to be used in agriculture'? Here, most respondents (63%) were willing to allow the use of this technology if it was regulated by Polish and EU law. Interestingly, 9% of respondents did not want the products of these techniques to be regulated by law, but wanted them to be treated like the products of classical breeding methods. The majority of participants of the EU consultation survey [6] believed that the existing provisions of GMO legislation are not appropriate for plants obtained by targeted mutagenesis or cisgenesis. According to the study published in 2021 by EC, based on targeted consultations on the status of NGTs in EU legislation and in the light of the Court of Justice's ruling in Case C-528/16, the main concerns were related to safety aspects raised by consumers, farmers, food producers and other stakeholders [5]. Most respondents expressed different, sometimes conflicting, views on the level of safety of NGT and their products, and on the need and requirements for risk assessment. Some ethical questions concerned the application of these techniques, not the techniques themselves.

In response to the question "In which economic sectors can NGTs be used?" 32% of respondents indicated medical purposes, which is in line with the results of similar work in the EU [17, 18]. However, a smaller number of respondents (2%) in our survey thought that NGTs should not be used at all or had no opinion (8%). A more personal question concerned the willingness to use existing NGT products if they were available in Poland. Most of the respondents (46%) had no opinion on this, 27% would use them, while 14% would not use NGTs products and 13% saw no use for these techniques. Women were more conservative, as only 16% would use them, while men were less sceptical (39%). The observed results are in line with the research of scientists from USA or Israel [19, 20]. They pointed out that women have a stronger aversion to innovation, a lower interest in technology, a greater concern for the environment and a higher perception of environmental risks.

In 2020, Müller et al. [20] summarised the total number of tweets (in English) ever published about CRISPR/Cas9 since the first information about the genome editing technology came out on the social media platform Twitter. The applied analysis model BERT (Bidirectional Encoder Representations from Transformers) generated groups of positive/neutral/negative sentiments expressed in n = 1,311,544 tweets about the CRISPR/Cas9 technology. The information conveyed in the tweets generally concerned unspecified organisms (51%) or humans (30%), and plants were represented in only 5% of the tweets. The study concluded that overall, thematic tweets were initially received very positively (52%) compared to natural (40%) and negative (7.5%) tweets, but decreased over time [20]. Since 2015, various ethical concerns have been raised, especially in the field of human genetics, which also shed a negative light on NGTs applied in the plant sector. Nevertheless, the topics of disease and medical treatment were considered important in the discussion, and was associated with a positive attitude among people, which was also evident in this study.

The further development of the new genomic techniques is dependent on legal decisions restricting or supporting the technology, but will likely depend as well, to a certain extent, on the acceptance and the decision that a citizen makes in an election and a consumer makes in a purchase. It seems that an analysis of public acceptance of these biotechnological solutions is important for predicting the economic development and economic prospects of companies and investors operating in this field [21].

5 Conclusions

The study aimed to answer the question of whether the new genomic techniques, referred to by the acronym NGTs, are known in Polish society, and if so, whether their use is accepted, in what areas and to what extent. Opinion polls, despite their limitations in terms of sampling, reliability and accuracy of conclusions, are an important means of obtaining data for comparative analysis.

The results of our survey, which analysed a total of 194 questionnaires, offer some new insights into the current awareness and perception of NGTs by Polish citizens. In general, it was found that concepts such as genome editing, CRISPR/Cas9 and TALEN are hardly known. The majority of respondents acknowledged the potential benefits of using the technology in agriculture or healthcare, but with appropriate regulations that would be guaranteed by the EU. Nevertheless, respondents are not convinced about the personal use of CRISPR/Cas9 if it were available in Poland. For most of the questions asked, there were no statistically significant differences in the answers, regardless of gender, age, place of residence, occupation or education.

In order to solve the problems of modern agriculture, healthcare or environmental protection, new biotechnology solutions should be developed and disseminated. Despite considerable interest in research on NGTs in the EU [5, 22], Poland, as part of the EU, is on the way to accepting and implementing this technology in agriculture or in the medical field. We believe that the dissemination of information and advisory services, along with research and development, are crucial factors for the promotion and adoption of any technology, especially in agriculture. The presented study has shown a big gap in knowledge, therefore more efforts should be made, starting with education at school and university level, easy access to comprehensive and neutral information in the mother tongue, including both technical and legal issues, to inform Polish citizens about the possibilities offered by NGTs.

Acknowledgements The authors thank COST Action CA18111 "Genome editing in plants – a technology with transformative potential" for the possibility of getting and exchanging knowledge about NGTs. We would like to thank Dr. Justyna Marchewka-Długońska (UKSW) for the valuable comments on the statistical data, Dr. Izabela Bukalska (UKSW) and Dr. Joanna Wróblewska-Skrzek (UKSW) for the help in the formation of the questionnaire.

References

1. Chen, K., Gao, C.: TALENs: customizable molecular DNA scissors for genome engineering of plants. J. Genet. Genomics. **40**(6), 271–279 (2013)
2. Baltes, N.J., Gil-Humanes, J., Cermak, T., Atkins, P.A., Voytas, D.F.: DNA replicons for plant genome engineering. Plant Cell. **26**(1), 151–163 (2014)
3. Purnhagen, K.P., Wesseler, J.H.: Maximum vs minimum harmonization: what to expect from the institutional and legal battles in the EU on gene editing technologies. Pest Manag. Sci. **75**, 2310–2315 (2019)
4. Van Der Meer, P., Angenon, G., Bergmans, H., Buhk, H.J., Callebaut, S., Chamon, M., et al.: The status under EU law of organisms developed through novel genomic techniques. Eur. J. Risk Regul. **20**, 1 (2021)
5. European Commission. Study on the Status of New Genomic Techniques under Union Law and in Light of the Court of Justice Ruling in Case C-528/16 (2021). Available at: https://food.ec.europa.eu/document/download/5135278b-3098-4011-a286-a316209c01cd_en?filename=gmo_mod-bio_ngt_eu-study.pdf. Accessed 5 Sept 2022
6. European Commission Legislation for plants produced by certain new genomic techniques. Ref. Ares 6392169-16/09/2022 (2022). Accessed 14 April 2023
7. Twardowska-Pozorska, A., Grajek, W., Twardowski, T.: Co Polacy wiedzą o biotechnologii w 2001r. Biotechnologia. **4**(55), 115–121 (2001)
8. Twardowski, T.: Opinia publiczna a GMO. Biotechnologia. **3**(78), 45–65 (2007)
9. Rzymski, P., Królczyk, A.: Attitudes toward genetically modified organisms in Poland: to GMO or not to GMO? Food Sec. **8**, 689–697 (2016)
10. Woźniak-Gientka, E., Tyczewska, A., Perisic, M., Beniermann, A., Eriksson, D., Vangheluwe, N., Gheysen, G., Cetiner, S., Abiri, N., Twardowski, T.: Public perception of plant gene technologies worldwide in the light of food security. GM Crops Food. **13**(1), 218–241 (2022)
11. Uchiyama, M., Nagai, A., Muto, K.: Survey on the perception of germline genome editing among the general public in Japan. J. Hum. Genet. **63**, 745–748 (2018)
12. Gatica-Arias, A., Valdez-Melara, M., Arrieta-Espinoza, G., Albertazzi-Castro, F.J., Madrigal-Pana, J.: Consumer attitudes toward food crops developed by CRISPR/Cas9 in Costa Rica. Plant Cell. **139**, 417–427 (2019)
13. Saher, M., Lindeman, M., Hursti, U.K.K.: Attitudes towards genetically modified and organic foods. Appetite. **46**, 324–331 (2006)
14. Heiman, A., Agmon, O., Fleisher, R., Zilberman, D.: Attitude and purchasing decisions regarding genetically modified foods based on gender and education. Int. J. Biotechnol. **12**, 50–65 (2011)
15. López, O.A.M., Pérez, E.F., Fuentes, E.E.S., Luna-Espinoza, I., Cuevas, F.A.: Perceptions and attitudes of the Mexican urban population towards genetically modified organisms. Br. Food J. **118**, 2873–2892 (2016)
16. Simis, M.J., Madden, H., Cacciatore, M.A., Yeo, S.K.: The lure of rationality: why does the deficit model persist in science communication? Public Underst. Sci. **25**(4), 400–414 (2016)
17. Lakomý, M., Bohlin, G., Hlavová, R., Machácková, H., Bergman, M., Lindholm, M.: Public attitudes to life sciences research in six European countries. ORION project Deliverable, (2.3) (2018)
18. Woźniak, E., Tyczewska, A., Twardowski, T.: A shift towards biotechnology: social opinion in the EU. Trends Biotechnol. **39**(3), 214–218 (2021)
19. Öz, B., Unsal, F., Movassaghi, H.: Consumer attitudes toward genetically modified food in the United States: are millennials different? J. Trans. Manag. **23**(1), 3–21 (2018)
20. Müller, M., Schneider, M., Salathé, M., Vayena, E.: Assessing public opinion on CRISPR-Cas9: combining crowdsourcing and deep learning. J. Med. Int. Res. **22**(8), e17830 (2020)
21. Poort, L.M., Swart, J.A.A., Mampuys, R., et al.: Restore politics in societal debates on new genomic techniques. Agric. Hum. Values. **39**, 1207–1216 (2022)

22. Parisi, C., Rodriguez Cerezo, E.: Current and Future Market Applications of New Genomic Techniques, EUR 30589 EN, Publications Office of the European Union, Luxembourg, ISBN 978-92-76-30206-3 (2021)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 30
Improving Science Communication About Genome Editing – Mitigating Strong Moral Convictions Through Shared Moral Goals

Gabi Waldhof

Abstract A large share of natural scientists considers the application of genetic engineering (GE) to be necessary for a sustainable transformation of agriculture. However, there is a stark divergence between scientific assessment and public perception of this technology, particularly in Germany. So far, science communication has not been able to reconcile this divergence. The chapter suggests that strong moral convictions hinder effective science communication and provides scientific evidence for this claim. Furthermore, the chapter presents research suggesting that strong moral convictions can be mitigated through shared moral goals. Building on this insight, recommendations for improved science communication about GE are formulated.

1 Introduction

In the coming decades, the agricultural and food industry will face major challenges such as climate change that will affect the cultivation of agricultural crops, for example through more extreme weather conditions [1, 2]. In addition, the food supply of the steadily growing world population must be secured [3]. Also, the European Commission's goal to make the European Union (EU) economy climate-neutral by 2050 [4] also contributes to the fact that people require a far-reaching transformation to enable a sustainable economy.

G. Waldhof (✉)
Faculty of Business, Economics and Social Sciences, Department of Socioeconomics, Universität Hamburg, Hamburg, Germany

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_30

1.1 Genetic Engineering as a Potential Tool

Such a sustainable economy, e.g. a bioeconomy, can in part be enabled by innovative, efficient and sustainable technologies. One such technology with high potential – that is even considered necessary by many experts [5] – is genetic engineering (GE). Here, GE refers to those genomic techniques that include gene transfer and gene editing [6]. In the broader debate, its products are often referred to as "genetically modified organisms", "GMO", "GM foods", or pejoratively "genetically manipulated organisms" [7]. Here, the term "genetically engineered" (GE) is used, because "genetically modified" can refer to any type of genetic modification, including conventional breeding [8].

Scientists involved in GE argue that GE and its products can have many beneficial trades: according to numerous scientific studies, GE can greatly increase crop yields and thus create economic opportunities for farmers in poorer countries [9]. One reason for this is its potential to make plants more resistant to environmental stress such as extreme weather conditions like drought [10, 11]. Moreover, GE can increase the soil compatibility of cultivation and protect biodiversity, e.g. by reducing the use of chemical pesticides [9]. It can also be used to develop healthier food. One of the most famous examples is Golden Rice, which is enriched with beta-carotene [12].

Furthermore, an extensive study by the National Academies of Sciences, Engineering, and Medicine [8] concludes that GE crops are not riskier than conventionally produced crops, but have generally favorable social, economic and ecological effects. In addition, among natural scientists, there is almost consensus on the safety of this technology: 88% of the members of the American Association for the Advancement of Science (AAAS) consider GE foods safe to eat [13]. German science academies are also uniformly in favor of the use of GE, for example, the Academies of Sciences Leopoldina or the Berlin-Brandenburg Academies of Sciences [14, 15]. In general, scientific findings convey a positive picture of the effects of GE. For example, in a joint statement, the German National Academy of Sciences Leopoldina, the German Research Foundation, and the Union of the German Academies of Sciences report increased yields and income for farmers through GE, as well as reduced pesticide use [16]. Qaim also notes that the technology can contribute substantially to sustainable agriculture and food security [17].

These study results signal that GE can address some of the current challenges, such as climate change, malnutrition, and economic hardship. For example, the German Ministry for Education and Research (BMBF) even considers GE necessary to manage such topics [18].

1.2 Public Perception Stands in Stark Contrast to the Scientific Assessment

Despite the quasi-consensus among scientific academies, there is a large discrepancy between the natural scientists and the public's opinions on GE, particularly in Germany.

Numerous studies on the societal acceptance of GE indicate people's general rejection of such biotechnologies. For example, a recent survey shows that about half of the German population is concerned about GE food [19]. In another survey, respondents were asked to name problems and risks associated with food: GE foods were amongst the most frequently named causes of worry [20]. Additionally, even when asked for spontaneous associations with food risks, GE foods were among the most frequently mentioned items (i.e. 9%) [20]. To assess the public perception of GE, the Author of this chapter conducted a series of studies with their colleagues. Among the population, rejection remains high. A survey that is representative for the German population according to age, gender, income, education and region (N = 619), showed a rejection rate of 67% [21]. In another representative survey from 2020, 75% of participants (N = 653) stated to be more likely to reject GE [22]. In another study, this rate (76%, N = 636) is replicated. Others even found a rejection rate of 83% in Germany [23, 24]. In 2022, a study by the Federal Institute for Risk Evaluation found that 64% of consumers (N = 1001) said that they were "somewhat" to "very" concerned about GE foods [25].

1.3 Public and Consumer Support Is Necessary for Adequate Policies and Their Adoption

The stable and stark rejection of GE by the German public stands in contrast to the assessments of scientists involved in GE research. This is problematic because scientific consensus on the technology does not suffice for widespread adoption. It also requires the support of the public and consumers because public rejection can lead to adverse policies, and consumer rejection additionally hinders adoption. Without public support there are serious challenges in terms of policy making, agricultural development and science, as GE products cannot be beneficially developed and implemented.

Studies show that bans on GE prevent their potential positive effects and can even have negative consequences such as high opportunity costs, vitamin deficiency, hunger, economic losses, disproportionate bureaucratic efforts, etc. [9, 11, 26–29]. A prominent example is the earlier mentioned Golden Rice, which could be part of the solution for famine in poor countries since it counteracts common malnourishment by supporting the human body to produce vitamin A [12]. However, the stark

public rejection has so far prevented the cultivation of Golden Rice on a large-scale. Since this technology cannot be used for food production in Europe, or rather in Germany, its advantages cannot be used either.

If the application of GE really brings all these positive effects for environment and society, urgent action is required. In order to contribute to solving this problem, the present chapter hypothesizes that a decisive driver for the stark divergence between scientific assessment and public perception was the way the German GE debate has been conducted in the public arena. Consequently, the present chapter suggests that this divergence can be mitigated by improving the public debate about biotechnologies, as well as its science communication.

1.4 Advocates in the German GE Debate Are Rarely Heard

In Germany, particularly the terms “Grüne Gentechnik” (green gene technology) and “GMO” – pejoratively understood by the public as “genetically manipulated organisms” – have become popular [30, 31]. Here, GE foods have been heavily criticized since their emergence. Probably no other foodstuff has ever been discussed as fiercely in Germany. The public debate has been ongoing since the late 1980s, and a settlement is not in sight. The arguments in the debate are manifold and often surprisingly contradictory. While opponents fear risks to human health [32], advocates report on health-promoting properties of such plants, e.g. through vitamin enrichment [12]. While proponents are convinced that increasing yields through GE increases the freedom and autonomy of farmers [33], opponents see poor smallholders in developing countries threatened by patents on GE products [34]. One might think that such contradictory views could be clarified by examining the facts and comparing the evidence. But the public discourse could not have been resolved so easily.

In the wake of this rejection, numerous demonstrations have been organized. The most prominent is the “Wir haben es satt!” [We are fed up.] event in Berlin, where approximately 30 thousand participants demonstrate against GE every year, among other issues.

A large number of Non-Governmental Organizations (NGOs) are involved in organizing this event, such as Bread for the World or the Nature and Biodiversity Conservation Union of Germany [35]. In general, NGOs position themselves homogeneously against GE in the public debate [36]. Organic associations such as “Demeter” also reject GE [37]. Organic organizations publicly represent this rejectionist stance prominently. For example, the initiative “Ich stehe auf Essen ohne Gentechnik” [I stand for food without GE] collected more than 100 thousand signatures in 2018 and handed them over to the Federal Environment Ministry [38].

This gives the impression that not only the majority of the public but also the majority of official organizations reject GE. This impression is created because proponents of GE are less present in the social debate. Although agricultural associations such as Deutscher Bauernverband position themselves in favor of GE [39],

it is unknown if they launched any media campaigns or initiatives. The situation is similar for biotechnology companies such as Syngenta or Bayer: they are in favor of GE, but publicly accessible information is brief and rare [40]. Political supporters also largely consider the debate to be settled – and are quite obviously withdrawing their involvement [41]. Thus, on the advocating side, for the most part, only scientists and science representatives are involved in the discourse, such as the Association for Biology, Biosciences and Biomedicine [42].

1.5 Moral Convictions Can Hinder Policy Debates and Science Communication

The current situation of the public debate about GE, as well as the stark public resistance against this technology, particularly in Germany, suggest that science communication about GE has not been effective. This chapter aims to expand on why this is happening and how to solve this problem.

Particularly, based on recent research, this chapter proposes that science communication has not been effective due to strong moral convictions. This chapter further proposes that these strong moral convictions can be mitigated by providing credible, fact-based, scientific reasons for GE that address people's dearest moral concerns.

Accordingly, this chapter takes the German debate about GE as an example to learn about the debate, improve science communication about GE more generally, and about newer genomic techniques such as "CRISPR"[1] [43] in particular.

For this purpose, this chapter proceeds as follows: First, the German GE debate is discussed. Based on this, as well as on research about judgement and opinion formation, it is argued that a closer look into moral convictions related to GE is sensible. Then, it is reported on research showing that GE attitudes in Germany are mainly based on such moral convictions. It is then suggested that a ban (or approval) of GE has become a moral goal in itself, rather than a tool. However, as this chapter argues, this strong moralization can be overcome if the focus can be turned towards common moral goals. It is reported on research that successfully tested this. Building on this, suggestions of how to improve science communication about GE are made.

[1] ""CRISPR" (pronounced "crisper") stands for Clustered Regularly Interspaced Short Palindromic Repeats, which are the hallmark of a bacterial defense system that forms the basis for CRISPR-Cas9 genome editing technology. In the field of genome engineering, the term "CRISPR" or "CRISPR-Cas9" is often used loosely to refer to the various CRISPR-Cas9 and -CPF1, (and other) systems that can be programmed to target specific stretches of genetic code and to edit DNA at precise locations, as well as for other purposes, such as for new diagnostic tools. With these systems, researchers can permanently modify genes in living cells and organisms [for example to make food crops more drought resistant] and, in the future, may make it possible to correct mutations at precise locations in the human genome in order to treat genetic causes of disease." [43]

2 GE Attitudes Are Likely Guided by Moral Intuitions

2.1 Complex Topics Such as GE Are Difficult to Assess as a Single Person with Time Constraints and Without Expert Knowledge

GE is a collective term for several methods which can be used for a variety of applications. From a theoretical perspective, these can result in applications with normatively desirable results as well as with normatively undesirable results. This chapter therefore takes the position that GE can yield many beneficial contributions to society and nature. At the same time, this also means that abuse of this technology is conceivable, and appropriate measures should be taken that help prevent such abuse.

For a single person not involved in the topic, and with their own time constraints, it is difficult to make an informed assessment of which applications should be used under which circumstances. Even experts involved in GE do not make any of these assessments alone. On the contrary, it usually takes different groups that can assess e.g. economic, social, or environmental aspects. Consumers, therefore, need an appropriate complexity reduction to formulate an informed opinion about GE.

