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1 Introduction

Genome editing technology offers tremendous potential for agricultural improvements including consumer health, increased productivity, and alleviating the growing food security crisis. Progress in developing and implementing site-directed nucleases (SDNs), in particular CRISPR/Cas9, has been rapid and exciting. In addition, many new approaches have been concurrently developed for delivery of CRISPR/Cas9 components to plant cells, from *Agrobacterium* infection to nanoparticles (Chen et al., 2019). However, advances in genome editing and delivery technologies continue to outpace our ability to efficiently produce edited events due to the lack of robust methods for regeneration of fertile plants for many crops (Altpeter et al., 2016). Historically, recovering transgenic plants, even at low frequency, has been considered a major success for many crops. However, genome

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editing applications comprising template-based modifications via homology-directed repair (HDR), often require production of hundreds of regenerated events to recover plants with the desired edit(s). This limitation in regeneration efficiency hinders full adoption of CRISPR/Cas9-mediated genome editing in agriculture.

The challenge of efficient regeneration influences many experimental options to develop a successful genome editing protocol and ultimately depends on how amenable a species is to *in vitro* manipulation. Given this prerequisite, easily cultured species such as *Arabidopsis thaliana* and *Nicotiana benthamiana* became the model for demonstrating multiple genome editing applications, using delivery to a variety of plant explants ranging from protoplasts (Li et al., 2013) to *in planta* (Maher et al., 2020). Conversely, many important row crop species (e.g. cereals, legumes, and woody perennials, among others) are difficult to manipulate *in vitro*, limiting the opportunities for successful application of genome editing technologies.

Recalcitrant species, which typically exhibit consistently low-regeneration frequencies, are usually suitable for simple mutagenesis-based knockouts, but not for more complex (and low frequency) template-based edits. While recalcitrance for a given species can sometimes be overcome by perseverance and patience through exploring tissue culture conditions, new approaches such as the use of morphogenic genes can be an important adjunct, aiding in both accelerating progress in certain species and expanding the range of accessible plant varieties.

To ensure success, choosing the optimal combination of CRISPR/Cas9 delivery method and tissue culture strategy requires thoughtful consideration for any given plant species. In this chapter we discuss delivery options, cell type or plant explant, and the potential benefits of using morphogenic genes.

2 Delivery of genome editing components into plant cells

Competence and regenerability are critical considerations in selecting an explant to be suitable for genome modification. Competence reflects the ability of a cell/tissue to be genetically modified by receiving exogenous biomolecules (e.g. DNA, RNA, protein etc.). Regenerability can be described as the plant cell's ability to switch toward a regenerative pathway resulting in the production of a full fertile plant capable of transmitting the intended genome modification to its progenies (Delporte et al., 2012).

Options for transformable explant material range from single cells, such as protoplasts or microspores, to embryonic tissues such as meristem, scutellum or cotyledons, to seedling-derived tissues such as apical/axillary meristems, hypocotyl or leaves, and finally *in planta* alternatives. In general, these tend to progress from methods involving extended lengths of *in vitro* culture (e.g.

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protoplast, embryogenic/organogenic callus), toward those that capitalize on more rapid plant development, including direct somatic embryo or meristem/shoot formation, and finally remaining entirely *in planta*. Following this trend is a concomitant reduction in the time required from delivery of DNA, RNA, and/or protein to recovery of a fertile genome-edited plant, conditions that have long been recognized as desirable to minimize tissue culture and reduce somaclonal variation (Côte et al., 2001). The ideal scenario would be a simple *in planta* method that worked across all plants, but currently many species require long *in vitro* manipulation while others, including many agronomically important crops, remain recalcitrant to any of the generic strategies outlined in Table 1.