Conversely, this means that potential benefits are difficult to be effectively communicated in the public debate: on the one hand, sweeping endorsements of GE lack credibility. Since the majority of the German population already rejects GE, such diametrically opposed blanket statements would only meet with even more resistance. On the other hand, differentiated, deliberative views may not entail the kind of complexity reduction that consumers, for example, need in their everyday, non-primary decision-making behavior. They are therefore difficult to communicate.

2.2 Public Debate Should Provide Guidance, But Mutual Accusations Make It Unclear Who to Trust

A public debate about new technologies can serve as a complexity reduction that informs the public or consumers and provides fact-based arguments about the risks and benefits in relation to the adoption of a technology. At the same time, public debates sometimes do not seem to move towards a consensus. This may leave the non-expert consumer with questions as to what to make of a technology.

In this, scientists – as the experts in their respective field or technology – have the task to make assessments about potential risks and benefits, and to make those publicly available. These assessments should provide guidance in trade-offs in public debates about new technologies. In fact, about GE, there are a number of such studies by scientific academies. The National Academies in the United States of America (USA) have evaluated the evidence available on GE and found no evidence that GE products are less safe for humans and animals than other products [8].

The World Health Organization states that no adverse health effects of GE are known to date [44]. Moreover, the German Ministry of Education and Research, BMBF reports on more than 300 studies that assess the safety of GE [45].

In their statements, the German national academies and the European Academies Science Advisory Council note the high potential of GE, as did NASEM, in their report [8, 16, 46]. In 2019, the German Research Foundation, the Union of the German Academies of Sciences and Humanities, and the Leopoldina repeatedly pointed to the opportunities offered by GE [14].

However, in the German GE debate, scientists and their official statements appear to not have been successful in contributing their expert-mediator role to the debate. One potential explanation for this is the situation of the German GE debate mentioned above, in which the opposition is large, strong, present, and well-organized [35, 36], while the proponents are not very present but almost non-existent in the public debate [47]. This led to the curious situation in which scientists involved in GE research are almost the only group publicly providing arguments in favor of GE.

This situation in turn – so it is argued in this chapter – has made it difficult to perceive scientists in their role as mediators. In the case of GE, they may often not be perceived in a neutral position as informing experts, but rather, as is argued here, as a **supporting party within the debate** [47]. This may make it difficult for a consumer who wishes unbiased guidance on a complex topic.

This problem is fortified by the way the debate is conducted – in part. For example, the molecular biologist Ralph Bock asks in one of his essays whether politics wanted to "bully" the researchers in Germany until they were driven to despair [48]. The geneticist Wolfgang Nellen speaks of "hysteria" and "ignorance" in one of his articles [49]. Another example can be found in an article by geneticist Reinhard Szibor: "Green genetic engineering apparently serves as a projection surface for all the fears and frustrations of this world." [50].

In 2016, this situation led 124 Nobel laureates to a rather extreme measure: they signed an official letter urging Greenpeace and the governments of the world to cease the campaigns against Golden Rice [51].

In this letter, the authors position themselves against opposition based not on scientific facts but on "emotion and dogma" [51]. They also call on governments to reject such campaigns. The criticism of Greenpeace in the letter is sharp: the NGO is accused of ignoring or misinterpreting scientific evidence, and of supporting field destruction. Implicitly, the Nobel laureates accuse Greenpeace of being responsible for the deaths of up to 500 thousand children annually. They also speak of a "crime against humanity" in this context. To date, 160 Nobel laureates signed this letter.

These examples show that scientists do not always maintain their neutrality as expert-mediator on the issue, but also argue with strong emotions and sharp criticisms against GE opponents. Such emotional debating does not strengthen the public's trust in scientific expertise but may make science appear biased.

As a further intensifier of the problem, it can be observed that some GE opposing organizations doubt the integrity of scientists. For example, in response to the above-mentioned statement of the German Research Foundation and the National Academies of 2019, Testbioech e.V. spoke of an obvious "interest in the application

of genetic engineering and its commercial exploitation." [52]. Similarly, in response to the Nobel laureates' letter, Greenpeace does not show the desired reaction, but insinuates bias: the NGO asked the lead author to be honest, and to disclose economic interests in the technology [53]. This reinforces an impression of partisanship.

At the same time, while science communication is necessary for progress and education, and is increasingly demanded and promoted, it is difficult for scientists to always argue in an unrestrictedly objective manner since they also want to defend their work, their community, and their identity. The current GE debate in Germany runs the risk to lead to further frustration and resignation among scientists.

On top of this, both sides are not involved in a dialogue, but address **topics that are unrelated** [36]. For example, while supporters of GE foods usually point out the technological advancements and the increased economic efficiency of GE, opponents of GE foods usually refer to potential environmental risks and health risks [54, 55].

Moreover, supporters and opponents also appear to use different **concepts of naturalness** in their arguments. On the opponents' side, nature is seen as something sacred and pure that should remain untouched [56]. For advocates of naturalness, the GE of plants feels like interfering with nature and contaminating our flora [57]. Thus, the advocates of this view reject any human processing in nature [58]. On the other side, supporters affirm that GE foods do not pose any risks to nature and can even have beneficial properties for nature [45]. While GE supporters attribute some characteristics to nature that should be sustained, such as biodiversity, GE opponents see nature as something sacred that does not allow human processing [59].

With reference to the previous point, the debating parties may **emphasize distinct values in their arguments**. For example, while opponents point out aspects of fairness in relation to GE foods, supporters may rather focus on loyalty. More specifically, opponents state that corporations involved in GE food products disregard the rules for fair competition by lobbying for their own interests [60]. Supporters on the other hand promise the commitment of scientists to the use of GE for the benefit of society [45].

2.3 People Likely Resort to Moral Values and Emotions as Guidance

The current situation of the public GE debate, as well as the fact that GE assessment requires expert knowledge and years of training, suggests the following assumption: the public and consumers resort to intuitive guidance such as moral values and moral emotions. And indeed, research on factors that influence people's opinion towards GE suggests that knowledge does not have a substantial influence on how people perceive this technology [61]. Rather, intuitions, such as emotions, play a much bigger role in GE acceptance [62].

The importance of emotions as intuitions for people's opinion formation can be explained by adopting the distinction between two modes of thought: System 1 and System 2 [63]. System 1 operates automatically and quickly, with little or no effort and no sense of voluntary control. This refers to anything that we do intuitively or with a lot of practice, such as recognizing a friend or understanding the facial expression of anger. System 2 comprises effortful mental activities, such as complex computations. Operating System 2 entails conscious reasoning and requires agency, choice, and concentration [63]. For example, we use it when we make plans or check a logical argument for its validity. System 1 may be an evolutionary adaptation [64]: in order to survive, emotions needed to signal us fast and reliably when we are facing a threat, such as rotten food. Since emotions lead to much quicker judgments than rational thinking, they serve as a guide in matters of immediate danger.

There is a special type of emotions, moral emotions, that are neatly intertwined with moral values. In general, these are such emotions that motivate us to behave morally, and that respond to violations of moral values [65]. Following moral values means setting aside pure self-interest and orientating behavior and judgment according to the interest of the greater social welfare [66]. In human nature, **moral values take priority over non-moral concerns** [67]. Since we are social beings and could not survive without the protection and help of a group, moral values also fulfil an adaptive task: they make us liable for our actions and hence make it possible for us to live in groups and resist temptations which may be destructive for the survival of the entire group [68].

Since we relied on emotions and moral emotions for survival, it seems plausible that we also listen to them when we form our opinions on overly complex issues such as new technologies that may have an unprecedented impact on our lives.

And indeed, there is scientific evidence that opinion on GE foods is guided by moral values and moral emotions. Research on public opinions on agriculture finds that GE easily evokes emotions such as anger [69] and is usually connected with normative demands [70]. Moreover, it could be found that knowledge about GE is rather low in the public and that perceived fairness and trust in food producers enhances GE acceptance [54]. Furthermore, as mentioned earlier, a perceived violation of nature deemed sacred seems to be one of the main reasons for the rejection of GE foods [59, 61]. This can be grounded in seeing nature as an intrinsic value that needs protection, or in religious reasons such as human interference in god's will [56].

2.4 For Many, Trying GE Is Not Worth the Risk

Above, it was proposed that the complexity of the issue, as well as the situation of the public GE debate, lead people to resort to moral intuitions in their opinion formation. And indeed, for example, people's individual predisposition towards risks plays a decisive role in opinion formation about GE: research has shown that

risk-averse individuals are significantly more likely to oppose GE than risk-averse individuals [21].

Many people tend to want to avoid future uncertainties in principle [71]. The introduction of new technology represents such a future uncertainty, as one deviates from the known status quo. It seems to be the case that the majority of the German public prefers to maintain the status quo with regard to GE, rather than risk unanticipated consequences as a result of the adoption of the technology. In this chapter it is suggested that this is possible because in Germany, for the average consumer, it comes at no perceptible cost to maintain the status quo because there are enough affordable alternatives. Simply, there is the opportunity to be against GE. Under these circumstances, it does not seem "worth the risk" to try something that is perceived as unknown, compared to conventional breeding.

This importance of moral intuition implies that a debating party will be more convincing if they speak to those moral emotions and values that are most relevant to the public. Arguments that are less relevant with regard to moral emotions and values have much less persuasive power.

And at the moment, GE opponents are more successful in addressing people's worries and risk aversion, by feeding into them. For example, a meta-analysis found that studies made by NGOs without scientific peer review appear to systematically reduce the estimated positive effects of GMOs [9]. Such measures reinforce the public perception that came to see these products as much more threatening than research would suggest.

Moreover, GE opponents address moral emotions such as disgust or anger, and thus further stimulate a rejection of GE foods in the public. For example, in some campaigns, GE foods have been associated with strong, disgust-provoking images, such as a woman eating 'Frankenfood', i.e. a genetically modified tomato which looks like a fish [72]. From eating this tomato, her skin is turning green. With that, the moral emotion of disgust is triggered, which relates to a violation of nature as an intrinsic value that no one should interfere with. So, moral opposition is the consequence.

Moreover, using language that is easy to grasp is another common strategy for many GE opponents [36]. Polemic terms such as "Gene-contamination" [31] or "Frankenfood" [73] produce memorable pictures and dramatize the topic. The consequence is predominantly negative associations with GE foods. Generally, some NGOs opposing GE make heavy use of emotionalized language. For example, in a book published by Earth Open Source, the authors describe GMO supporters as 'unpleasant, angry, and hostile' [74]. Another recent booklet states that GE 'failed' [31].

In contrast, arguments put forward by GE supporters seem to have less intuitive appeal [64].

Moreover, media reporting on GE likely contributes to this focus on risks associated with GE. Scientific experiments show that consumers prefer negative news and react more strongly to it [75, 76]. Negative news are therefore easier to sell, which means that they are likely overrepresented in the media landscape [77]. This is where the media's incentive to report on potential risks of GE – rather than on potential benefits of the products – becomes apparent.

3 Moral Intuitions About GE Have Resulted in Strong Moral Convictions

As proposed above, the stark rejection of GE in the public and the fierce debate have led to a moralization of the topic. In our representative surveys, the majority of participants indicated that the question of GE was a moral one for them [21]. Furthermore, in recent survey (N = 619; N = 636), up to 90% of the opponents stated that they reject GE, no matter how great the benefits [21, 23]. This is in line with previous research by Scott et al. [59], who measured GE attitudes in the USA. Of their representative sample, 64%, were opposed. Of this opposition, 71% (i.e. 46% of the entire sample) stated opposition regardless of the consequences, claiming that GE (in this study: GMOs) "should be prohibited no matter how great the benefits and minor the risks from allowing it" were [59]. Other studies achieved similar results [24]. This means that participants state to maintain their attitude regardless of possible consequences. Many in Germany have become consequence-insensitive with regard to their GE position.

Taken literally, this means that the perceived benefit from a ban of GE, or approval, respectively, is infinite [59, 78]. Thus, there is no cost-benefit trade-off regarding the use of GE. Rather, their GE position has been elevated to a sacred value worthy of protection [79].

Interestingly, research has shown that attitudes toward GE are consequence-insensitive for a significant proportion of supporters as well [22, 23]. Here, it is important to emphasize that, contrary to popular belief, GE is not only a moral issue for opponents. Rather, for a significant proportion of supporters, their GE position has become a moral issue as well.

Such strong moral conviction makes it hard to engage in a constructive discourse about the risks and benefits of the technology and its potential applications. Here likely lies one reason for why science communication about GE is not effective at the moment. Rozin refers to such phenomena as "moral piggybacking" [80]. Here, objects or facts that are in themselves value-free are morally charged, as can be observed, for example, among vegetarians on the topic of meat consumption [80, 81]. For many, GE opposition, but also GE support, has become a moral goal in itself.

3.1 In the Public Eye, GE Has Become a Moral Goal, Rather Than a Means to Moral Goals

Among consumers and the public, the moralization of GE has grown so strong that a ban or approval of it now has an intrinsic moral value, good or evil. This means that the public does not perceive the properties of the technology, but the technology itself as the central interest in the discourse. Rather than looking at potential

consequences of the technology, GE has become the moral goal of the debate. Here, moral goals are confused with their potential means [82]. GE has become the moral goal in itself [47].

This normativity of GE in the population is readily taken up by the parties to the discourse and forms the central problem of the conflict: in a generalized normative ban or command on GE are mutually exclusive, there is no room for agreement.

It can be observed that positions in the public debate are generally quite generalized. These general evaluations create two fronts of “right/good” and “wrong/evil”, in which the goals of each side appear incompatible: it appears that one can either be in favor or against GE. The fact that many arguments in the debate are not weighed up in a differentiated way creates the impression that one can only decide for or against one side. This creates a moral conflict of objectives for the public in which the potential positions appear mutually exclusive: It appears as if one can position oneself either for a ban on GE, or an approval of GE.

In the case of mutually exclusive demands, however, it is not possible to resolve the discourse to the benefit of both sides. Since it is then not in the interest of either side to deviate from their positions, the discourse is stuck [47].

In this situation, the debating parties perceive the debate as a tradeoff thinking with little room for a solution. The debate resembles a social dilemma: the strategies of the participants lead to a mutually worse position because this stalemated debate benefits no one and is tiresome for all participants and observers [82]. This stuck discourse puts pressure on policymakers and ultimately leads to legislation that reflects the goal-means confusion. As an institutional framework for research and development, this legislation blocks innovation. The results are opportunity costs because possible solutions cannot be found and urgent problems remain unsolved [29, 83].

3.2 Moral Convictions Are Especially Hard to Mitigate

These strong moral convictions provide another explanation for why science communication and arguments of scientists involved in the debate do not seem to reach the public: research in moral psychology has shown that moral judgments are much more stable and long-term than other judgments [84]. This is because, for humans, moral concerns take priority over non-moral concerns [67]. Such strong moral beliefs have real effects on daily actions: For example, people with different moral beliefs are more likely to be avoided, and they are also less likely to be believed [85, 86]. Over time, some moral beliefs can become so strongly attached to a person’s core moral constitution that they become linked to that person’s identity [80, 81, 85].

And indeed, in recent research, a large proportion of subjects view their position towards GE as central to their personal moral beliefs [87].

4 Can Strong Moral Convictions Be Mitigated?

If science communication has difficulties reaching the majority of the German public because of strong moral convictions about GE, a solution that mitigates these strong moral convictions and allows GE to be discussed as a possible means, rather than a moral goal is necessary.

Similar suggestions can also be found in "Ethical Tensions from New Technology" [88]. It is not the technology that should be discussed, but its applications. And this with regard to questions such as: What problems do we face? For which of these problems can GE be an appropriate tool? Under what conditions? What are the risks? Are there sensible alternatives? Similarly, scientific academies in Germany are already calling for it [14].

However, if the rejection of GE is moral, is there a way to overcome this moral rejection of this technology?

Since the beginning of modernity, many technological innovations have enriched society. For example, medical diagnostics was able to make great progress through X-ray or MRI technologies. Everyday life has also been made easier by household appliances such as the washing machine or the dishwasher. Some technologies are quickly adapted because of their obvious high utility (e.g., X-ray machine, household appliances). New technologies are not always greeted with openness; in some cases, their development is viewed skeptically. For example, Kenneth Olsen saw no reason why anyone would need a computer at home [89]. Sir Preece, the chief engineer at the British Post Office, thought the telephone was unnecessary because there were enough letter carriers [90]. Thus, some technologies experience initial skepticism, but then prevail because of their advantages.

In the eighties and nineties, there were very heated debates in Germany about the medical application of GE [91]. The focus here was particularly on human insulin, which has been used since the 1980s to treat diabetes. Concerning this, negative reports circulated in the media. A newspaper called "TAZ" poke of a "gateway drug" [92] and reported on side effects and dubious competitive practices surrounding the drug [93]. In 1991, a report of the public television broadcasting service "ARD" spoke of frequent and serious risks to patients from human insulin [94]. These reports were contradicted by physicians and scientists. For example, in the German Medical Journal, the German Medical Association confirmed the successful and problem-free treatment of patients since 1983 [94].

By now, this great initial skepticism has dissolved into acceptance. In the meantime, Germany is one of the world's leading producers of genetically engineered pharmaceuticals, which are no longer an issue in the public debate [91].

Thus, it is proposed here that consumers and the public need to be provided with tangible, credible reasons to try GE foods or adopt GE technology in spite of their worries related to the technology. Specifically, the advantages of GE have to become salient and tangible enough for people to outweigh perceived potential risks.

4.1 Moral Goals Can Mitigate Strong Moral Convictions

Recently, it was tested whether there are such consequences of GE that would make the public and consumers abandon their moral convictions about the technology [87]. Specifically, in an online experiment in Germany in 2020 (based on own research), representative of the population (N = 1900) according to age, gender, and level of education, it was tested whether respondents could identify consequences that are so important to them, that they would abandon their moral convictions towards GE. This experiment only included people who indicated a strong moral conviction towards GE. This led to the inclusion of around 1500 GE opponents who stated that they would reject GE regardless of the consequences. Additionally, this also led to inclusion of around 400 GE supporters who stated they approve of GE regardless of the consequences.

These "consequence-insensitive" participants were randomly assigned to four probes that tested their moral conviction. Here, two of those probes are presented.

In the open-ended reflection task, consequence-insensitive participants were asked whether they could think of any circumstances that would make them abandon their position towards GE. Respondents could then provide their answers in free-text boxes. Table 30.1 shows examples of circumstances stated, the moral goals these address, as well as their frequencies.

In this probe, a little less than 50% did not state any consequence that would make them abandon their consequence-insensitive position towards GE – but more than 50% did!

In another, the counterexamples probe, consequence-insensitive participants were provided with a list of moral goals and asked them to select all of those that would make them abandon their consequence-insensitive position towards GE. Table 30.2 shows how frequently each moral goal has been selected.

In this probe, almost every opponent (361/369; 97.8%) and supporter (100/101; 99%) selected at least one option.

The results of these probes show that nearly everyone who previously proclaimed consequence insensitivity indicated that there are circumstances for which they would abandon their position.

After these probes, participants were asked to state their positions towards GE again. Interestingly, a significant proportion of those who initially stated to maintain their position regardless of the consequences abandoned this strong moral conviction after the probes. Moreover, the attitudes towards GE among those previously morally convicted also became less extreme after the probes [87].

Therefore, while moral convictions about GE are both common and hard to change, still the majority of consequence-insensitive participants state that there are at least some circumstances that would make them abandon their position. This was particularly true when common moral goals were made salient to them. Examples of such moral goals that are dearest to participants are life, health, and environmental protection.

Table 30.1 Open-ended reflection (share of consequence-insensitive respondents mentioning a topic)

Opposition status	Coded categories	Paraphrased topics (Examples)	Share of consequence-insensitive respondents
Consequence-insensitive opponents	Reaffirmation of position	Reasons against GE crops	16% (60)
	Stated they cannot think of anything	Don't know, don't care, nothing	23% (84)
	No response	No response	9% (33)
	Food security	Famine, poverty, overpopulation, food	23.2% (85)
	Health	Fight diseases, medicine / health	12.8% (47)
	Environment	Dying species, environment protection, sustainability	11.2% (41)
	Emergency	Fight wars and crises, save lives, no alternative	11.2% (41)
	Resilience	Fight pests / plant diseases, adapt to climate or soil conditions	9.8% (36)
	Efficiency	Higher yield, product optimization	2.5% (9)
	Safety	If proven to be harmless, strict labelling, separation of GMO / non-GMO	1.9% (7)
	Research	For research purposes	1.4% (5)
Consequence-insensitive supporters	Reaffirmation of position	Reasons in favor of GE crops	3% (3)
	Stated they cannot think of anything	Don't know, don't care, nothing	17% (16)
	No response	No response	23% (21)
	Health	Medical risks, unhealthy food	29.3% (27)
	Safety	Danger, mutations, loss of control	17.4% (16)
	Environment	Risks for animals, risks for environment	17.4% (16)
	Abuse	Use for war, weapons, moral concerns, human trials	14.1% (13)

Note. Totals do not match the number of respondents (and percentages do not sum to 100) because participants could provide up to five responses [87]

This means that consensus in the GE debate is possible and that it is possible for science communication to reach even the most morally convicted – if people's actual moral goals would become the focus of the debate, and GE would then be discussed as a potential means of these goals, rather than the goal itself.

Table 30.2 Counterexamples (percentage of consequence-insensitive respondents selecting each counterexample)

Opposition status	Genetic engineering of plants is acceptable if it…	Selected by
Consequence-insensitive opponents	Saves human lives.	63.7% (235)
	Prevents a global problem.	44.2% (163)
	Prevents more of the same thing.	24.7% (91)
	Improves living conditions.	18.2% (67)
	Has economic benefits.	13.3% (49)
	Is for a good moral cause.	12.2% (45)
	Genetic engineering of plants is NOT acceptable if it…	
Consequence-insensitive supporters	Reduces the quality of life.	49.5% (50)
	Kills people.	47.5% (48)
	Causes a global problem.	46.5% (47)
	Has economic disadvantages.	35.6% (36)
	Causes more of the same thing.	23.8% (24)
	Is bad for moral reasons.	19.8% (20)

Note. Totals do not match the number of respondents (and percentages do not sum to 100) because participants could select multiple responses [87]

5 Improved Science Communication About GE in Public Debate

5.1 Provide Credible and Tangible Advantages That Address Common Moral Goals

The abovementioned study indicates that in many cases, both supporters and opponents of GE actually share moral goals, such as life, health and environmental protection. This is also one result of another content analysis [95]. Moreover, our studies indicate that strong moral convictions about GE can be mitigated if people are provided with **credible, tangible moral reasons** to try GE products in spite of their worries.

If the common moral goals are identified, it is possible to examine whether GE is a suitable means for reaching these goals. Here, appropriate measurement tools can be used to clarify this perceived conflict. These are, for example, scientific studies, meta-analyses, risk analyses, or long-term observations of the plants under discussion.

Then, science communication about GE is likely effective if science communicators provide credible evidence that GE can be a tool to reach these moral goals.

For example, one of the common moral goals that Waldhof identified is effective development aid [95]. However, at the moment, some public statements against GE

state that the technology causes disadvantages for small farmers [31]. Evidently, many conclude from such statements that if one wants to pursue effective development aid, GE would be a hindrance. Consequently, the moral goal development aid would be a reason to ban GE.

Therefore, at the moment, the argumentation can be presented as follows [47]:

1. Normative assumption: It is a moral goal to provide effective development aid.
2. Positive assumption: GE causes economic disadvantages for small farmers.
3. Normative conclusion: Therefore, GE should be banned.

However, a meta-study on the effects of GE found that the technology especially benefits farmers in developing countries [9].

Similarly, Ahmed et al. concluded in their study in Bangladesh that farmers save their costs, obtain more yield, and likewise protect their health through reduced pesticide use by using a GE eggplant [26]. This scientific evidence requires that the positive assumption (2) needs to be changed: GE benefits small farmers in developing countries, particularly. As a consequence, the conclusion (3) also changes: GE should be used for development aid. Now, the argumentation is as follows:

1. Normative assumption: It is a moral goal to provide effective development aid.
2. Positive assumption: GE benefits small farmers in developing countries, particularly.
3. Normative conclusion: GE should be used for development aid.

The same procedure can also be applied to the moral goal of nature conservation. GE crops are often associated with increased pesticide use [96]. Pesticides can be a burden on soils and can also put insects at excessive risk. For this reason, Brookes and Barfoot examined pesticide use associated with GE from 1996 to 2015 [97]. They found that pesticide use decreased by 8.1% due to the use of green genetic engineering. Overall, their study concludes that biotechnology has a positive impact on the environment. GE thus contributes to nature conservation.

5.2 Include Credible and Salient Science Communicators That Truly Care About the Moral Goals

It is argued above that scientists involved in GE research are currently not perceived as expert mediators that can solve the discourse block, but as – a somewhat biased – party within the debate. It was argued that this may be in part because of the way the debate is conducted, and because scientists and scientific organizations are the only ones bringing forward supporting arguments for GE. Consequently, scientists alone cannot resolve the strong moral conviction and the resulting discourse block. They need support. In the same vein, in a newsletter of the Leopoldina, Hans-Georg Dederer, demands more support from politicians [41].

It is argued here that, alongside science-based and moral arguments, the debate about GE needs additional actors that truly care about the moral goals, and not about the technology or its ban. By now, more and more such actors are joining the debate. One example is Professor Urs Niggli, who was the director of the Research Institute for Organic Agriculture for 30 years since 1990 and is also known as the "organic pope" [98, 99]. He is considered one of the world's leading scientists in organic agriculture and is known for his commitment to sustainable food and farming systems [99, 100]. According to him, agriculture is at a turning point where a stronger orientation towards technology is necessary [101, 102]. For him, this also includes the use of biotechnological processes in agriculture. Niggli believes that the use of CRISPR means that goals such as food security and biodiversity no longer have to be mutually exclusive, but can be achieved together [99].

The NGO "Progressive Agrarwende" takes a similar view. This young group of scientists, students and politicians, founded in 2019, is pursuing the goal of implementing an agricultural turnaround toward socially and ecologically sustainable agriculture according to the latest scientific and technological standards [103]. They observe that many environmentally conscious people act against their own goals by a blanket rejection of modern agricultural techniques. For this reason, they have founded a dialogue platform to address applications, regulations and patenting of agricultural technologies in an open-ended and transparent manner. To this end, Progressive Agrarwende regularly writes articles, organizes events or reports on scientific findings.

More and more actors that call for a science-based evaluation of products developed from GE technology are joining the debate or gaining more attention. These are for example maiLab (youtube.com/@maiLab), Grain Club (grain-club.de/) or transGEN (transgen.de/). Coalitions with such professional science communicators help scientific evidence gain salience in the debate. They will also help scientists to communicate their findings in an understandable and enjoyable way that is easy to follow.

Moreover, the call for a science-based assessment of GE is receiving more media attention. For example, the TV show MaiThink X dedicated an entire episode to arguing for a science-based evaluation of GE [104]. Similarly, in December 2022, the TV show Heute Show [Today Show] argued that a general fear of GE is not science-based and should be reconsidered [105].

Moreover, recent research has shown that in many arguments, the debate deviates from discussing the risks and benefits of the technology to discussing the behavior of the actors involved [95]. For example, while GE opposition criticizes unfair behavior of corporations, GE supporters claim loyal commitment of scientists to use GE research for the common welfare. However, ensuring the trustworthiness of scientists seems unlikely to solve reservations towards corporations or the market. Understandably, scientists involved in GE research cannot speak to the governance of involved actors. Here, it would help if social scientists joined the debate to discuss market mechanisms and the behavior of companies and other actors.

Furthermore, self-binding mechanisms offer a further opportunity to increase the quality of discourse. As described above, organizations involved in trade-offs also have a long-term interest in objectifying the discourse because of the loss of reputation that can otherwise be expected. If NGOs join forces and commit themselves to collectively agreed standards and fair discourse processes, they can signal the high quality of their contributions [106]. Corresponding initiatives already exist, such as Accountable Now! [83, 107, 108]. The NGOs involved regularly submit reports that are reviewed and commented on by a panel.

Generally, it seems that the German GE debate is changing, moving away from a generalized debate towards a more differentiated debate about what GE can and cannot do. In this, science-based arguments appear to be gaining increasing salience in the debate.

6 Summary

A large share of natural scientists consider the application of GE to be necessary for a sustainable transformation of agriculture. However, there is a stark divergence between scientific assessment and public perception of this technology, particularly in Germany. So far, science communication has not been able to resolve this divergence. Research on technology acceptance shows that people have a general tendency to follow their emotions and intuitions when forming their opinions on technologies [61]. The present chapter suggests that strong moral convictions hinder effective science communication and provides scientific evidence for this claim. This is because moral convictions are more stable than other judgements. This led to a situation, in which the approval or rejection of GE are perceived as a moral goal in itself. The analysis thus suggests that a way has to be found in which GE is perceived as a potential tool for moral goals, rather than a goal in itself. For this purpose, the chapter also reports on research which suggests that strong moral convictions can be mitigated through shared moral goals. Building on this insight, recommendations for improved science communication about GE are formulated.

Generally, GE should be perceived as a potential tool to reach moral goals such as food security or sustainability. With every tool, there are advantages and disadvantages. This is what makes thorough risk assessments, and a debate that weighs potential risks and benefits, indispensable. One result of such debates would be procedures that reduce potential risks of new technologies such as GE. At the same time, potential risks should be addressed symmetrically:

> The use of [new genomic techniques] NGTs raises ethical concerns but so does missing opportunities as a result of not using them. […] Any further policy action should be aimed at reaping benefits from innovation while addressing concerns. A purely safety-based risk assessment may not be enough to promote sustainability and contribute to the objectives of the European Green Deal […] [109].

References

1. Ali, S.M., Khalid, B., Akhter, A., Islam, A., Adnan, S.: Analyzing the occurrence of floods and droughts in connection with climate change in Punjab province. Pakistan. Nat. Hazards (Dordr.). **103**, 2533–2559 (2020)
2. Woodward, A.J., Samet, J.M.: Climate change, hurricanes, and health. Am. J. Public Health. **108**, 33–35 (2018)
3. Kc, K.B., et al.: When too much isn't enough: does current food production meet global nutritional needs? PLoS One. **13**, e0205683 (2018)
4. European Commission: A European strategic long-term vision for a prosperous, modern, competitive and climate neutral economy. Retrieved February 27, 2020, from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52018DC0773 (2018)
5. Bundesministerium für Ernährung und Landwirtschaft: Nationale Politikstrategie Bioökonomie, Nachwachsende Ressourcen und biotechnologische Verfahren als Basis für Ernährung, Industrie und Energie, BMEL (2014)
6. Qaim, M.: Possible socioeconomic implications of plant genome editing. Keynote presentation at the 3rd PlantEd conference of the COST Action CA18111 "Genome Editing in Plants", in Düsseldorf, Germany (06.09.2022)
7. Evanega, S., Conrow, J., Adams, J., Lynas, M.: The state of the "GMO" debate – toward an increasingly favorable and less polarized media conversation on ag-biotech? GM Crops Food. **13**, 38–49 (2022)
8. National Academies of Sciences, Engineering, and Medicine: Genetically Engineered Crops: Experiences and Prospects. The National Academies Press, Washington, DC. https://doi.org/10.17226/23395 (2016)
9. Klümper, W., Qaim, M.: A meta-analysis of the impacts of genetically modified crops. PLoS One. **9**, e111629 (2014)
10. Qaim, M.: What kind of agriculture do we need for future world food security?; Presentation at the InnoPlanta Forum Gatersleben (06.09.2017)
11. Qaim, M.: Role of new plant breeding technologies for food security and sustainable agricultural development. Appl. Econ. Perspect. Policy. **42**, 129–150 (2020)
12. Rauner, M.: Sind Sie auch gegen Genfood [Are you also against genetically modified food]? Zeit Wissen. https://www.zeit.de/zeit-wissen/2017/04/gentechnik-genfood-pflanzen-ernaehrung-gesundheit (18.07.2017)
13. Funk, C.: Public and scientists' views on science and society. Pew Research Center Science and Society. https://www.pewresearch.org/science/2015/01/29/public-and-scientists-views-on-science-and-society/ (2015)
14. Nationale Akademie der Wissenschaften Leopoldina, Union der Deutschen Akademien der Wissenschaften, Deutsche Forschungsgemeinschaft: Wege zu einer wissenschaftlich begründeten, differenzierten Regulierung genomeditierter Pflanzen in der EU https://www.leopoldina.org/uploads/tx_leopublication/2019_Stellungnahme_Genomeditierte_Pflanzen_web.pdf (2019)
15. Berlin-Brandenburgische Akademie der Wissenschaften [Berlin-Brandenburg Academy of Sciences and Humanities, BBAW]: Vierter Gentechnologiebericht. Bilanzierung einer Hochtechnologie [Fourth Gene Technology Report. Review of a High-tech sector]. https://www.nomos-elibrary.de/10.5771/9783845293790/vierter-gentechnologiebericht (2018)
16. Nationale Akademie der Wissenschaften Leopoldina, Deutsche Forschungsgemeinschaft, Acatech, Union der Deutschen Akademien der Wissenschaften: Chancen und Grenzen des genome editing [Opportunities and limitations of genome editing]. https://www.leopoldina.org/uploads/tx_le-opublication/2015_3Akad_Stellungnahme_Genome_ Editing.pdf (2015)
17. Qaim, M.: Genetically Modified Crops and Agricultural Development. Palgrave Macmillan US, New York (2016)

18. Wissenschaftlicher Beirat für Agrarpolitik beim Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz. [Scientific Council of the BMBF]: Koexistenz Gentechnik in der Land- und Ernährungswirtschaft, https://www.bmel.de/SharedDocs/Downloads/Ministerium/Beiraete/Agrarpolitik/Stellungnahme-Koexistenz-Gentechnik.pdf?__blob=publicationFile (2010)
19. Bundesinstitut für Risikobewertung: BfR-Verbrauchermonitor 8|2019. ISBN 978-3-948484-01-9 (2019)
20. European Commission: Special Eurobarometer 354 – Food-related risks. https://ec.europa.eu/commfrontoffice/publicopinion/archives/ebs/ebs_354_en.pdf (2010)
21. Waldhof, G.: Emotional and Intuitive Response to GMOs as a Topic of Debate. Unpublished Manuscript, University of Hamburg (2020)
22. Waldhof, G., Inbar, Y.: Evidence for Motivated Reasoning in the German GMO Debate. Unpublished Manuscript, University of Hamburg (2021)
23. Jauernig, J., Uhl, M., Waldhof, G.: Genetically Engineered Foods and Moral Absolutism: A Representative Study from Germany [Manuscript submitted for publication]. Department of Structural Change, Leibniz Institute of Agricultural Development in Transition Economies (IAMO) (2022)
24. Scott, S., Inbar, Y., Rozin, P.: In Europe and the United States, Most GE Food Opposition is Moral and Responds Specifically to Moral Countering [Manuscript submitted for publication]. Olin Business School, Washington University in St. Louis (2019)
25. Bundesinstitut für Risikobewertung [BfR]: BfR-Verbrauchermonitor 08|2022 [BfR Consumer Monitor 08|2022]. https://www.bfr.bund.de/cm/350/bfr-verbrauchermonitor-08-2022.pdf (2022)
26. Ahmed, A.U., Hoddinott, J., Abedin, N., Hossain, N.: The impacts of GM foods: results from a randomized controlled trial of bt eggplant in Bangladesh. Am. J. Agric. Econ. **103**, 1186–1206 (2021)
27. Lassoued, R., Macall, D.M., Hesseln, H., Phillips, P.W.B., Smyth, S.J.: Benefits of genome-edited crops: expert opinion. Transgenic Res. **28**, 247–256 (2019)
28. Biden, S., Smyth, S.J., Hudson, D.: The economic and environmental cost of delayed GM crop adoption: the case of Australia's GM canola moratorium. GM Crops Food. **9**, 13–20 (2018)
29. Van Eenennaam, A.L., De Figueiredo Silva, F., Trott, J.F., Zilberman, D.: Genetic engineering of livestock: the opportunity cost of regulatory delay. Annu. Rev. Anim. Biosci. **9**, 453–478 (2021)
30. Umwelt Institut München e.V.: Warum der Anbau genmanipulierter Pflanzen problematisch ist. https://umweltinstitut.org/landwirtschaft/gentechnik-bei-pflanzen/ (2023)
31. Greenpeace E.V.: Zwei Jahrzehnte des Versagens. Die gebrochenen Versprechen der Agro-Gentechnik [Two decades of failure. The broken promises of agro-genetic engineering]. https://www.greenpeace.de/publikationen/zwei-jahrzehnte-versagens (2015)
32. Then, C., Bauer-Panskus, A.: Russisches Roulette mit der biologischen Vielfalt [Russian roulette with biodiversity]. Testbiotech e.V. https://www.testbiotech.org/content/russisches-roulette-mit-der-biologischen-vielfalt (2017)
33. Nüsslein-Volhard, C.: Grüne Gentechnik und die Freiheit der Forschung [Green genetic engineering and freedom of research]. [Speech]. Gregor Mendel Stiftung. https://www.gregor-mendel-stiftung.de/fileadmin/files/downloads/2011-04-04_Vortrag_Nuesslein-Volhard.pdf (04.04.2011)
34. Callenius, C., Tanzmann, S.: Die Welternährung braucht keine Gentechnik [Feeding the world does not need genetic engineering]. Brot für die Welt, Aktuell. **37**. https://www.brot-fuer-die-welt.de/fileadmin/mediapool/2_Downloads/Fachinformationen/Aktuell/Aktuell_37_Welternaehrung_braucht_keine_Gentechnik.pdf (2014)
35. Kampagne Meine Landwirtschaft: Demo für gutes Essen & gute Landwirtschaft: Wir haben es satt! [Demo for good food & good agriculture: We are fed up!]. https://www.wir-haben-es-satt.de/ (2022)

36. Freitag, B.: Die Grüne-Gentechnik-Debatte: Der Einfluss von Sprache auf die Herstellung von Wissen. Springer, Fachmedien Wiesbaden (2013)
37. Demeter: Wahlfreiheit und Vorsorge sichern: Gentechnik muss gesetzlich geregelt bleiben [Ensuring freedom of choice and precaution: genetic engineering must remain regulated by law]! https://www.demeter.de/aktuell/gentechnik-muss-gesetzlich-geregelt-bleiben (2018)
38. Overmann, M.: Unternehmen und Bürger: Gemeinsam gegen Gentechnik. https://www.br.de/nachrichten/bayern/unternehmen-und-buergergemeinsam-gegen-gentech-nik, Qy57Pgm (2018)
39. Bauernverband, D.: Chancen der „Neuen Züchtungsmethoden“ nutzbar machen. https://www.bauernverband.de/presse-medien/pressemitteilungen/pressemitteilung/chancen-der-neuen-zuechtungsmethoden-nutzbar-machen (2019)
40. BAYER: Verborgenes Potenzial nutzen – Biotechnologie und GVO, https://www.bayer.com/de/crop-science/biotechnologie-und-gvo (2020)
41. Dederer, H.: Verfahrensbezogene Regulierung ist wissenschaftlich nicht begründbar, Leopoldina aktuell 6/2020. https://newsletter.leopoldina.org/mag/0455328001607545280/p8 (2020)
42. VBIO – Verband Biologie, Biowissenschaften und Biomedizin Deutschland: Genome Editing: Faktenbasierte Regulierung durch differenzierte Betrachtungsweise. https://www.vbio.de/fileadmin/user_upload/wissenschaft/pdf/200203_Impulse_VBIO_WGG2.pdf (2020)
43. Broad Institute: Questions and Answers about CRISPR. https://www.broadinstitute.org/what-broad/areas-focus/project-spotlight/questions-and-answers-about-crispr (2023)
44. World Health Organization: Q&A Detail/Food, genetically modified. https://www.who.int/news-room/q-a-detail/food-genetically-modified (2014)
45. Bundesministerium für Bildung und Forschung [Federal Ministry of Education and Research, BMBF]: 25 Jahre BMBF-Forschungsprogramme zur biologischen Sicherheitsforschung [25 years of BMBF research programs on biological safety research]. https://www.bmbf.de/SharedDocs/Publikationen/de/bmbf/7/30957_BMBF-Forschungsprogramme_zur_biologischen_Sicherheitsforschung.pdf?__blob=publicationFile&v=3 (2014)
46. European Academies Science Advisory Council: Genome editing: scientific opportunities, public interests and policy options in the European Union; EASAC policy report **31**; ISBN: 978-3-8047-3727-3 (2017)
47. Pies, I., Waldhof, G., Valentinov, V.: Diskursblockaden in der Debatte um grüne Gentechnik — Analysen und Reformempfehlungen aus ordonomischer Sicht [Discourse Blockages in the Debate on Green Genetic Engineering – Analyses and Reform Recommendations from an Ordonomic Perspective]. Green Deal — Was kommt auf die Land- und Ernährungswirtschaft zu? Schriftenreihe der Rentenbank, Band **37**, Frankfurt a.M., S. 93–146 (Edmund Rehwinkel-Stiftung der Landwirtschaftlichen Rentenbank (2021)
48. Bock, R.: Die systematische Selbsttäuschung beim Thema Gentechnik; Okkultismus statt Aufklärung? – Deutschland am Scheidweg, FGV-Broschüre, http://www.gruenevernunft.de/sites/default/files/Broschuere_Okkultismus_statt_Aufklauml%20rung%20%283%29.pdf (2015)
49. Nellen, W.: How the Green Party-Led Anti-Biotechnology Movement Captured German Policy and Why it Endangers Germany's Future Innovation in Gene Editing; https://geneticliteracyproject.org/2018/06/27/how-green-party-led-anti-biotechnology-movement-captured-german-policy-endangers-germanys-future-innovation-gene-editing/ (GMO Beyond The Science, 2018)
50. Szibor, R.: Grüne Gentechnik: Das Vokabular des Schreckens. Ein Sieg von Greenpeace & Co. gegen die Naturwissenschaft in Europa. http://www.gruenevernunft.de/sites/default/files/meldungen/Broschuere_Vokabular_des_Schreckens_final.pdf (2013)
51. Roberts, R. J.: Letter from Nobel Laureates in Support of Precision Farming (Genetically Modified Organisms, GMOs)]. [White Paper]. https://www.supportprecisionagriculture.org/german_letter.doc (Support Precision Agriculture, 2016)