The cell types or tissues used for successful CRISPR/Cas9-mediated editing include examples such as lettuce protoplasts (Woo et al., 2015), leaf tissue in tobacco (Gao et al., 2015), immature embryos in cereals (Svitashev et al., 2015, 2016; Shi et al., 2017; Liang et al., 2017; Hamada et al., 2018), embryogenic callus in rice (Xu et al., 2015; Sun et al., 2016), and immature cotyledons in soybean (Li et al., 2015). Many alternative explants such as callus, cotyledons, true leaves, microspores, axillary meristems, and hypocotyl (or stem) have been used to produce transgenic plants (Table 1) and remain a viable starting material for future CRISPR/Cas9-mediated genome editing. For many crops, explant preference can change over time with concomitant reductions in tissue culture. Cowpea is one example of a recalcitrant crop in which the choice of explant and culture route has had a profound impact on the outcome. Accordingly, researchers have recognized the benefit of moving away from organogenic or embryogenic cultures as a transformation target, which results in prolonged periods of tissue culture. Instead, they have shifted to T-DNA delivery into such explants as cotyledons (Raveendar and Ignacimuthu, 2010) or cotyledonary nodes (Chaudhury et al., 2007), and the more direct route of *Agrobacterium*-mediated delivery into the embryo axis (Popelka et al., 2006; Bett et al., 2017), which reduced time in culture. Finally, by targeting the apical meristem (Bett et al., 2017) or cotyledonary/axillary meristems (Che et al., 2019), followed by direct shoot formation, the selection of transgenic events in cowpea has become simpler (Bett et al., 2017) or both simpler and more efficient (Che et al., 2019).

In planta methods that take advantage of normal growth and reproduction with a minimal perturbation to the system, are the quintessence of transformation approaches for all plant species. Floral-dip in Arabidopsis has been widely used for transformation over many years, and more recently has been extended to delivery of CRISPR/Cas9 components for mutagenesis and template-based genome editing (Table 1). In a more recent *in planta* report, the axillary meristem has been targeted for *Agrobacterium*-mediated delivery to demonstrate genome editing (Maher et al., 2020).

Table 1 Explants and growth strategies used to recover transgenic and/or edited plants

			Route to heritable transmission						
General strategy	Target explant	Delivery method	Callus	Regenerable structure	Regenerated plantlets	Fertile plant	Transgenic	**Genome editing**	Illustrative references
In vitro via somatic embryo	Protoplasts	TF	Yes	Somatic Embryo (SE)	Limited**	Limited**	Yes**	**Mut, Ins**	**Puchta 1996, Wright 2005, Li 1990, Park 2019, Lin 2018, Murovec 2018, Woo 2015**
	Callus	Agro	Yes	SE	Yes	Yes	Yes	–	Bevan 1994, Komari 1990, Hatanaka 1999, Sidorov 2006, Du 2019
	Zygotic embryo	PG, Agro	Yes	SE	Yes	T0	Yes	**Mut, Edit & Ins**	Wan 1995, Lowe 2016, **Svitashev 2015, others**
	Cotyledon	PG, Agro	Yes	SE	Yes	T0	Yes	–	Parrott 1998, Yan 2000
	True leaf	PG, Agro	Yes	SE	Yes	T0	Yes	–	Matsuda 2005, Lowe 2016
	Zygotic embryo	PG, Agro	⇨	SE	Yes	T0	Yes	**Deletion**	Lowe 2018, Hoerster 2020, **Gao 2020**
	Somatic embryo	PG, Agro	⇨	Secondary SE	Yes	T0	Yes	**Mut, Ins**	Li 2009, **Jacobs 2015, Li 2015**
	Microspore	PG, Agro	⇨	SE	Yes	Limited**	Yes**	–	Brew-Appiah 2013

In vitro via shoots	Callus	PG, Agro	⇨	Shoot	Yes	T0	Yes	–	Hong 2007
	Cotyledon node	PG, Agro	⇨	Shoot	Yes	T0	Yes	–	Meurer 1998, Olhoft 2007
	Embryo axis (meristem)	PG, Agro	⇨	Shoot	Yes	T0	Yes	**Mut**	Pigeaire 1997, Dutt 2007, Paz 2006, Sitther 2018, **Curtin 2011, Haun 2014, Demorest 2016**
	Isolated Axil MERISTEM	Agro	⇨	Shoot	Yes	T0	Yes	–	Matsuda 2005, Fujita 2009, Qi 2014, Mayavan 2015
	Hypocotyl/ stem	PG, Agro	⇨	Shoot	Yes	T0	Yes	–	Cardoza 2003, Zhao 2017
In planta	Floral dip	Agro	⇨	T1 Seed	⇨	T1	Yes	**Mut, Edit & Ins**	**Lloyd 2005, Zhang 2010, de Pater 2009 2013, Forner 2015**
	Axillary meristem	Agro	⇨	Shoot	⇨	T0	–	**Mut**	**Maher 2020**