52. Then, C., Bauer-Panskus, A.: Testbiotech comment on the Statement „Towards a scientifically justified, differentiated regulation of genome edited plants in the EU“ published by the National Academy of Sciences (Leopoldina), German Research Foundation (DFG) and Union of the German Academies of Sciences and Humanities. Testbiotech e.V. https://www.testbiotech.org/content/testbiotech-comment-statement-leopoldina#:~:text=Testbiotech_background_statement_leopoldina_dfg_2020.pdf (2020)
53. Johnston, P..: https://supportprecisionagriculture.org/greenpeace-response.html (2016)
54. Siegrist, M., Connor, M., Keller, C.: Trust, confidence, procedural fairness, outcome fairness, moral conviction, and the acceptance of GM field experiments: trust, fairness, and acceptance of GM field experiments. Risk Anal. **32**(8), 1394–1403 (2012). https://doi.org/10.1111/j.1539-6924.2011.01739.x
55. Dürnberger, C.: Normative concepts of nature in the GMO protest. A qualitative content analysis of position papers criticizing green genetic engineering in Germany. Food Eth. **4**(1), 49–66 (2019). https://doi.org/10.1007/s41055-019-00046-4
56. Scott, S.E., Inbar, Y., Wirz, C.D., Brossard, D., Rozin, P.: An overview of attitudes toward genetically engineered food. Annu. Rev. Nutr. **38**, 459–479 (2018)
57. Rozin, P.: The meaning of “natural”: process more important than content: process more important than content. Psychol. Sci. **16**, 652–658 (2005)
58. Gen-ethisches Netzwerk e.V.: 25 Jahre Widerstand [25 years of resistance]. https://shop.gen-ethisches-netzwerk.de/faltblatt/26-25-jahre-widerstand.html (2015)
59. Scott, S.E., Inbar, Y., Rozin, P.: Evidence for absolute moral opposition to genetically modified food in the United States. Perspect. Psychol. Sci. **11**, 315–324 (2016)
60. Gen-ethisches Netzwerk e.V.: Gentech-Konzerne Macht euch vom Acker [Genetic engineering companies get off the field]! https://shop.gen-ethisches-netzwerk.de/faltblatt/1-gentech-konzerne-macht-euch-vom-acker.html (2017)
61. Connor, M., Siegrist, M.: Factors influencing people’s acceptance of gene technology: the role of knowledge, health expectations, naturalness, and social trust. Sci. Commun. **32**, 514–538 (2010)
62. Siegrist, M., Bernauer, T.: Meinung, Konsum, Kommunikation und Akzeptanz. In Leitungsgruppe des NFP 59 (Eds.), Nutzen und Risiken der Freisetzung gentechnisch veränderter Pflanzen – Programmsynthese des Nationalen Forschungsprogramms 59, 198–219. vdf Hochschulverlag AG (2012)
63. Kahneman, D.: Thinking, Fast and Slow. Farrar, Straus and Giroux (2011)
64. Blancke, S., Van Breusegem, F., De Jaeger, G., Braeckman, J., Van Montagu, M.: Fatal attraction: the intuitive appeal of GMO opposition. Trends Plant Sci. **20**, 414–418 (2015)
65. Haidt, J.: The moral emotions. In: Davidson, R.J., Scherer, K.R., Goldsmith, H.H. (eds.) Handbook of affective sciences, pp. 852–870. Oxford University Press (2003)
66. Gewirth, A.: Ethics. In: Goetz, P.W. (ed.) The Encyclopedia Brittanica, vol. 6, 15th edn, pp. 976–998. Encyclopedia Britannica, Chicago (1984)
67. Hare, R.M.: Moral thinking: Its levels. method, and point. Oxford University Press (1981)
68. Frank, R.H.: The strategic role of the emotions. Emot. Rev. **3**, 252–254 (2011)
69. Šorgo, A., Jaušovec, N., Jaušovec, K., Puhek, M.: The influence of intelligence and emotions on the acceptability of genetically modified organisms. Electron. J. Biotechnol. **15** (2012)
70. Kantar Emnid: Das Image der deutschen Landwirtschaft. https://media.repro-mayr.de/79/668279.pdf (2017)
71. Slovic, P.: Perception of risk. Science. **236**, 280–285 (1987)
72. Mirchandani, A.: The Original Frankenfoods: Origins of Our Fear of Genetic Engineering; Genetic Literacy Project. https://geneticliteracyproject.org/2015/02/10/the-original-frankenfoods/ (2015)
73. Der Spiegel: Frankenfood im Tiefkühlfach. https://www.spiegel.de/spiegel/print/d-13688892.html (1993)
74. Fagan, J., Antoniou, M., Robinson, C.: GMO Myths and Truths, earthopensource, https://earthopensource.org/wordpress/downloads/GMO-Myths-and-Truths-edition2.pdf (2014)

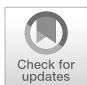
75. Trussler, M., Soroka, S.: Consumer demand for cynical and negative news frames. Int. J. Press Polit. **19**, 360–379 (2014)
76. Soroka, S., Fournier, P., Nir, L.: Cross-national evidence of a negativity bias in psychophysiological reactions to news. Proc. Natl. Acad. Sci. U. S. A. **116**, 18888–18892 (2019)
77. Pinker, S.: The media exaggeraters negatie news. This distortion has consequences. https://www.theguardian.com/commentisfree/2018/feb/17/steven-pinker-media-negative-news (2018)
78. Baron, J., Spranca, M.: Protected values. Virology. **70**, 1–16 (1997)
79. Baron, J., Leshner, S.: How serious are expressions of protected values? J. Exp. Psychol. Appl. **6**, 183–194 (2000)
80. Rozin, P.: The process of moralization. Psychol. Sci. **10**, 218–221 (1999)
81. Feinberg, M., Kovacheff, C., Teper, R., Inbar, Y.: Understanding the process of moralization: how eating meat becomes a moral issue. J. Pers. Soc. Psychol. **117**, 50–72 (2019)
82. Pies, I.: Ordonomik als Methode zur Generierung von Überbietungsargumenten – Eine Illustration anhand der Flüchtlings(politik)debatte. Z. Wirtsch. Unternehmensethik. **18**, 171–200 (2017)
83. Hielscher, S., Pies, I., Valentinov, V., Chatalova, L.: Rationalizing the GMO debate: The ordonomic approach to addressing agricultural myths. Int. J. Environ. Res. Public Health. **13** (2016)
84. Ellemers, N., van der Toorn, J., Paunov, Y., van Leeuwen, T.: The psychology of morality: a review and analysis of empirical studies published from 1940 through 2017. Personal. Soc. Psychol. Rev. **23**, 332–366 (2019)
85. Skitka, L.J., Bauman, C.W., Sargis, E.G.: Moral conviction: another contributor to attitude strength or something more? J. Pers. Soc. Psychol. **88**, 895–917 (2005)
86. Skitka, L.J., Hanson, B.E., Morgan, G.S., Wisneski, D.C.: The psychology of moral conviction. Annu. Rev. Psychol. **72**, 347–366 (2021)
87. Inbar, Y., Waldhof, G.: Mitigating consequence insensitivity for genetically engineered crops. J. Exp. Psy. App. https://doi.org/10.31234/osf.io/bnq8c (2022)
88. James, Harvey, Ed.: Ethical tensions from new technology: the case of agricultural biotechnology. Wallingford, Oxfordshire (2018)
89. The Guardian: Ken Olsen obituary. https://www.theguardian.com/technology/2011/feb/09/ken-olsen-obituary (2011)
90. Cellan-Jones, R.: The future of technology….who knows? https://www.bbc.com/news/technology-17510101 (2012)
91. Wieland, T.: Rote Gentechnik und Öffentlichkeit: Von der Grundlegenden Skepsis zur Differenzierten Akzeptanz. in Biotechnologie-Kommunikation 69–111. Springer, Berlin Heidelberg (2012)
92. Thurau, M.: Der Fall Humaninsulin Hoechst. Taz.de https://taz.de/Der-Fall-Humaninsulin-Hoechst/!1844898/ (1988)
93. Schellenberg, S.: Vor dem Profit kommt der Bedarf. Taz.de https://taz.de/!1834612/ (1988)
94. Deutscher Ärzteverlag GmbH: Stellungnahme zur ARD-Sendung über Humaninsulin. Deutsches Ärzteblatt https://www.aerzteblatt.de/archiv/93150/Stellungnahme-zur-ARD-Sendung-ueber-Humaninsulin (1992)
95. Waldhof, G.: Opponents and supporters of genetically engineered foods emphasize distinct moral foundations and exhibit a clash of two diverging worldviews [Manuscript submitted for publication]. Department of Structural Change. (Leibniz Institute of Agricultural Development in Transition Economies, 2023)
96. Hofstetter, M., Krautter, A., Oeck, S., Toewe-Rimkeit, S.: Hintergrund Landwirtschaft. Landwirtschaft. Was wollen wir essen? Gift und Gentechnik – nein danke [Background Agriculture. Agriculture. What do we want to eat? Poison and genetic engineering – no thanks]! Greenpeace.de. https://www.greenpeace.de/publikationen/greenpeace-hintergrund-landwirtschaft-h03032.pdf (Greenpeace, 2011)

97. Brookes, G., Barfoot, P.: Environmental impacts of genetically modified (GM) crop use 1996–2015: impacts on pesticide use and carbon emissions. GM Crops Food. **8**, 117–147 (2017)
98. Awater-Esper, S.: Niggli übergibt FiBL an ein Team. https://www.topagrar.com/oekolandbau/news/niggli-uebergibt-fibl-an-ein-team-12029472.html (2020)
99. Jötten, F.: Wir essen buchstäblich den Planten auf. https://www.fr.de/zukunft/storys/ernaehrung/bio-experte-urs-niggli-nachhaltige-landwirtschaft-konsum-afrika-90053854.html (2020)
100. Achermann, B., Jäggi, S.: Urs Niggli. Psssssssss – und weg ist das Unkraut!, https://www.zeit.de/2020/04/urs-niggli-biologische-landwirtschaft-rente-oekologie-gentechnik-pestizide (2020)
101. Hardegger, A.: Schweizer Bioforscher: „Historisch ist es mit der Gentechnik schiefgelaufen" https://www.nzz.ch/schweiz/urs-niggli-mit-der-gentechnik-ist-es-historisch-schiefgelaufen-ld.1550799 (2020)
102. Grefe, C.: Ökolandbau. „Modernes Bio für alle!" https://www.zeit.de/2014/22/oekolandbau-bio-urs-niggli (2014)
103. Progressive Agrarwende: Progressive Agrarwende. https://progressive-agrarwende.org/ (no date)
104. MaiThinkX: Grüne Gentechnik – MAITHINK X vom 27. März 2022 mit Dr. Mai Thi Nguyen-Kim [Green Genetic Engineering – MAITHINK X of March 27, 2022 with Dr. Mai Thi Nguyen-Kim]. https://www.zdf.de/show/mai-think-x-die-show/maithink-x-folge-10-102.html (2022)
105. Heute Show: Heute-show [Today-show of 2nd December 2022]. https://www.youtube.com/watch?v=6bwxeM8fndo&ab_channel=ZDFheute-show (02.12.2022)
106. Hielscher, S., Winkin, J., Crack, A., Pies, I.: Saving the moral capital of NGOs: identifying one-sided and many-sided social dilemmas in NGO accountability. VOLUNT. Int. J. Volunt. Nonprofit Organ. **28**, 1562–1594 (2017)
107. Hielscher, S., Winkin, J., Pies, I.: The interdependence of ideas and institutions in NGO accountability: Toward a social learning approach of collective self-regulation. in 29th Annual Meeting of the International Association of Business and Society (IABS) (2018)
108. Accountable Now We are transforming accountability throughout the civil society sector. https://accountablenow.org/ (2022)
109. European Commission: Executive Summary. Commission staff working document. Study on the status of new genomic techniques under Union law and in light of the Court of Justice ruling in Case C-528/16 (para.16,21) https://food.ec.europa.eu/system/files/2021-04/gmo_mod-bio_ngt_exec-sum_en.pdf (2021)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 31
The Citizens' Awareness and Concerns During the Transition from Genetically Modified to Genome Edited Plants in Europe About Their Use in Agriculture and Food Production



Mihael Cristin Ichim

Abstract The genome edited crops and foods are commercially cultivated and marketed already at global level, rapidly expanding towards new applications and plant species, and successfully complementing the genetically modified ones. In the European Union, the genome edited plants have to follow the two-decade-old regulatory framework for genetically modified organisms. The decrease of both, number of notifications for field trials, and hectarage and countries commercially cultivating genetically modified plants, registered in the last decade in the European Union, has been closely followed by lower levels of awareness and concerns expressed by the European Union citizens about their use in farming and food production, as recorded by a long time series of Eurobarometers surveys. In contrast, in the four recent years only, the awareness about genome editing among the European Union citizens has significantly increased, reaching more than the half of the one about genetically modified products, along with the number of concerned European Union citizens about genome editing which also has doubled. The public opinion about genome edited crops and food products needs to be monitored further as it decisively influences the new regulatory framework to be proposed by the European Commission and therefore the extent to which the European consumer will benefit from the new biotechnologies.

M. C. Ichim (✉)
"Stejarul" Research Centre for Biological Sciences, National Institute of Research and Development for Biological Sciences, Piatra Neamt, Romania
e-mail: cichim@hotmail.com

© The Author(s) 2024
A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_31

1 From Genetically Modified to Genome Edited Plants: The Global Context

The global production status of genetically modified (GM) crops has steadily grew since their commercial adoption in 1996, to reach a 112-fold increase [1] to around 190 million hectares in 2019, almost equally split between 21 developing countries and five industrial countries [2]. In terms of the share of the main crops in which transgenic traits have been commercialized, GM traits accounted for 47% of the global plantings to soybeans, maize, cotton, and canola in 2020 [3]. The global economic benefits, over the period 1996 to 2020, have been significant with farm incomes for those using the GM crop technology, having increased by 261.3 billion USD [4]. All the above are based on a sharp increase in the approval of the number of plant species with GM varieties. At global level, 44 countries, plus the European Union (EU) Member States (MS), have approved a total of 46 commercial GM traits introduced, as single or stacked transformation events, in 32 GM crop species for use in commercial cultivation, food, and feed.[1]

Genome editing (GE) was being applied to more than 40 crops across 25 countries [5]. The GE plant varieties in advanced development pipeline span now a wide range of crops, including alfalfa, camelina, canola, citrus, flax, maize, pennycress, potato, lettuce, tomato, and watermelon, and are suggesting the flexibility of the new breeding techniques (NBTs) in crop improvement [6]. Despite the apparent potential, however, only six GE crop traits - in soybean, canola, rice, maize, mushroom and camelina - have been approved for commercialization to date [7]. The United States (US) farmers are growing from 2016 a oligonucleotide-directed mutagenesis (ODM)- based GE sulfonylurea tolerant weed control canola (Cibus), a transcription activator-like effector nucleases (TALEN)- based genome edited soybean with modified oil composition (Calyxt) [5] but also a Clustered Regularly Interspaced Short Palindromic Repeats -associated protein 9 (CRISPR–Cas9) edited waxy corn (Corteva Agriscience) [8]. The hectarage cultivated with Calyxt genome edited soybean alone has increased significantly, to approximately 40,000, from 17,000 in 2019 [5, 9]. The two genome editing pioneering companies, Cibus and Calyxt, have recently announced they had entered into a definitive merger agreement [10]. Genome edited food was first sold on the open market in 2021, when the Sicilian Rouge tomatoes, genetically edited to contain high amounts of γ-aminobutyric acid (GABA), was sold direct to consumers in Japan by Sanatech Seed [11]. Two years before that, was marketed the first genome edited food product, the high-oleic soybean oil Calyno™, obtained from plants that have been edited to produce fewer saturated fats and zero trans fats (Calyxt) [9]. To date, the GE crops and products have still limited commercial prevalence because they are new and unfamiliar to consumers relative to other breeding techniques and the regulatory process is ill defined and shifting in many countries [12].

[1] ISAAA: ISAAA GM Approval Database, https://www.isaaa.org/gmapprovaldatabase/. Accessed: 14 May 2023.

To reach the market, each GM plant event must undergo a long and costly process. Two decades ago, it was estimated that 8–12 years of research and development were needed prior to commercialization of GM plants [13]. A recent agri-biotech market survey has reported that the cost of discovery, development and authorization of a new plant biotechnology derived single genetic trait has declined from 136 million USD in the 2008–2012 period to the current value of 115 million USD, but the time required to complete the process has increased from 13.1 to 16.5 years [14]. This data suggest a twice as long period needed nowadays, compared with the early 2000s, for a new GM single trait plant to reach the commercialization phase. On the other hand, early commercial applications of genome editing for crop improvement appear to confirm the inherent speed and overall cost efficiency of genome editing [6]. For comparison, the cost for GE crops development to commercialization were estimated to cost ten million USD and the time needed is 5 years [15]. The genome editing is faster and cheaper approach to market than conventional GM technology [16].

Consumers response to GM products is largely influenced by the decision of the governments to ban or approve the GM crops cultivation and between acceptance for cultivation of GM crops and market exists a wide gap across countries, a comprehensive bibliometric analysis has concluded [17]. On the other hand, a recent systematic global overview has concluded that GE foods are often more accepted than the GM foods, which is partially due to the fact that genome editing is perceived as more natural [16, 18].

2 The Commercial Cultivation of Genetically Modified Plants in the EU

In 1998, only two years after the GM crops were first marketed worldwide [3], their cultivation in the EU started, being among the first territories to commercially adopt the cultivation of biotech crops at global level [19] (Fig. 31.1), well in advance of countries such as Brazil, India, Paraguay, and Pakistan, each of them cultivating millions of hectares with GM crops nowadays [20].

Only two GM maize varieties, both producing Cry1Ab toxin, insecticidal protein from *Bacillus thuringiensis*, have been authorized for commercial cultivation in EU: Bt176 (1998–2005), and MON810 (since 2003). The maize hybrids derived from event Bt176 were officially withdrawn from the European market in 2006 because this event contained an ampicillin resistance gene as selectable marker [23]. It took 15 years to reach the cultivation peak, in 2013, with 148,013 ha, on all growing YieldGard™ maize (single trait event MON-ØØ81Ø-6) which, since 2006, continues to be the only GM plant commercially cultivated in the EU [20]. Yet, this GM maize line is one of the 49 insect resistant (IR) transgenic events approved for food

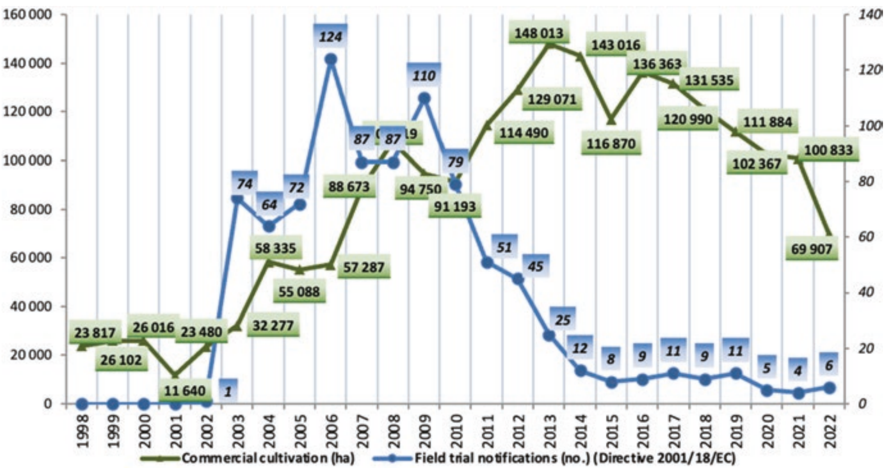


Fig. 31.1 Commercial cultivation of GM crops and GM plant field trial notifications in EU (1998–2022) [19, 21, 22]

and feed use in the EU Register of authorised GMOs.[2] Since then, the hectarage cultivated with GM maize has gradually decreased to approx. 70,000 ha in 2022, a comparable value with the one register 15 years ago. Last year was also registered the biggest reduction of the cultivated area, with approx. 30%, the most abrupt yearly variation ever recorded in EU [21, 22].

The gradually decrease of the cultivated area with GM plants in Europe was accompanied by a reduction of the countries growing these biotech crops, as farmers from Romania (in 2015) [24], Czech Republic, and Slovakia (in 2017), have voluntarily stopped the commercial cultivation of GM maize [19]. Only two countries, Spain [22] and Portugal [21], are commercially cultivating GM crops in Europe for the last 5 years.

Worth mentioning that the provisions of the EU GMO opt-out Directive 2015/412 [25], which have allowed to a significant number of EU MSs to officially restrict the cultivation of genetically modified organisms (GMOs) in their territory, did not changed the *status quo* of the EU-28 countries, as 11 countries have officially chosen to legally allow the commercial cultivation of GM crops [19]. Yet, in 2018 the Court of Justice of the EU (CJEU) ruled (case C-528/16) that organisms obtained by mutagenesis, as can be achieved using New Plant Breeding techniques (NPBTs), including genome editing techniques, are genetically modified organisms (GMOs) as defined in the European Directive 18/2001/EC [26]. This decision, imposing for the genome edited plants to follow exactly the same authorization procedure as the GM plants, was expected to delay their development, testing, approval and

[2] European Commission: EU Register of authorised GMOs, https://webgate.ec.europa.eu/dyna2/gm-register/. Accessed: 14 May 2023.

availability of these new plant varieties on the EU market and their commercial cultivation [19].

3 The Notifications for Field Trials with Genetically Modified and Genome Edited Plants in the EU

The field trial under field conditions is a preliminary, but essential step for developing GM plants, especially if they are intended for commercial cultivation. In the EU, a new GM crop event has to be compared to its closest non-GM counterpart as a corner stone of the pre-market risk assessment. This is a critical final test to monitor their effects on the receiving environment (e.g. non-target organisms) and also detect possible occurrence of differences, caused by intended and unintended effects, in composition, as well as in agronomic, phenotypic and molecular characteristics [27]. The EU continues to have the broadest and most stringent regulations in the world governing these field trials as an essential part of the risk assessment before market approval [28].

During the regulatory framework set by the Directive 90/220/EEC (1991–2001) [29] 1687 field trial notifications were registered and their number dropped by 76% between 1998 and 2001, mainly due to the *de facto* moratorium in place since 1999 [13]. The notifications documented by the dedicated European Commission' GM Plants Register,[3] containing the list of summary notifications (SNIFs) submitted to the competent authority of the EU MS under the Directive 2001/18/EC (from 2002) [30], has also dramatically dropped by 91% in the last 9 years (Fig. 31.1).

The CJEU's decision from 25 July 2018, stating that plants obtained with the new genome editing techniques are GMOs from the regulatory point of view, has immediately changed the legal status of the first field trial of a CRISPR-Cas-9 genome edited crop, *i.e*., *Camelina sativa*, in Europe (EU-28) [31], that began on 5 June the same year at Rothamsted Research (UK) [32]. Yet, the first notification of a field trial with a genome edited plant in Europe recorded in the dedicated GMO Register was carried out in 2018 by the Flemish Institute for Biotechnology (VIB) (Ghent, BE) with maize with an impaired DNA-repair mechanism and maize with modified growth characteristics (Fig. 31.2).[3]

That first field trial with a GE plant has immediately raised a strong concern that limiting their feasibility by expanding the complexity of the regulatory process and the associated financial burden of dedicated experimental sites will certainly hinder research [32].

The evolution of the field trial notification with GE plants since than (2018–2022) (Fig. 31.2) has fully confirmed, to date, that assumption: their yearly total number being maximum 5 (in 2019).

[3] European Commission: Eurobarometer – Public opinion in the European Union, https://europa.eu/eurobarometer/screen/home. Accessed: 14 May 2023.

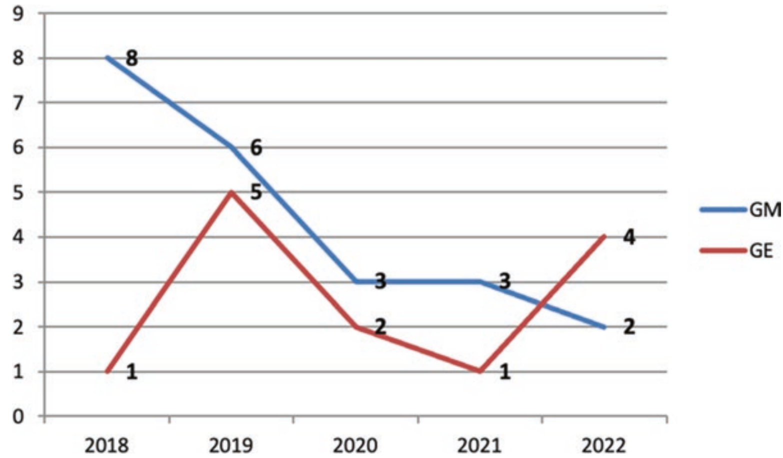


Fig. 31.2 The notification for field trials with GM and GE plants under Directive 2001/18/EC (2018–2022)

The field trial notifications with GM plants in EU proved to be a sensitive indicator of the overall research and development activities in the past [33]. Moreover, the dramatic reduction of field trials in the EU has also coincided with increasing safety demands, decreases in funding, and changes in the European directives [34]. Under the regulatory framework set by the Directive 2001/18/EC, the last 20 years can be generally divided into two separate periods: the first years characterized by a sharp increase of both number of field test notifications and hectarage of commercial cultivation followed by a decrease of both of them (Fig. 31.1). This data suggests a high degree of interdependence of their overall trend in the EU. While the notifications for field tests with GM plants do follow a clear descendent trend in the last 4 year, the notifications for GE are variable but their status in 2022, compared with the total number, was bigger than of the field trials with GM plants, for the first time (Fig. 31.2), offering hopes for an ascendant trend to be expected in the coming years, hopefully translated into their commercial cultivation status in EU. Worth remembered that all the field trials, including with GE plants, are carried out following the regulatory framework developed more than two decades ago [30], well before the NBTs to be proposed, developed and commercially validated, as it is the situation nowadays. The current regulatory regime for field trials was found ill-fitted to breeding activities [35].

4 The Awareness and Concerns of the EU Citizens About the Use of Genetically Modified and Genome Edited Plants in Agri-Food

Eurobarometer is the polling instrument used by the European Commission (EC), and other EU institutions, to monitor regularly the state of public opinion in Europe on issues related to the EU as well as attitudes on subjects of political or social nature.[4] It is a cross-temporal and cross-national comparative program of regularly repeated cross-sectional surveys [36]. Additionally, special Eurobarometers are used to monitor if citizens want the EU to act on a specific policy topic or support the EC's solution to a policy problem [37]. In order to guarantee the representativeness of results, Eurobarometer surveys rely on a randomly selected sample of persons and the total sample is weighted to ensure demographic and geographical representativeness and the EU average is calculated taking into account the relative weight of each country.[4]

4.1 The Concern of the EU Citizens About the Use of GM Plants for Farming

The attitude of EU citizens toward the release of GMOs into the environment was assessed through a special series of four Eurobarometers during a 9-year period (2002–2011). In 2002, when they were first asked about, the "use of GMOs" was a concern ranked 18th, from 25 different environmental issues, representing 30% from the worries expressed by the EU citizens [38]. The following three surveys (2004, 2007, 2011) have specifically asked for concerns related to the use of GMOs for farming [39–41]. The percentage of worried EU citizens about the use of GM plants in the environment, for farming purposes, has decreased, constantly, down to 19% (Fig. 31.3).

4.2 The Awareness and Concern of the EU Citizens About the Use of GM and GE Ingredients in Food

A series of four Eurobaromer surveys (2005, 2010, 2019, and 2022), initiated by the European Food Safety Agency (EFSA), aimed to investigate Europeans' perceptions of and attitudes towards food safety by exploring the Europeans' interest in

[4] European Commission: GMO Register / Part B notifications (experimental releases) – GM Plants, https://webgate.ec.europa.eu/fip/GMO_Registers/GMO_Part_B_Plants.php. Accessed: 14 May 2023.

Fig. 31.3 Concerns expressed by the EU citizens toward the use of GM plants in farming (2004–2011)

food safety-related topics and factors affecting food-related decisions and awareness of and main concerns about food-safety topics [42], including "genetically modified ingredients in food or drinks" (2005, 2010, 2019, and 2022) and, more recently, "genome editing" in food (2019, and 2022).

4.2.1 The Awareness of the EU Citizens About the Use of GM and GE Ingredients in Food

Awareness of food safety topics remains high among EU citizens [42]. The awareness among the EU consumers of the use of both GM and GE in food production was investigated in the last two Special Eurobarometers [42, 43], offering a valuable insight on how the two plant biotech technologies, GMOs, used for almost three decades for farming, and food and feed production in Europe, and GE, only recently has entered into the EU consumer's attention, but with no farming and food production applications in Europe, yet, are perceived and aware off at the consumers' level (Fig. 31.4).

As expected, in 2019, the awareness level about GM were considerably higher (three fold) than about GE-food associated issue, but only 3 years later, due to a marginal decrease (4%) of the declared awareness about GMO, and a significant increase (38%), of the GE among the EU citizens, the difference between the two food safety-associated topics decreased by one third from the previous levels. Notably, in 23 of the 27 EU MSs, awareness of the use of new biotechnology in food production, *i.e.*, GE, has risen since 2019 [42].

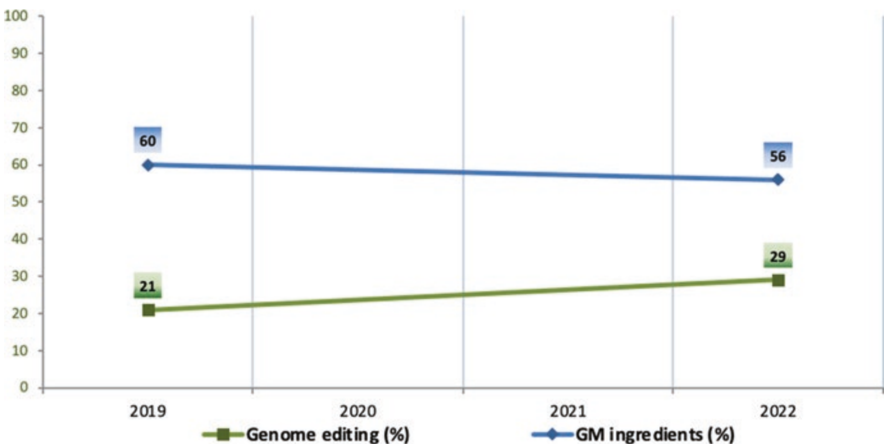


Fig. 31.4 Awareness expressed by the EU citizens about the use of GM and GE in food production

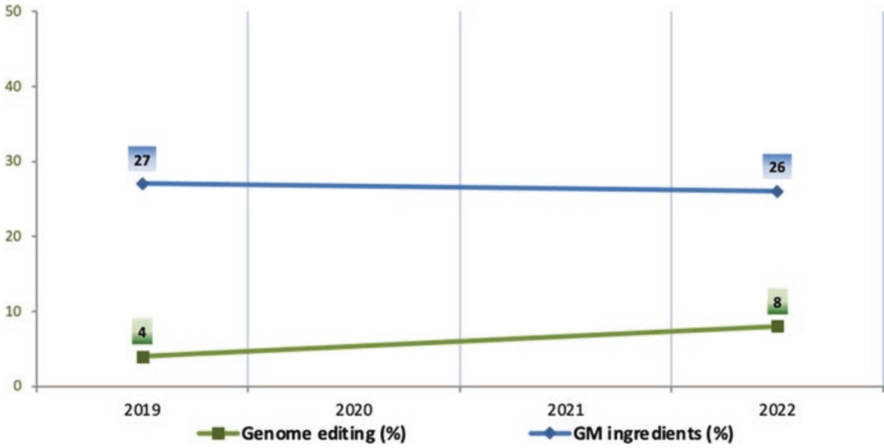


Fig. 31.5 Concerns expressed by the EU citizens about the use of GM ingredients and genome editing in food and drinks

4.2.2 The Concerns Expressed by EU Citizens About the Use of GM and GE Ingredients in Food

Around one quarter (26%) of the EU citizens indicate the presence of genetically modified ingredients in food or drinks as a concern in 2022 while only a smaller proportions (8%), the use of new biotechnology in food production, i.e. genome editing (Fig. 31.5).

Compared with 2019, for most of the concerns expressed by EU consumers, listed in the Special Eurobaromer survey, there have been increases in the

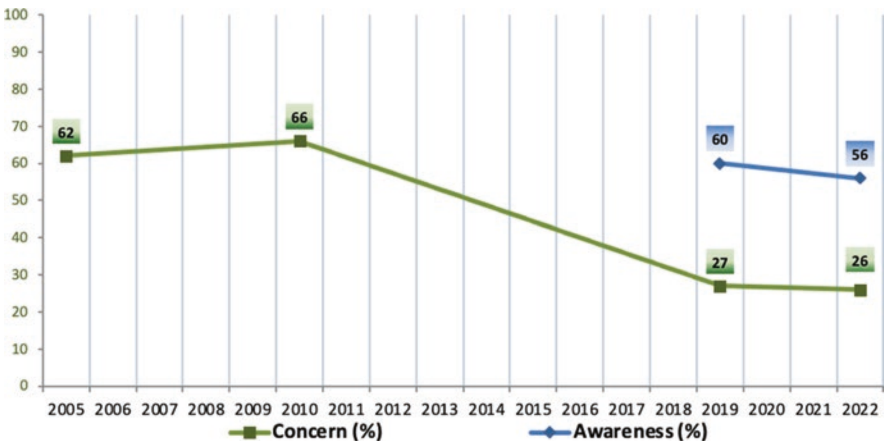


Fig. 31.6 Trends of the awareness and concern expressed by the EU citizens about the use of GM ingredients in food

proportions and this is particularly the case for the use of GE in food production which has doubled, from 4% to 8% from the total, among the EU citizens [42].

4.2.3 Trends and Evolution of the Awareness and Concerns Expressed by the EU Citizens About the Use of GM and GE in Food Production

The time series of Special Eurobaromer surveys on Food Safety, spanning over 18 years (2005–2022), offer a consistent and clear overview on the opinions the EU citizens have on the most relevant food-safety related issues. Included right from the start among the investigated topics, the opinion about the concerns about the presence of GM ingredients in food was recorded and it reveals a clear and significant reduction of them, from approximately 2/3 (62–66%) of the citizens to less than 1/3 (27–26%) (Fig. 31.6).

4.3 The EU Citizens' Awareness and Concerns During the Transition from GM to GE Plants

Gathered in the last three decades, scientific evidence about the perception of GM plants and derived food products is large enough, the EU especially being one of the territories well represented in such analyses [16]. Comprehensive analyses have concluded that EU consumers have more negative perception and less purchase intention toward GM foods in contrast to the consumer perception in North America while in developing nations, the positive perception arises owing to the persistent demand for food [17].

In Europe, during the first decade of the new millennium (1999–2010) a 12% increase was recorded, by a series of Special Eurobarometers, among the EU citizens optimistic about biotechnology [44]. Unfortunately, this share of optimism was not reflected towards the use of GM plants in agri-food in EU. The awareness levels, recorded lately among the citizens regarding the use of GM ingredients in food, were at medium levels and have even decreased slightly [45]. This could be correlated with the abrupt decrease of both GM crop testing and commercial cultivation in Europe. The lower level of awareness was inevitably followed by the proportion of EU citizens concerned about the use of GM crops in farming and for food production, as shown by a long time series of Special Eurobarometers.

The available data about the perception from the public and relevant stakeholders of GE is very limited [16] and, in this context, the recent surveys of the awareness and concerns among the EU citizens regarding the use of GE in food production is all the more relevant considering the large territory covered and the trends revealed [42]. In only 4 years (2019–2022), the awareness about GE among the EU citizens has significantly increased, reaching more than the half of the one about GM products – a three decade old technology in Europe though. Moreover, the number of concerned EU citizens about GE has doubled in the same period, as well as the proportion compared with the level of concerns about the GM ingredients.

NBTs and their products tend to be valued more highly than their GMO counterparts [12, 16] but because large differences occurs within target groups, regions and products [16] a large, highly and multidimensional heterogeneous territory, as the EU proved to be along the years with respect to biotechnology, is scientifically and practically relevant for all stakeholders. It is critically important the public attitude towards new breeding techniques, and their field testing, commercial cultivation and products, to be extensively investigated as they could be misperceived and rejected even though they hold much promise to improve food supply chains' sustainability, foster better health outcomes for consumers and the environment [12] and their acceptance will be a key factor for policy support [16].

5 Conclusion and Future Perspectives

Relevant time series of Eurorometers have surveyed the awareness and concerns of the EU citizens about the use of GM crops for farming and food production. The public opinion has shown a steady decrease of both indicators along with the notification for field trials and commercial cultivated of GM plants in the EU. More recently, both the awareness level and concerns expressed by the EU citizens about the use of GE in food production has increased significantly in only 4-year period.

The public opinion is fundamental for the acceptance and adoption of any new technology, including NBTs, in Europe. The long term evolution of the public attitude and acceptance of the GE plants and foods is particularly relevant considering the substantial opposition to GMOs recorded in the past, among the European citizens. The current level of awareness and concerns about GE, and their short term

evolution, is important as a new regulatory framework is in preparation by the European Commission.

Acknowledgments This work was supported by a grant of the Ministry of Research, Innovation and Digitization through Program 1 - Development of the National R&D System, Subprogram 1.2 - Institutional Performance - Projects for Excellence Financing in RDI, contract no. 2PFE/2021.