Arrows indicate a step in the process that has been eliminated for a particular method.
References in bold font indicate nuclease-mediated modifications.
TF = polyethylene glycol- or electroporation-mediated transfection.
PG = Particle gun delivery.
Mut = mutagenesis.
Edit = Oligonucleotide-mediated edit.
Ins = targeted insertion.
NT* = Nanotubules have been successfully used in delivery of DNA.
** = Restricted genotype range.
HDR = Homology-Dependent Repair.

3 Delivery methods for genome editing reagents: delivery into single cells

The presence of a rigid glycan-rich cell wall surrounding the cell membrane makes the task of delivering exogenous material to plant cells extremely challenging. Current delivery options for genome editing reagents can be divided into three main categories: physical, chemical, and biological (Que et al., 2019). The method by which genome modification reagents are introduced is dictated by the explant type and its regenerative capacity for any given plant species. For most species, more than one delivery system can be used. In turn, these available alternatives allow delivery of different genome editing reagents (e.g. DNA, RNA, proteins, or mixtures). Among physical delivery options, particle bombardment or biolistic (Klein et al., 1987) and electroporation (Fromm et al., 1985; Shillito et al., 1985) are most common, predominantly used for delivery into either whole cells/tissue or into protoplasts, respectively. Polyethylene glycol (PEG) has been the most widely used chemical agent for protoplast transformation (Kofer et al., 1998). In terms of biological delivery, all mainstream methods rely on *Agrobacterium tumefaciens* (Gelvin, 2003; 2017), but other methods such as the use of viral particles have been attempted (Grimsley et al., 1987).

Protoplasts are plant cells from which the cell wall has been mechanically (Von Klercker, 1892) or enzymatically removed (Cocking, 1960; Takebe et al., 1968; Power and Cocking, 1970). They can be isolated directly from different parts of *in vitro* or greenhouse-grown plants, with the young leaf mesophyll being the preferred tissue. Alternatively, protoplasts can be isolated from *in vitro* cultured tissues, with cell suspension cultures derived from friable embryogenic callus being the most common source. Plant growing conditions and plant age exert a major influence on yield and viability of the isolated protoplasts (Shepard and Totten, 1977; Kao and Michayluk, 1980). In addition, *in vitro* cultured material provides a more reliable source for consistent high-quality protoplasts if the cells can be maintained at maximum growth rates and utilized at an early log phase (Ishii, 1988). The viability of freshly isolated protoplasts is also influenced by enzyme composition and concentration, pH, and osmotic pressure used for isolation (Uchimiya and Murashige, 1974). Both electroporation and PEG-mediated delivery are efficacious, with the success depending on the species and tissue source used to produce protoplasts as well as the physical parameters used during delivery.

Electroporation methods have been developed for protoplasts from a wide range of both monocotyledonous and dicotyledonous plants (Hauptmann et al., 1987). Biological parameters known to impact electroporation efficiency include cell diameter and cell density (Rouan et al., 1991; Rao et al., 1995). Other important factors include strength and duration of the electric pulse (Fisk

and Dandekar, 2004), ion types, and their concentration in the buffer (Saunders et al., 1995; Niedz et al., 2003).

Chemical transformation of protoplasts commonly relies on the use of PEG (Krens et al., 1982; Paszkowski et al., 1984). PEG is an inert hydrophilic polymer of ethylene oxide and in combination with various salts facilitates the uptake of molecules into cells. Factors that influence the transfection efficiency of this process include the concentration and molecular weight of PEG, the pH of the transfection buffer, and the type and concentrations of the cations (Bittencourt et al., 1995; Maas and Werr, 1989).