References

1. Abdul Aziz, M., Brini, F., Rouached, H., Masmoudi, K.: Genetically engineered crops for sustainably enhanced food production systems. Front. Plant Sci. **13**, 1027828 (2022). https://doi.org/10.3389/fpls.2022.1027828
2. Turnbull, C., Lillemo, M., Hvoslef-Eide, T.A.K.: Global regulation of genetically modified crops amid the gene edited crop boom – a review. Front. Plant Sci. **12** (2021). https://doi.org/10.3389/fpls.2021.630396
3. Brookes, G.: Genetically modified (GM) crop use 1996–2020: environmental impacts associated with pesticide use change. GM Crops Food. **13**, 262–289 (2022). https://doi.org/10.1080/21645698.2022.2118497
4. Brookes, G.: Farm income and production impacts from the use of genetically modified (GM) crop technology 1996–2020. GM Crops Food. **13**, 171–195 (2022). https://doi.org/10.1080/21645698.2022.2105626
5. Menz, J., Modrzejewski, D., Hartung, F., Wilhelm, R., Sprink, T.: Genome edited crops touch the market: a view on the global development and regulatory environment. Front. Plant Sci. **11**, 586027 (2020). https://doi.org/10.3389/fpls.2020.586027
6. Kalaitzandonakes, N., Willig, C., Zahringer, K.: The economics and policy of genome editing in crop improvement. Plant. Genome. **16**(2), e20248 (2022). https://doi.org/10.1002/tpg2.20248
7. Pixley, K.V., Falck-Zepeda, J.B., Paarlberg, R.L., Phillips, P.W.B., Slamet-Loedin, I.H., Dhugga, K.S., Campos, H., Gutterson, N.: Genome-edited crops for improved food security of smallholder farmers. Nat. Genet. **54**, 364–367 (2022). https://doi.org/10.1038/s41588-022-01046-7
8. Gao, H., Gadlage, M.J., Lafitte, H.R., Lenderts, B., Yang, M., Schroder, M., Farrell, J., Snopek, K., Peterson, D., Feigenbutz, L., Jones, S., St Clair, G., Rahe, M., Sanyour-Doyel, N., Peng, C., Wang, L., Young, J.K., Beatty, M., Dahlke, B., Hazebroek, J., Greene, T.W., Cigan, A.M., Chilcoat, N.D., Meeley, R.B.: Superior field performance of waxy corn engineered using CRISPR–Cas9. Nat. Biotechnol. **38**, 579–581 (2020). https://doi.org/10.1038/s41587-020-0444-0
9. Voigt, C.A.: Synthetic biology 2020–2030: six commercially-available products that are changing our world. Nat. Commun. **11**, 6379 (2020). https://doi.org/10.1038/s41467-020-20122-2
10. NASDAQ: Calyxt And Cibus To Merge In All-stock Deal, https://www.nasdaq.com/articles/calyxt-and-cibus-to-merge-in-all-stock-deal
11. Waltz, E.: GABA-enriched tomato is first CRISPR-edited food to enter market. Nat. Biotechnol. **40**, 9–11 (2021). https://doi.org/10.1038/d41587-021-00026-2
12. Beghin, J.C., Gustafson, C.R.: Consumer valuation of and attitudes towards novel foods produced with new plant engineering techniques: a review. Sustainability. **13**, 11348 (2021). https://doi.org/10.3390/su132011348
13. Lheureux, K., Menrad, K.: A decade of European field trials with genetically modified plants. Environ. Biosaf. Res. **3**, 99–107 (2004). https://doi.org/10.1051/ebr:2004009
14. CropLife International: Time and Cost of Bringing a Biotech Crop to Market. (2022)

15. Bullock, D.W., Wilson, W.W., Neadeau, J.: Gene editing versus genetic modification in the research and development of new crop traits: an economic comparison. Am. J. Agric. Econ. **103**, 1700–1719 (2021). https://doi.org/10.1111/ajae.12201
16. Strobbe, S., Wesana, J., Straeten, D.V.D., Steur, H.D.: Public acceptance and stakeholder views of gene edited foods: a global overview. Trends Biotechnol. **41**, 736–740 (2023). https://doi.org/10.1016/j.tibtech.2022.12.011
17. Sendhil, R., Nyika, J., Yadav, S., Mackolil, J., Rama Prashat, G., Workie, E., Ragupathy, R., Ramasundaram, P.: Genetically modified foods: bibliometric analysis on consumer perception and preference. GM Crops Food. **13**, 65–85 (2022). https://doi.org/10.1080/21645698.2022.2038525
18. Woźniak-Gientka, E., Tyczewska, A., Perisic, M., Beniermann, A., Dennis, V., N., Gheysen, G., Cetiner, S., Abiri, N., Twardowski, T.: Public perception of plant gene technologies worldwide in the light of food security. GM Crops Food. **13**, 218–241 (2022). https://doi.org/10.1080/21645698.2022.2111946
19. Ichim, M.C.: The Romanian experience and perspective on the commercial cultivation of genetically modified crops in Europe. Transgenic Res. **28**, 1–7 (2019). https://doi.org/10.1007/s11248-018-0095-9
20. ISAAA: Global Status of Commercialized Biotech/GM Crops: 2019 – ISAAA Brief 55-2019. (2019)
21. DGAV (Direção-General de Alimentação e Veterinária): Regras para o Cultivo de Plantas Geneticamente Modificadas – DGAV, https://www.dgav.pt/plantas/conteudo/autorizacoes-de-cultivo/variedades-geneticamente-modificadas/regras-para-o-cultivo-de-plantas-geneticamente-modificadas/
22. MAPA (Ministerio de Agricultura, Pesca y Alimentación): Registro Público de Organismos Modificados Geneticamente (OMG), https://www.mapa.gob.es/es/agricultura/temas/biotecnologia/omg/registro-publico-omg/default.aspx
23. García, M., García-Benítez, C., Ortego, F., Farinós, G.P.: Monitoring insect resistance to Bt Maize in the European Union: update, challenges, and future prospects. J. Econ. Entomol. **116**, 275–288 (2023). https://doi.org/10.1093/jee/toac154
24. Ferrante, M., Lövei, G.L., Magagnoli, S., Minarcikova, L., Tomescu, E.L., Burgio, G., Cagan, L., Ichim, M.C.: Predation pressure in maize across Europe and in Argentina: an intercontinental comparison. Insect Sci. **26**, 545–554 (2019). https://doi.org/10.1111/1744-7917.12550
25. European Commission: Directive (EU) 2015/412 of the European Parliament and of the Council of 11 March 2015 amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory, https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32015L0412, (2015)
26. Callaway, E.: CRISPR plants now subject to tough GM laws in European Union. Nature. **560**, 16 (2018). https://doi.org/10.1038/d41586-018-05814-6
27. Slot, M.M., van de Wiel, C.C.M., Kleter, G.A., Visser, R.G.F., Kok, E.J.: The assessment of field trials in GMO research around the world and their possible integration in field trials for variety registration. Transgenic Res. **27**, 321–329 (2018). https://doi.org/10.1007/s11248-018-0076-z
28. Gómez-Galera, S., Twyman, R.M., Sparrow, P.A.C., Van Droogenbroeck, B., Custers, R., Capell, T., Christou, P.: Field trials and tribulations—making sense of the regulations for experimental field trials of transgenic crops in Europe. Plant Biotechnol. J. **10**, 511–523 (2012). https://doi.org/10.1111/j.1467-7652.2012.00681.x
29. European Commission: Council Directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms, https://eur-lex.europa.eu/eli/dir/1990/220/oj, (1990)
30. European Commission: Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms, https://eur-lex.europa.eu/eli/dir/2001/18/oj, (2001)

31. DEFRA: Genetically Modified Organisms: Rothamsted Research (18/R08/01), https://www.gov.uk/government/publications/genetically-modified-organisms-rothamsted-research-18r0801
32. Faure, J.-D., Napier, J.A.: Europe's first and last field trial of gene-edited plants? elife. **7**, e42379 (2018). https://doi.org/10.7554/eLife.42379
33. Mitchell, P.: Europe sees sharp decline in GMO research. Nat. Biotechnol. **21**, 468–469 (2003). https://doi.org/10.1038/nbt0503-468
34. Nausch, H., Sautter, C., Broer, I., Schmidt, K.: Public funded field trials with transgenic plants in Europe: a comparison between Germany and Switzerland. Curr. Opin. Biotechnol. **32**, 171–178 (2015). https://doi.org/10.1016/j.copbio.2014.12.023
35. Zimny, T.: Regulation of GMO field trials in the EU and new genomic techniques: will the planned reform facilitate experimenting with gene-edited plants? Biotechnologia. **104**, 75–83 (2023). https://doi.org/10.5114/bta.2023.125086
36. Moschner, M.: Eurobarometer. In: Michalos, A.C. (ed.) Encyclopedia of Quality of Life and Well-being Research, pp. 2006–2009. Springer, Dordrecht (2014)
37. Haverland, M., de Ruiter, M., Van de Walle, S.: Agenda-setting by the European Commission. Seeking public opinion? J Eur. Public Policy. **25**, 327–345 (2018). https://doi.org/10.1080/13501763.2016.1249014
38. European Commission: Eurobarometer 58.0. (2002)
39. European Commission: Special Eurobarometer 217/Wave 62.1. (2005)
40. European Commission: Special Eurobarometer 295/Wave 68.2. (2008)
41. European Commission: Special Eurobarometer 365/Wave 75.2. (2011)
42. European Commission: Special Eurobarometer/Wave EB97.2. (2022)
43. European Commission: Special Eurobarometer Wave EB91.3. (2019)
44. Woźniak, E., Tyczewska, A., Twardowski, T.: A shift towards biotechnology: social opinion in the EU. Trends Biotechnol. **39**, 214–218 (2021). https://doi.org/10.1016/j.tibtech.2020.08.001
45. Ichim, M.C.: The more favorable attitude of the citizens toward GMOs supports a new regulatory framework in the European Union. GM Crops Food. **12**, 18–24 (2021). https://doi.org/10.1080/21645698.2020.1795525

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 32
What Is the Problem with Europe in a Philosophical Point of View?

Marcel Kuntz

Abstract It is well known that developing plant biotechnological products is far more difficult in Europe than in the United States, for example. Of course, the different regulatory rationales impact technological development in both cases. This chapter discusses the reasons for such a difference, in relation with the historical background of Europe *vs*. USA, and in the philosophical context of 'postmodernism'. The latter is influent in both the European Union (EU) and the USA, but does not politically express itself in the same way. The central pillar of the doctrine currently dominant in the EU being to prevent repetition of the tragedies of the past, especially wars, which includes avoiding becoming a political power in the old sense. This chapter proposes that this political thought has also influenced the way technological risks are considered (Precautionary Principle) while benefits are sometimes ignored, such as those of plant biotechnology. The April 2021 EU Commission report on gene editing is discussed as an example of postmodern framing.

1 Introduction

Comparing how the transgenic plant innovation has been dealt with in the European Union (EU) *vs*. in the USA is of high interest in order to understand the possible perspective of genome editing in Europe.

While the transgenic plant technologies were co-invented in the early 80's by laboratories in both Europe and the USA [1, 2], the EU was clearly the centre of the plant biotechnology backlash which started in the middle of the 90s [3]. Earlier, in 1990, the EU, through its 90/220/EEC Directive, has created a new judicial object

M. Kuntz (✉)
Laboratoire de Physiologie Cellulaire et Végétale, Université Grenoble Alpes, CNRS, CEA, INRAE, Grenoble, France

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_32

called a 'genetically-modified organism' (GMO).[1] Although this Directive was replaced by Directive 2001/18/EC,[2] it retained what many consider as a scientific meaningless definition of a GMO [4]. A central element of the definition of a GMO according to the EU is that it is an "*Organism in which the genetic material has been altered in a way that does not occur naturally*". Such a concept has become highly damaging for public acceptance in the wake of the 'mad cow' crisis in Europe (when what was considered as an unnatural way of feeding cattle led to bovine spongiform encephalopathy, a disease potentially transmittable to humans). The temporal coincidence of the media coverage of this disease and the arrival on the market of the first GMO harvests provided anti-GMO activists the opportunity to spread fears about food derived from GMOs. As a political response, the above-mentioned new Directive was adopted in 2001. It states: "*In accordance with the precautionary principle, the objective of this Directive is to approximate the laws, regulations and administrative provisions of the Member States and to protect human health and the environment*". In other words, in the view of the EU, GMOs are intrinsically different from traditionally bred organisms and are therefore more risky, thus justifying a precautionary approach.

In the USA, the Federal government established a formal biotechnology policy as early as 1986, known as the 'Coordinated Framework for Regulation of Biotechnology'. It has since been updated [3] but remains based on existing laws, not a law in itself, in contrast with the path taken by the EU. Although one may consider that this Coordinated Framework has limited the deployment of transgenic crops to some extent, it did not have an inhibiting effect as laws in the EU had. Activists also attempted to propagate fears in the USA, which eventually lead to Public Law 114–216 on GMO labelling in 2016. However, the latter had only minimal labelling requirements, in contrast to what occurred in the EU under the 2001 Directive.

The advent of gene editing techniques gave rise to the question whether or not such a type of mutagenesis should be subject to specific biosafety regulatory provisions. Many papers discussed the possible 'natural' occurrence of such 'edited' mutations [5]. It should also be noted that the 2001 GMO Directive lists 'mutagenesis' in "*Techniques/methods of genetic modification yielding organisms* to be excluded *from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules*…".

However, in July 2018 the judgment of the Court of Justice of the European Union (CJEU) ruled that "*Organisms obtained by mutagenesis are GMOs and are, in principle, subject to the obligations laid down by the GMO Directive*" unless they

[1] Council Directive 90/220/EEC of 23 April 1990, https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A31990L0220. Accessed 12 october 2023.

[2] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001, https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A32001L0018. Accessed 5 June 2023.

[3] EPA (USA), Update to the Coordinated Framework for the Regulation of Biotechnology, 2017, https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/update-coordinated-framework-regulation-biotechnology. Accessed 5 June 2023.

"*have a long safety record*".[4] This excludes gene editing, with an explicit reference to "*the precautionary principle which that directive seeks to implement*". The CJEU reasoning was that gene editing is closer to transgenesis than to conventional mutagenesis ("*since the direct modification of the genetic material of an organism through mutagenesis makes it possible to obtain the same effects as the introduction of a foreign gene into the organism (transgenesis) and those new techniques make it possible to produce genetically modified varieties at a rate out of all proportion to those resulting from the application of conventional methods of mutagenesis*").

This view again clearly differentiates the EU from the USA. In the latter country, simple mutations, including those obtained by gene editing are usually considered as not necessitating regulatory oversight [5].

The question arising from these considerations is why are there such fully opposed approaches in the EU *vs*. the USA for the same biotechnologies? To address this question, this chapter will attempt to highlight the underlying ideological views and proposes that the latter are linked to a broader historical background.

2 A Brief Look Back at the History of Europe During the Twentieth Century

Alongside undeniable progress (in civil rights, social conditions, medicine, etc.), Twentieth century Europe is characterized by two World Wars, with destruction at levels never seen before, and the mass crimes of two totalitarian states, including their respective concentration camps and genocides. Drawing lessons from the past, Europe has undertaken to avoid the repetition of such tragic events. One of the political instruments of this project was the European integration, which progressed from the Treaty of Rome in 1957 up to the current EU.[5]

The aim of the 1957 treaty was "*To work towards integration and economic growth, through trade*" and had 'specific goals', amongst them to "*Pool their resources to* preserve and strengthen peace *and liberty*". The Consolidated Version of the Treaty on European Union (in 2016) goes further in presenting its 'values'.[6] Its Preamble states: "*Recalling the historic importance of the ending of the division of the European continent and the need to create firm bases for the construction of the future Europe*", and "*Confirming their attachment to the principles of liberty, democracy and respect for human rights and fundamental freedoms and of the rule of law*".

---

[4] Court of Justice of the European Union, press release No 111/18, 25 July 2018, https://curia.europa.eu/jcms/upload/docs/application/pdf/2018-07/cp180111en.pdf. Accessed 5 June 2023.

[5] Treaty of Rome (EEC), https://www.europarl.europa.eu/about-parliament/en/in-the-past/the-parliament-and-the-treaties/treaty-of-rome. Accessed 5 June 2023.

[6] Consolidated version of the Treaty on European Union (2016), https://eur-lex.europa.eu/resource.html?uri=cellar:9e8d52e1-2c70-11e6-b497-01aa75ed71a1.0006.01/DOC_2&format=PDF. Accessed 5 June 2023.

In other words, these values are now the 'Big Principles' on which the EU has been founded, in order to avoid the repetition of the disasters of the past. Significantly, "*The main goal of the European Union is to defend these values in Europe and promote peace and the wellbeing of the citizens*".[7]

3 'Big Principles' Have Also Been Applied to Science and Technologies

Although the contributions of science and technologies to improving the human condition were considerable during the twentieth century, accidents and disasters did occur [6], with science being used to develop weapons of mass destruction. For the German-born philosopher Günther Anders and for others, the main events of the twentieth century were 'Auschwitz' and 'Hiroshima' [7].

The ideology on which the EU is founded, namely a political dream of 'no tragedy' has also encouraged an utopia of 'no technological risk', illustrated by the 'Precautionary Principle'. Together with what can be seen as a Principle of Participation (of 'stakeholders', 'citizens'…), which will not be developed here (see below and for more details see [8]), these represent new 'Big Principles' which were designed to avoid repetition of accidents caused by technologies.

In concrete terms, this precautionary ideology inspired the drafting of the GMO Directives (the General Principles of the 2001 Directive state: "*In accordance with the precautionary principle, the following general principles should be followed…* [for risk assessment]"). Gene editing is a new biotechnology which was not anticipated in these Directives, thus requiring an *ex-post* legal interpretation. In other words, the *letter* of the Directive being unclear, the CJEU (see above) reasoned within the *spirit* of these Directives, namely applying the Precautionary Principle.

4 Postmodernism as a Philosophical Background

If one wishes to analyse further what has been described above in a philosophical context, the concept of postmodernism seems relevant, despite the fact that it is a polysemic term (it is also used in art, for example). Here, postmodernism refers to a type of thought that is largely a 'deconstruction' of the general Enlightenment philosophical viewpoints and values. The latter became dominant in the Western world progressively from the eighteenth century to the mid twentieth century. Regarding postmodernism, one can cite two French philosophers; Emmanuel Lévinas who criticized the Enlightenment philosophy as a "*totalizing*" system of thought and

[7] European Parliament in plain language: Values, https://europarlamentti.info/en/values-and-objectives/values/. Accessed 5 June 2023.

Jean-François Lyotard who defined postmodernism as the rejection of (western) "*metanarratives*" of human biological, historical, and social development. More generally, the modern 'universalist' thoughts have been accused to impose western conformity on other perspectives, thereby oppressing them, which is supposed to justify the 'deconstruction' of the European Enlightenment (for more details see [9]). The term 'deconstruction' is actually a translation of the concept of "*Abbau*" developed by German philosopher Martin Heidegger [10].

In a more recent phase, the concepts of 'deconstruction' and postmodernism moved from what was an intellectual debate to a form of more general rejection of the Western past. Such thoughts were largely fed by the tragic events mentioned above, to which one may add for some countries; colonialism, slavery and, more generally, discriminations of any type. From the 80's onwards, postmodernism insidiously became the dominant ideology of the European 'Elites'. It is largely influenced by what can be termed 'Western Guilt' [11], which can manifest itself in various forms, from repentance to self-hatred. Its political project is a form of new redeemed society.

Such a redeemed society would not only be a 'no tragedy' one, but can also be extended to a 'post-history' one in the postmodern dream. It is worth mentioning here the book by the American political scientist Francis Fukuyama, entitled *The End of History and the Last Man*. Interestingly, its author commented the following: "*I believe that the European Union more accurately reflects what the world will look like at the end of history than the contemporary United States. The EU's attempt to transcend sovereignty and traditional power politics by establishing a transnational rule of law is much more in line with a 'post-historical' world than the Americans' continuing belief in God, national sovereignty, and their military*" [12].

This is not to say that postmodern thoughts have not reached the USA; they are simply different (the past inspiring guilt and rejection is rather linked to racial issues in that country). It has provided the intellectual background of 'identity politics'. The broad implications of this politics will not be discussed here, only its implication for science.

5 Postmodernism Effects on Science

Regarding science, 'identity politics' clash with Enlightenment epistemology which values open debates and merit in science. In contrast, postmodern views justify 'affirmative action' in favour of minorities and more generally politicization of science. This has led to an outcry from some scientists [13].

The general ideas of postmodernism ('deconstruction' of the Western past) have been adopted by various sociological fields, leading to the creation of new 'studies', such as 'cultural studies', 'gender studies', 'science and technology studies' (STSs), etc. [14].

While the aim of science within modern framing is to determine what is true and what is false, within the STSs field some deconstructionist thinkers questioned the validity of claims of scientific truth and criticized scientific method. In their opinion, scientific objectivity is reduced to 'claims' that are the expression of one community (the scientists) which shares preconceptions. Thus, science is simply one 'construct' of reality amongst many others (for more details, taken from the GMO case, see [15]). In such a relativist framing, politically-constructed claims by anti-GMO activists should have the same validity as science-based risk assessment of GMOs. For example, how it permeated the European Parliament was discussed [15].

Such postmodern views encourage a 'participative' approach in science, which draws scientists into the political arena, with no observed benefits for science and risk assessment as far as the GMO dispute is concerned [16]. Regarding interactions between science and society, the move from modernism to postmodernist can be summarized by a shift from the concept that 'the public (in its own interest) should rely on the judgments of expert scientists' to a reverse one, namely that 'scientists should listen to society'. In addition, concepts of 'justice', 'fairness', 'democratic deliberation', etc., will compete with the truth seeking approach of science (for an example taken in the USA, namely a report on 'gene drive' by the National Academy of Science, see [17]).

In summary, the transgenic technology has been affected by postmodernism in different manners: the Precautionary approach and cognitive relativism, which could not be attenuated due to the simultaneous 'deconstruction' of certain modern values, such as distinguishing truth and falseness, and trust in scientific progress.

6 Fundamental Differences Between USA and Europe Regarding the Use of Technology

Although the USA was involved in both World Wars experiencing great human loss, mass devastation and the above-mentioned tragedies actually occurred in Europe, not in the USA. These facts provide a likely historical explanation for the dream of 'no tragedy' in Europe but not in the USA (the outcomes in other countries such as Japan or Russia will not be considered here). In addition, the USA became the leading power in the world. To maintain this status, their authorities will always privilege their national interest and hence their industries [18].

In parallel, the former European 'imperialist' powers disappeared and the *raison d'être* of the EU was not to recreate a new bellicose empire, but was just the opposite. The EU's ambition is limited to a soft power which attempts to export its well-thinking norms and 'values' to other regions of the world, when the large size

of its market allows it to do so (for discussion on the EU soft power, see resource notes.[8,9,10,11] It also export its fears, as the GMO case has shown.

Based on good intentions and decked with its moral values, the EU has given a greater importance to consumers and the environment than to 'realpolitik' (for references on consumer protection and environmental policies in the EU, see notes).[12,13,14,15] These EU priorities justify its reasoning on the necessity of regulation that others judge excessive (*e.g.* on biotechnologies).

However, one can note that the EU is embracing 'realpolitik' when it can hardly do otherwise. For example, it imports more than 20 million tons of GM soybeans per year to feed part of its livestock, thus supporting the production of transgenic plants in South America, while European farmers cannot do so because of the Precautionary Principle.

Besides the influences of postmodernism, the ban on GMO cultivation was also the product of demagogy and short-term electoral alliances of certain governments in Europe, which will not be discussed here (for Germany, see [19]; for France, see [20, 21]). However, the fact that such political manoeuvres could have the adhesion of so many people is linked to the dissemination of the postmodern ideology beyond the 'Elites'.

7 The Implication for 'Poor' Countries

The rejection of GMO cultivation by some 'developing' countries, such as some African countries which could benefit from plant biotechnology, can have different causes, from a concern of their leaders to preserve export opportunities to the EU, to the importation of European health fears by these countries. This situation should not be viewed as the adoption of 'postmodernism' by these countries, since the latter type of thought is inseparable from the 'Western Guilt'. It seems rather to be the consequence of a 'modern' type of reasoning (although not science-based), such as: 'why should we, the poor, adopt a technology that is rejected by the rich'.

---

[8] https://www.eeas.europa.eu/sites/default/files/eugs_review_web_0.pdf. Accessed 5 June 2023.

[9] https://www.unav.edu/documents/16800098/17755721/DT-05-2018_EU-soft-power.pdf. Accessed 5 June 2023.

[10] https://www.academia.edu/30914935/Is_there_a_European_Soft_Power. Accessed 5 June 2023.

[11] https://cepa.org/article/europe-too-soft-not-enough-power/. Accessed 5 June 2023.

[12] https://european-union.europa.eu/priorities-and-actions/actions-topic/environment_en. Accessed 5 June 2023.

[13] https://commission.europa.eu/live-work-travel-eu/consumer-rights-and-complaints/sustainable-consumption_en. Accessed 5 June 2023.

[14] https://www.efta.int/eea/policy-areas/flanking-horizontal-policies/consumer-protection. Accessed 5 June 2023.

[15] https://www.beuc.eu/blog/the-eu-has-become-an-environmental-policy-champion-time-to-go-the-last-mile/. Accessed 5 June 2023.

8 The EU Commission Report on Gene Editing as an Example of Postmodern Framing

On 29th April 2021, the European Commission published a report regarding the status of what is called 'New Genomic Techniques' (NGTs, *i.e*. gene editing) under Union law.[16] This report includes excellent overviews of research and innovation, as well as of risk assessment in this field.

Interestingly, it also provides an illustration of the postmodern views dominant in the EU. This report appears rather positive about the potential benefits of the gene editing technology, but is not considered an asset for some kind of European power (industrial, agricultural…). Instead, gene editing is viewed as a potential contributor to its own pre-set goals: "*Several of the plant products obtained from NGTs have the potential to contribute to the objectives of the EU's Green Deal and in particular to the 'farm to fork' and biodiversity strategies and the United Nations' sustainable development goals (SDGs) for a more resilient and sustainable agri-food system*". Similarly, when benefits for farmers are mentioned, such as "…*plants more resistant to diseases*…", it is in a context "*of reduced use of agricultural inputs (including plant protection products)*", *i.e.* more constrains imposed on farmers by the EU policy.

The following sentence also illustrates the framing of the Commission: "*The Communication on the 'farm to fork' strategy stated that new innovative techniques, including biotechnology and the development of bio-based products, may play a role in increasing sustainability,* provided they are safe for consumers and the environment *while bringing benefits for society as a whole*". One can wonder whether a technology can be proven "*safe*" and whether any technology in its initial phase has ever brought "*benefits for society as a whole"*…

The following sentence illustrates that the Precautionary Principle is not viewed as a guiding tool for risk assessment, but as an aim in itself: "*Directives 2001/18/EC and 2009/41/EC* [on the contained use of genetically modified micro-organisms] *share very similar aims (protection of health and the environment, application of the precautionary principle)*". The report does not mention that NGTs could be important for 'European power', which as discussed above is not part of the EU ideology.

In Sect. 4.6.2, the report summarizes the view of "*Stakeholders that see benefit in NGTs*" and, in Sect. 4.6.3, those of "*Stakeholders that do not see benefits in NGTs*". It concludes that "*Stakeholders are divided on the need to maintain the current legislation and reinforce its implementation, or to adapt it to scientific and technological progress and the level of risk of NGT products*." In a somewhat utopic view, the report states that "…*efforts should be made to reconcile opposing views in order to find common ground to address the issues identified in this study*".

---

[16] EC study on new genomic techniques (2021), https://food.ec.europa.eu/plants/genetically-modified-organisms/new-techniques-biotechnology/ec-study-new-genomic-techniques_en. Accessed 5 June 2023.

In a relativist (postmodern) framing (but it could simply be a political tactic), the report does not attempt do separate what is true and what is false. It simply wishes that "*Finally, more effort should be made to inform and engage with the public on NGTs and assess their views*".

Recently, a document from the European Commission proposes new approaches to regulating NGT plants. This proposal states that "*the Union GMO legislation is not fit for the purpose of regulating the deliberate release of plants obtained by certain NGTs*".[17] In line with the 2021 report by the Commission, gene editing is viewed as "*a possible tool to increase sustainability*", that is to say its own pre-set well-thinking goals. However, a certain recognition of the reality of the world can now be observed: "*the Covid-19 pandemic and Russia's war of aggression against Ukraine have also revealed the EU's external dependencies*". It also mentions that "*The Union risks being excluded to a significant extent from the technological developments and economic, social and environmental benefits that these new technologies can potentially generate*". In addition, regarding Sustainable Development Goals (SDGs), SDG9 (industry, Innovation and Infrastructure) is now specifically mentioned. Furthermore, the general objectives of this draft include to "*enhance the competitiveness of the EU agri-food sector*", while keeping (as expected with regard to the above-mentioned domination of a postmodern framing in the EU) the reference to the Precautionary Principle, the Green Deal and the Farm to Fork and Biodiversity strategies. Whether these economic objectives will clash with sustainability objectives remains to be seen.

9 Conclusions and Perspectives

As discussed previously, "*The EU has thus given the absolute priority to consumers and perceived environmental care, based on good intentions and moral values, but to do so it has indulged itself in excessive regulations for ideological reasons*" [8].

In this context, it is unlikely that the EU will take into account scientific facts presented in a 'modern' framing of truth. This implies that scientists, in future narratives, should also put forward 'values' not merely scientific facts, bearing in mind that in the foreseeable future modifying the EU directives will not be possible if this contradicts the spirit of the European 'Big Principles' and primarily the Precautionary Principle.

[17] EC (2023). Proposal for a regulation of the European Parliament and of the Council on plants obtained by certain new genomic techniques and their food and feed, and amending Directives 68/193/EEC, 1999/105/EC, 2002/53/EC, 2002/55/EC, and Regulation (EU) 2017/625. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52023PC0411. Accessed 12 October 2023.

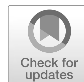
References

1. Angenon, G., Van Lijsebettens, M., Van Montagu, M.: From the tumor-inducing principle to plant biotechnology and its importance for society. Int. J. Dev. Biol. **57**, 453–460 (2023). https://doi.org/10.1387/ijdb.130295ga
2. Grunewald, W., Bury, J., Inzé, D.: Thirty years of transgenic plants. Nature. **497**, 40 (2013). https://doi.org/10.1038/497040a
3. Hundleby, P.A.C., Harwood, W.A.: Impacts of the EU GMO regulatory framework for plant genome editing. Food Energy Secur. **8**(2), e00161 (2019)
4. Tagliabue, G.: The meaningless pseudo-category of "GMOs". EMBO Rep. **17**, 10–13 (2016)
5. Custers, R., Casacuberta, J.M., Eriksson, D., László, S., Schiemann, J.: Genetic alterations that do or do not occur naturally; consequences for genome edited organisms in the context of regulatory oversight. Front. Bioeng. Biotechnol. **6** (2019). https://doi.org/10.3389/fbioe.2018.00213
6. Schlager, N.: When Technology Fails: Significant Technological Disasters, Accidents, and Failures of the Twentieth Century. Thomson Gale (1994)
7. Anders, G.: The Obsolescence of man, volume II, On the destruction of life in the epoch of the third industrial revolution, first published in German by C.H. Beck under the title *Die Antiquiertheit des Menschen* (1956). https://files.libcom.org/files/ObsolescenceofManVol%20IIGunther%20Anders.pdf
8. Kuntz, M.: Technological risks (GMO, Gene editing), what is the problem with Europe? A broader historical perspective. Front. Bioeng. Biotechnol. **8** (2020). https://doi.org/10.3389/fbioe.2020.557115
9. Duignan, B.: Postmodernism. Encyclopeadia Britannica (2023). https://www.britannica.com/topic/postmodernism-philosophy
10. Moran, D.: 55. – Destruction (*Destruktion, Zerstörung*) and Deconstruction (*Abbau*). In: Wrathall, M.A. (ed.) The Cambridge Heidegger Lexicon. Cambridge University Press (2021)
11. Bruckner, P.: The Tyranny of Guilt: An Essay on Western Masochism. Princeton, Princeton University Press (2010)
12. Fukuyama, F.: The End of History And the Last Man. Hamish Hamilton Ltd (1992)
13. Abbot, D., et al.: In defense of merit in science. J. Controversial Ideas. **3**, 1 (2023)
14. Felt, U., Fouché, R., Miller, C.A., Smith-Doerr, L. (eds.): The Handbook of Science and Technology Studies, 4th edn. MIT Press (2016)
15. Kuntz, M.: The postmodern assault on science. If all truths are equal, who cares what science has to say? EMBO Rep. **10**, 885–899 (2012). https://doi.org/10.1038/embor.2012.130
16. Kuntz, M.: Why the postmodern attitude towards science should be denounced. EMBO Rep. **14**(2), 114–116 (2013)
17. Kuntz, M.: Scientists should oppose the drive of postmodern ideology. Trends Biotechnol. **34**(12), 943–945 (2016)
18. The White House: National Security Strategy. https://www.whitehouse.gov/wp-content/uploads/2022/10/Biden-Harris-Administrations-National-Security-Strategy-10.2022.pdf (2022)
19. Katzek, J.: At the end of the day everything boils down to politics: the evolving of German policy toward GMO crops and the existing stagnation. GM Crop. Food. **5**(3), 178–182 (2014). https://doi.org/10.4161/21645698.2014.945881
20. Kuntz, M., Davison, J., Ricroch, A.E.: What the French ban of *Bt* MON810 maize means for science-based risk. Nat. Biotechnol. **31**(6), 498–500 (2013)
21. Kuntz, M.: The GMO case in France: politics, lawlessness and postmodernism. GM Crop. Food. **5**(3), 163–169 (2014). https://doi.org/10.4161/21645698.2014.945882

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Chapter 33
NGT Plant Products in the EU. The Postulates, The Outlooks, and Possible Consequences of a Regulatory System Reform in the Context of Legislative Reforms in Third Countries and Detection Requirements



Tomasz Zimny

Abstract This chapter presents potential consequences of the adoption of strict detection, identification or traceability requirements in the EU legislation regarding NGT products featuring single nucleotide variants. The context considered encompasses changes in the biosafety legislations not only in countries which were traditionally accepting of modern biotechnology products, but also in countries which were reluctant to use classic GMOs. Due to shifts in the approach to the regulation of NGT products not featuring stable inserts of foreign DNA, the EU risks becoming an isolated market with provisions not harmonized with those of its various trade partners or falling into a situation where regulated products officially not present on the market will enter due to a lack of efficient detection and identification methods and enforcement systems. Recent changes in the laws of such countries as Nigeria, Kenya or Japan are presented, as well as the recent jurisprudence of the Court of Justice of the EU.

1 Introduction

Following its study on the legal status of new genomic techniques' (NGT) products in the EU [1], the European Commission (EC) embarked on a mission to reform the current legislation on GMOs in order to facilitate the development and marketing of NGT products and to introduce an act, in which the level of regulation would be proportional to the risks related to the use of a given NGT product. The study considers NGT products as GMOs including: products of directed mutagenesis techniques, products of cisgenesis and intragenesis, as well as products featuring

T. Zimny (✉)
Institute of Law Studies, Polish Academy of Sciences, Warsaw, Poland
e-mail: tzimny@inp.pan.pl

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_33

interventions in the genotype without changing the nucleotide sequence (epigenetic changes). The situation occurred in the aftermath of the C-528/16 judgment [2] of the Court of Justice of the European Union (CJEU), where the court opted for a narrow interpretation of the exemption of mutagenesis products from the GMO legislation, limiting it only to products of such methods, which were widely used at the time of adoption of the 2001/18/EC Directive on the deliberate release of GMOs [3]. After the publication of the study of the EC, the CJEU was tasked with deciding yet another legal problem resulting from the sentence of the former judgment – namely whether the mutagenesis exception in the 2001/18/EC Directive applies to products of random mutagenesis *in vitro* as well as *in vivo*. The response of the CJEU states that the exception should be interpreted "as meaning that organisms obtained through the application of a technique/method of mutagenesis which is based on the same processes of modification, by the mutagenic agent, of the genetic material of the organism concerned as a technique/method of mutagenesis which has conventionally been used in a number of applications and has a long safety record, but which differs from that second technique/method of mutagenesis by virtue of other characteristics, shall, in principle, be excluded from the exemption laid down in that provision, provided that it is established that those characteristics are likely to lead to modifications of the genetic material of that organism which differ, by their nature or by the rate at which they occur, from those obtained by the application of that second technique/method of mutagenesis. However, the effects inherent in in vitro cultures do not, as such, justify the exclusion from that exemption of organisms obtained by the in vitro application of a technique/method of mutagenesis which has conventionally been used in a number of in vivo applications and has a long safety record with regard to those applications" [4].

In other words, the CJEU stated that the rule of thumb is that if a mutagenesis technique deviates in terms of efficiency or potential for modification from methods conventionally applied in 2001, then its products shall not be exempted from the GMO legislation. **However, the fact that random mutagenesis was performed *in vitro* does not seem to justify such a notion.**

It is not the goal of this study to perform a thorough and rigorous interpretation of the judgment. Rather it serves as another iteration of conceptual problems the EU institutions need to solve, when faced with the matter of the current GMO legislation [5]. The criteria for exemption of certain products of mutagenesis from the legislation seem to be connected with the time of the development of the method that brought about a particular mutation and the efficiency of the method. Given the fuzzy nature of such criteria, it is not surprising that the EC has decided to amend the current legislation. The recently published project of a new regulation on the matter, which envisages a confirmation procedure for products featuring minor changes (NGT type 1 plants) and their exclusion from organic production, as well as the questions regarding traceability and detection of NGT products posed by the EC in a recently closed public opinion poll [6], and also other activities, such as the recently closed Horizon Europe call for the development of new detection methods on products derived from new genomic techniques for traceability, transparency and innovation in the food system [7], suggest that the institution wishes to introduce a

system that would at least feature some sort of detection and identification mechanism, possibly also labelling, even if the provisions relating to the introduction of certain NGT product to the market were to be relaxed. While such solutions might be based on a wish to honor consumer choice, and address safety concerns, the technical difficulties connected with the performance of detection and identification of NGT products featuring point mutations, together with the regulatory tendencies in third countries, might lead to some undesired consequences. The success of any reform of the current legislation is also not guaranteed, since the recently leaked internal EC documents show [8] that the Commission is concerned about several issues connected with the reform, such as its influence on organic farming, the public rejection of GMOs in general and other issues, which might become an obstacle to the adoption of any amendment of the current laws.

2 Problems with Detection and Identification of NGT Products

If the EC fails to introduce a reform of the legislation, which would somehow exempt NGT products from authorization procedures, or if the reform will contain a relaxation of the current provisions but with the maintenance of detection, identification, traceability or labelling requirements, the compliance with such requirements might be technically challenging. Some researchers advocate a rather strict approach to the use of such techniques, including case-by-case risk assessment and *inter alia* whole genome sequencing for the detection of potential unintended consequences of editing [9]. Others seem much more skeptical as to the feasibility of such postulates, particularly, when it comes to detection or identification of products featuring single nucleotide variants, as well as the ability to prove that a given mutation (even if detected) was caused by a regulated technique rather than by an exempted one (e.g. random mutagenesis) or was spontaneous.

Detection of single nucleotide mutations in plant material, using PCR methods, depending on the method used, might require previous knowledge about the edit or might require data about the sequence surrounding the edit [10, 11]. It is also pointed out that even whole genome sequencing supported by bioinformatics and database access might be prone to errors especially for heterogenous samples, and that the efficiency of such detection methods also depends on the size of the genome that is being sequenced, making detection of potential contaminations in samples of such species as wheat or maize, less feasible [12].

These problems will gain practical significance with a broader adoption of NGT products featuring single nucleotide variants or even lacking changes in the nucleotide sequence, worldwide. The lack of applicability of existing laboratory methods for enforcement has already been stressed by the ENGL. It needs to be noted that, should EU legislation not change, unauthorized NGT products in the EU will be treated as unauthorized GMOs and essentially banned from the market. Should some requirements regarding detection, identification, labelling, coexistence with

conventional varieties remain, these will need to be enforced somehow, in a situation of lack of easily accessible, efficient and economic detection methods. Such outcomes, in the context of trade exchange with the currently biggest producers of GMO products imported to the EU have already been described [13]. It seems however that the problems might also apply to exchange in agricultural goods with other countries, which were hitherto reluctant to introduce classic GMOs, but have decided to relax the legislation or even exempt products featuring single nucleotide variants or not featuring stable foreign DNA inserts.

3 Situation in Third Countries

Countries, which are already well known for excluding certain NGT products from their GMO legislations, such as the USA, Canada, Argentina or Brazil [14, 15] have introduced changes in their legislations that will be difficult to harmonize with an EU solution that will require the authorization of such products and more importantly their identification through molecular methods. Also, several countries, which were so far reluctant to adopt products of genetic engineering, are changing their policies in such a way that they allow products featuring single nucleotide variants to be less regulated than classic GMOs.

Some African nations have been reluctant to adopt the GMO technology [16], partially due to the restrictive policies of the EU, a major trading partner in agricultural goods. However when it comes to NGT products, some African countries are adopting policies, which are more permissive for plants featuring single nucleotide variants or more generally: mutations akin to those achievable through random mutagenesis or conventional breeding. For instance Nigeria issued new guidelines regarding the procedures for administrative handling of certain NGT products in December 2020 [17], according to which the applicant shall receive a biosafety approval if the method used for obtaining a product does not involve recombinant DNA or if such DNA is not present in the final product [see also 18].

Kenya issued an interpretation of its existing legislation [18, 19],: "modifications by inserting genes from sexually compatible species and where regulatory elements (promoters and terminators) are also from the same species; deletions/knock outs provided that there is no insertion of foreign genetic material in the end-product; processed products whose inserted foreign genetic material cannot be detected; – do not fall under the Biosafety Act, which would otherwise require them to undergo an authorization procedure. The applicant is expected to submit an Early Consultation Form to the competent authority, in order to determine whether their product will fall under the biosafety legislation or not."

Japan is another example of a country introducing a leeway for products of directed mutagenesis without stable inserts of foreign DNA [20]. Relatively recent amendments to the biosafety policies provide that food products derived through a gene editing technology that do not contain remnants of foreign DNA fragments (e.g. SDN-1 products) fall under a notification rather than authorization procedure,

hence do not require to go through a safety assessment procedure. Conventional crosses with such plants do not require to be notified anymore. This solution, based on a preemptive confirmation of status, results in a release of certain products of gene editing and their progeny, to the market, without a requirement for traceability or any sort of identity preservation. Hence their products can freely circulate on the market, once they were initially notified to the competent authority. This step means that SDN-1 products would not fall under the legislation [21, 22]. In Japan classic GMOs can be authorized for cultivation, but their use would subsequently be thwarted through the decisions of regional governments, who had the last voice in the matter [23]. The currently adopted solution allows developers to avoid the administrative burdens to a large extent and some gene editing products were actually already accepted according to the new provisions, most notably fish [24] and tomatoes [25], which were already placed on the market [26].

Similar provisions were also adopted in high volume GMO trading countries. In Argentina and Brazil, it is the introduction of a stable construct of foreign DNA, which determines the regulatory status of the product [13, 14]. In the USA exemptions are *inter alia*:

– products featuring changes resulting from the cellular repair of a targeted DNA break in the absence of an externally provided repair template;
– single-base-pair substitutions or the introduction of a gene known to occur in the plant's gene pool
– a change in the targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool.

The consultation or notification of the authorities is not mandatory [27, 28].

England and Wales have also introduced relaxed provisions regarding field trials and marketing of "precision bred organisms", which could have been obtained from traditional processes [29].

Such solutions, as the ones presented above will likely result in products derived through e.g. SDN-1 techniques to fall out of the legislation, and are not required to meet the provisions associated with classic GMOs, such as traceability or development of detection or identification methods.

4 A Global Conceptual Shift

The situation presented above – where not only countries that were liberal towards cultivation and other use of traditional GMOs relax their legislation, but also countries, which used to oppose the use of such products for various reasons – constitutes a conceptual shift, where the need for regulation is altered significantly.

The EU is a trendsetter in the case of many technological standards, in that entrepreneurs from third countries tend to comply with the EU-set standards, due to the size of the common market and the purchasing power of EU citizens. Complying with such standards seems economically more viable than being effectively locked

out of such a market, due to lack of compliance. A recent example of this phenomenon was observed with the introduction of the USB-C port as the common charger port for all phones and tablets [30], which resulted in a decision to install such ports in iPhones, a step hitherto resisted by the manufacturer. In the case of "classic" GMOs the EU was also a *de facto* trendsetter for many third countries, which would prohibit the cultivation and often other uses of such products in order to prevent accidental influx of such products to the EU and so as to not to endanger their exports to the Union [16]. This seems not to be the case anymore, at least when it comes to NGT products featuring single nucleotide variants or lacking stable inserts of foreign DNA. In this sense the EU instead of being a standard setter is at risk of becoming an isolated island surrounded by regions with a fundamentally different approach to NGT products, namely a permissive rather than a precautionary one. This scenario is more likely to be realized if no changes are made in the current legislation or if the changed legislation will still require detection, identification, labelling or coexistence measures for such products. In the latter 2 cases, two not mutually exclusive scenarios can be presented for the behavior of foreign exporters, local importers or operators of those products. Firstly, they might attempt to adhere to the restrictive provisions, which might result in increased costs as well as lowering the competitive position of their products on the market. The development of a rigorous documentation system for all the steps of the production chain, capable of creating a reliable paper trail for each imported product would probably be required to support compliance with such provisions. Secondly, some entrepreneurs might choose to ignore such requirements, counting on the lack of rapid detection methods and a general lack of efficiency of the enforcement authorities in the detection of NGT products. The latter scenario might result not only in formally "unauthorized GMOs" circulating in the common market, or even authorized products not being properly labelled, but might cause damage further down production chains, if unauthorized products get eventually detected at the later stages of processing or marketing of processed products. A situation, where such plant material is used in breeding activities and is only later detected as a component of registered "conventional" or organic varieties needs also to be considered.

5 Conclusions

While the final shape of the new EU legislation regarding the development and use of NGT products is unknown and its fate also remains uncertain, the latest CJEU jurisprudence did not bring about any significant change to the existing status quo and the proposed legislative changes still feature authorisation procedures and labelling of some products, such as reproductive material of NGT type 1 plants. Concurrently a conceptual shift in the approach to NGT products featuring single nucleotide variants takes place in third countries, in that such products are no longer considered to be GMOs requiring strong regulation. This phenomenon becomes characteristic not only for nations, which were traditionally accepting of modern

biotechnology products, but also in countries, which were reluctant when it came to the adoption of classic GMOs. This shift might result in serious practical problems for EU entrepreneurs and enforcement authorities alike, due to practical problems with the development of efficient detection methods and also might lead to a situation of legal fiction, where certain products, although officially regulated, will circulate on the market due to the aforementioned deficiencies in cost- and technically-efficient detection and identification methods.

This work was financed from a National Science Centre Grant no: UMO-2020/39/D/HS5/03144, Transformation of biosafety legislation in agriculture in the EU law and the laws of its selected trade partners, with respect to the scientific development.

Literature

1. European Commission: Study on the status of new genomic techniques under Union law and in light of the Court of Justice ruling in Case C-528/16. European Commission, Brussels (2021)
2. CJEU C-528/16: Judgment of CJEU in the case C-528/16 Confédération paysanne and others v. Premier ministre Ministre de l'agriculture, de l'agroalimentaire et de la forêt. (2018)
3. Directive 2001/18/EC: Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. (2001)
4. CJEU C-688/21: Judgment of CJEU in the case C-688/21 Confédération paysanne and others v. Premier ministre, Ministre de l'Agriculture et de l'Alimentation,. (2023)
5. Van Der Meer, P., Angenon, G., Bergmans, H., Buhk, H.J., Callebaut, S., Chamon, M., Eriksson, D., Gheysen, G., Harwood, W., Hundleby, P., Kearns, P., Mcloughlin, T., Zimny, T.: The status under EU law of organisms developed through novel genomic techniques. Eur. J. Risk Regul. **14**(1), 93–112 (2021). https://doi.org/10.1017/err.2020.105
6. European Commission: Legislation for plants produced by certain new genomic techniques. https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/13119-Legislation-for-plants-produced-by-certain-new-genomic-techniques_en, last Accessed 13 Aug 2022
7. European Commission: New detection methods on products derived from new genomic techniques for traceability, transparency and innovation in the food system. https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/horizon-cl6-2023-farm2fork-01-11;callCode=null;freeTextSearchKeyword=food;matchWholeText=true;typeCodes=1,2,8,0;statusCodes=31094501,31094502;programmePeriod=null;programCcm2Id=43108390;programDivisionCode=null;focusAreaCode=null;destinationGroup=null;missionGroup=null;geographicalZonesCode=null;programmeDivisionProspect=null;startDateLte=null;startDateGte=null;crossCuttingPriorityCode=null;cpvCode=null;performanceOfDelivery=null;sortQuery=sortStatus;orderBy=asc;onlyTenders=false;topicListKey=topicSearchTablePageState, last Accessed 16 Apr 2023
8. Willard, M.: GMO Deregulation Delayed – Where are we at, where might we be going?. https://www.arc2020.eu/gmo-deregulation-delayed-where-are-we-at-where-might-we-be-going/, last Accessed 16 Apr 2023
9. Eckerstorfer, M.F., Dolezel, M., Heissenberger, A., Miklau, M., Reichenbecher, W., Steinbrecher, R.A., Waßmann, F.: An EU perspective on biosafety considerations for plants developed by genome editing and other New Genetic Modification Techniques (nGMs). Front. Bioeng. Biotechnol. **7**, 31 (2019)
10. European Network of GMO Laboratories (ENGL): Detection of food and feed plant products obtained by new mutagenesis techniques. (2019)

11. Shillito, R.D., Whitt, S., Ross, M., Ghavami, F., De Vleesschauwer, D., D'Halluin, K., Van Hoecke, A., Meulewaeter, F.: Detection of genome edits in plants—from editing to seed. In Vitro Cell. Dev. Biol. -Plant. **57**, 595–608 (2021). https://doi.org/10.1007/s11627-021-10214-z
12. Grohmann, L., Keilwagen, J., Duensing, N., Dagand, E., Hartung, F., Wilhelm, R., Bendiek, J., Sprink, T.: Detection and identification of genome editing in plants: challenges and opportunities. Front. Plant Sci. **10**, 236 (2019). https://doi.org/10.3389/fpls.2019.00236
13. Zimny, T., Sowa, S.: Potential effects of asymmetric legal classification of gene edited plant products in international trade, from the perspective of the EU. EFB Bioecon. J. **1**, 100016 (2021). https://doi.org/10.1016/j.bioeco.2021.100016
14. Hamburger, D.: Comparative Analysis: The Regulation of Plants Derived from Genome Editing in Argentina, Australia, Canada, the European Union, Japan and the United States. In: Dederer, H.-G., Hamburger, D. (eds.) Regulation of Genome Editing in Plant Biotechnology, pp. 313–363. Springer International Publishing, Cham (2019). https://doi.org/10.1007/978-3-030-17119-3_8
15. Zimny, T.: New genomic techniques and their European Union reform. Potential policy changes and their implications. Front. Bioeng. Biotechnol. **10**, 1019081 (2022). https://doi.org/10.3389/fbioe.2022.1019081
16. Mabaya, E., Fulton, J., Simiyu-Wafukho, S., Nang'ayo, F.: Factors influencing adoption of genetically modified crops in Africa. Dev. South. Afr. **32**, 577–591 (2015). https://doi.org/10.1080/0376835X.2015.1044078
17. NBMA: National Guidelines for Gene Editing (Nigeria). https://nbma.gov.ng/wp-content/uploads/2022/03/NATIONAL-GENE-EDITING-GUIDELINE.pdf, last Accessed 08 June 2023 (2020)
18. Ongu, I., Olayide, P., Alexandersson, E., Mugwanya Zawedde, B., Eriksson, D.: Biosafety regulatory frameworks in Kenya, Nigeria, Uganda and Sweden and their potential impact on international R&D collaborations. GM Crops Food. **14**, 1–17 (2023). https://doi.org/10.1080/21645698.2023.2194221
19. NBA Kenya: Guidelines for determining the regulatory process of genome edited organisms and products in Kenya. https://www.biosafetykenya.go.ke/images/GENOME-EDITING-GUIDELINES-FINAL-VERSION-25th-Feb-2022-03.pdf, last Accessed 08 June 2023 (2022)
20. Councillor for Environmental Health and Food Safety: food hygiene handling procedures for food and additives derived from genome editing technology. https://www.mhlw.go.jp/content/000550824.pdf last Accessed 19 Mar 2023
21. Tsuda, M., Watanabe, K.N., Ohsawa, R.: Regulatory status of genome-edited organisms under the Japanese Cartagena Act. Front. Bioeng. Biotechnol. **7**, 387 (2019)
22. Councillor for Environmental Health and Food Safety of Japan: Japanese Gene Editing (GE) Guidelines. https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/shokuhin/bio/genomed/index_00012.html, last Accessed 19 Mar 2023
23. Dederer, H.-G., Hamburger, D. (eds.): Regulation of Genome Editing in Plant Biotechnology: A Comparative Analysis of Regulatory Frameworks of Selected Countries and the EU. Springer International Publishing, Cham (2019). https://doi.org/10.1007/978-3-030-17119-3
24. Japan embraces CRISPR-edited fish: Nat Biotechnol. **40**, 10–10 (2022). https://doi.org/10.1038/s41587-021-01197-8
25. ISAAA: Japan launches world's first genome-edited tomato. https://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=18668, last Accessed 15 Apr 2023
26. ISAAA: Japan's three genome-edited food products reach consumers. https://www.isaaa.org/blog/entry/default.asp?BlogDate=1/19/2022, last Accessed 15 Apr 2023
27. USDA: SECURE rule. https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/biotech-rule-revision/secure-rule/secure-text/sr-text, last Accessed 09 June 2022
28. Hoffman, N.E.: Revisions to USDA biotechnology regulations: The SECURE rule. PNAS. **118**, e2004841118 (2021). https://doi.org/10.1073/pnas.2004841118

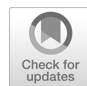
29. UK Parliament: Genetic Technology (Precision breeding) Bill – Parliamentary Bills – UK Parliament. https://publications.parliament.uk/pa/bills/cbill/58-03/0011/220011.pdf, last Accessed 08 June 2023 (2023)
30. EU Parliament and the Council: Directive (EU) 2022/2380 of the European Parliament and of the Council of 23 November 2022 amending Directive 2014/53/EU on the harmonisation of the laws of the Member States relating to the making available on the market of radio equipment (Text with EEA relevance). (2022)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

Part V
Future Outlook

Chapter 34
Prospects for Plant Genome Editing



Agnès Ricroch, Dennis Eriksson, Dragana Miladinović, Jeremy Sweet, Katrijn Van Laere, and Ewa Woźniak-Gientka

Genome editing – the focus of this book – is a set of methods that provide opportunities to precisely and efficiently improve crop traits by tailoring genes and regulatory domains. In combination with genetic modifications (GM), RNA interference (RNAi), epigenetics and the range of -omics technologies, they offer multiple methods for enhancing crop production, crop protection, crop quality and climate change adaptation. Combining these technologies with traditional breeding and careful management of crop cultivation methods in integrated systems can make major contributions to improving the sustainability of agricultural production, particularly in response to climate change. These technologies could also contribute to achieving United Nations' Sustainable Development Goals and national/EU policy objectives for agriculture, food safety, food security and the environment.

A. Ricroch (✉)
The Jean-Monnet Faculty. Idest Laboratory, Paris-Saclay University, Sceaux, France
e-mail: agnes.ricroch@universite-paris-saclay.fr

D. Eriksson
Department of Plant Breeding, Swedish University of Agricultural Sciences, Alnarp, Sweden

D. Miladinović
Institute of Field and Vegetable Crops, National Institute of Republic of Serbia, Novi Sad, Serbia

J. Sweet
Sweet Environmental Consultants, Cambridge, UK

K. Van Laere
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Melle, Belgium

E. Woźniak-Gientka
Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland

© The Author(s) 2024

A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_34

1 Technology

Genome editing in plants is often referred to as precision breeding due to the precise nature of the methods used compared with some other genetic modification and mutation breeding techniques. Genome editing technologies exceeded initial expectations as a tool in plant science and breeding and are quickly adopted in most life-science laboratories as a precise and high-throughput tool for targeted crop improvement. They became the method of choice in many research studies. The future of genome editing relies on expanding the technology toolbox with more efficient multiplexing and high-throughput strategies, applications towards chromosomal rearrangements and epigenetic changes together with more tools for efficient delivery of editing components and regeneration of edited cells.

2 Crop Improvement

Genome editing has already been applied for numerous trait improvements in crops. By now, there are more than 100 applications of genome editing on at least 28 different plant species, some of which have been described in this book. In planta genome editing methods still need to be optimized in a broader range of plant species before to achieve a significant commercial impact. This especially stands for time and costs related to the development of specialized regeneration protocols for individual plant lines. It is therefore important to broaden the targets for crop improvement by gaining knowledge about biological processes and genes involved, as well as the interaction of biological processes with the environment.

Although being known as a very precise technology, off-target, pleiotropic and other unintended effects have occasionally been reported. The regular plant breeding procedures, however, are expected to manage this by careful screening and testing of new breeding lines through several generations prior to multiplication and commercialisation.

Technical innovations will expand the genome editing toolbox and further strengthen its technical and economic advantages in crop improvement. However, genome editing will still mostly complement rather than replace conventional plant breeding methods in crop improvement. Further applications of genome editing technologies in crop improvement depend largely on the economic and legal framework, as well as public perceptions of the technology itself.

3 Regulations

Government regulation for new genomic techniques (NGTs) that comprise, among others, genome editing must be science-based, predictable, risk-proportional, and harmonized with international trading partners. Harmonization and predictability

would reduce investment risk and increase spending in R&D whereas risk-proportionality would balance the costs of respective innovation with the social benefits secured through risk avoidance. That is why it is important that appropriate and science-based policies and regulations are in place that allow rapid and thorough assessment of any risks associated with the products of.

A recent draft European Commission proposal in July 2023 defines an NGT (Category 1) plant as a plant obtained by directed mutagenesis, cisgenesis, and intragenesis, that is equivalent to a conventionally bred plant and does not contain any genetic material imported from outside the breeders' gene pool and contains a very limited number of changes to the plants genome. Therefore, the Commission defines NGT plants as GMOs that do not contain any "foreign" DNA. For NGT crops, the Commission introduces two categories that are regulated differently:

1. Category 1 NGT plants do not differ from plants from conventional breeding and/or could have arisen through natural or conventionally induced mutations. They are considered GMOs equivalent to conventionally grown plants provided that they meet the criteria further defined in Annex I.
2. Category 2 NGT plants are all other NGT plants that do not correspond to Category 1. The European Commission proposal specifies that plants which have acquired a herbicide-tolerance through gene editing always fall under Category 2.

According to the draft document Category 1 NGT plants are exempt from the existing GMO regulations. From this it can be deduced that Category 1 NGT plants are not subject to any specific risk assessments for health and the environment and will not require monitoring, labelling or traceability along supply chains. However, Category 1 NGT plants (and their products) are not unregulated. They will require notification to the relevant competent authority in order to allow confirmation of the categorisation before release or marketing. In addition, regulations governing activities with conventionally bred plants apply to them and -depending on the type of modification- other obligations such as imposed by the Novel Food Regulation (EU) 2015/22 must be met.

For Category 2 NGT plants, there are different procedures for release and placing on the market. The approval process is the usual GMO procedure based on the guidelines for genetically modified organisms (VO (EC) 1829/2003) with associated detection methods and traceability.

This proposal would potentially bring the EU more in line with regulatory authorities from other parts of the world and facilitate the commercialisation of many genome edited crops. This is especially important for controlled release of products of new breeding systems and technologies in field trials to assess the performance and net contribution that new varieties can make to sustainable farming systems and for environmental impact assessments. NGT varieties could also be assessed for their contribution towards managing crop production in relation to climate change and other externalities influencing food production and supply chains. However, while this proposal finally provides a concrete basis for an adapted regulatory framework, it will take several years before the new Regulation will be

finalized and become effective, meanwhile stifling the dynamic research, development and market environment.

4 Public Perception

Public perception is one of the critical parameters influencing the development and commercialization of plants produced with the use of NGTs. General positive attitudes towards genome editing, both in the public and stakeholder acceptance, can support the implementation of relevant regulations in different countries. Like the legal situation and state of genome editing that is diverse worldwide, the public perception of plant gene technologies differs across regions. These differences in opinions are not grounded in science but rather in politics, psychological, social, cultural, personal and economic factors.

Engaging citizens in the development of innovations in life sciences is critical, and there is a potential advantage in communicating biotechnology and genome editing to society. The engagement of scientists and experts in public debates about the future of NGTs is crucial and may encourage scientists to make more effort in public debates regarding the benefits of genome editing products. Scientists, policymakers and entrepreneurs should create more opportunities for the public to participate in relevant meetings and activities (e.g., citizen science projects). Moreover, these interactions facilitate monitoring shifts in the acceptance of NGTs by the public.

5 PlantEd

The COST Action PlantEd (CA 18111) has since 2019 brought together scientists, plant breeders and other stakeholders to discuss many aspects of plant genome editing and this book reflects many to the issues considered during this Action. In a survey circulated among the 608 experts involved in PlantEd to estimate the value and impact of the network, 90% agree (completely or somewhat) that they have obtained new ideas and knowledge about plant genome editing by being part of the PlantEd network, 86% agree that they have obtained new connections and potential collaborators through the network, and 88% agree that PlantEd has contributed to the development of plant genome editing in Europe and beyond. This emphasizes the importance of the Action itself, as well as the importance of constant delivery of broad knowledge about genome editing technology to general public and stakeholders. This should facilitate the adoption of NGTs in crop improvement and agricultural production that should further contribute to food security and sustainability of agricultural production in changing climate and unstable market conditions.

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.