Both electroporation and PEG-mediated delivery into protoplasts have been successfully used for transient and stable CRISPR/Cas9-mediated genome editing in multiple crop species (Feng et al., 2013; Xie and Yang, 2013; Gao et al., 2015; Bhowmik et al., 2018). The use of protoplasts for transient evaluation of editing reagents represents the latest application of this versatile single cell–based platform allowing the production of thousands of independent events and high-throughput screening (Dlugosz et al., 2016; Lin et al., 2018).

Isolated protoplasts are theoretically capable of dedifferentiation, re-entry into the cell cycle, and development into callus from which plants may be regenerated by somatic embryogenesis or organogenesis (Xu and Xue, 1999). The first report of plants regenerated from protoplasts was in *Nicotiana tabacum* (Takebe et al., 1971) followed by multiple other crops (Baset et al., 1991; Binding et al., 1978; Rhodes et al., 1988). The ability to regenerate plants has been demonstrated to be under genetic control in different species (Koornneef et al., 1987; Mórocz et al., 1990; Chupeau et al., 2013) and recalcitrance to regeneration has also been associated to physiological factors like the increase of intracellular level of polyamine (Papadakis et al., 2005) or oxidative stress induced during protoplast isolation and culture (Benson and Roubelakis-Angelakis, 1994; de Marco and Roubelakis-Angelakis, 1996; Papadakis et al., 2001). Improvement of regeneration efficiency has been achieved in some species by manipulation of the culture systems (Pati et al., 2005; Niedz, 2006; Kiełkowska and Adamus, 2012) and media manipulation (Grzebelus et al., 2012). However, regeneration can be efficiently obtained only in few genotypes (Eeckhaut et al., 2013; Hu et al., 1999) limiting the practical utility of protoplast-based systems for crop improvement (Table 1).

4 Delivery methods for genome editing reagents: delivery into intact tissues

Delivery of genome editing reagents to intact plant tissues has relied predominantly on particle bombardment and *Agrobacterium* with emphasis inexorably shifting toward the latter option. In addition, use of *Agrobacterium*-mediated delivery of viral-based replicons into leaf tissue of various dicot species

is also increasing the efficiency of CRISPR/Cas9 gene modification (Baltes et al., 2014; Čermák et al., 2015; Butler et al., 2016). Direct delivery of Cas9 and guide RNA (gRNA) ribonucleoprotein (RNP) complexes into rice zygotes has also been reported (Toda et al., 2019). Most recently, the use of nanoparticles is bringing greater attention back to physical delivery methods. Specific examples from within these broad topics are presented in the next section.

4.1 Particle bombardment

The biolistic method relies on the ability of heavy-metal carriers (e.g. tungsten or gold) coated with cargo biomolecules to penetrate plant cells when accelerated using high-pressure helium in a partial vacuum. Once inside the cell the cargo can elute from the microparticles and either integrate into the genome (stable expression) or remain as extrachromosomal material (transient expression). This transformation system can transform not only the nucleus but also plastid and mitochondria (Sanford, 2000). Furthermore, biolistics can be used with diverse cell types and delivers a high number of biomolecules (Altpeter et al., 2005). One additional advantage of this delivery system is that multiple genome editing components can be co-delivered on separate plasmids, simplifying vector design and assembly. The success of biolistic experiments depends on numerous parameters including acceleration of particles, selection of target tissues, size of microparticles, and ratio of DNA to particles. Disadvantages of this method include limited control over distribution and penetration of microparticles and potential cell lethality. In addition, chromosome damage and complex multicopy vector DNA integration reduce the proportion of usable genome-edited events (Svitashev et al., 2000; Svitashev et al., 2002; Liu et al., 2019). Biolistic delivery has been employed for genome modification application in numerous species including maize (Ainley et al., 2013; Svitashev et al., 2015, 2016; Shi et al., 2017), wheat (Liang et al., 2017; Hamada et al., 2018), soybean (Jacobs et al., 2015; Li et al., 2015), and rice (Sun et al., 2016).

4.2 Electroporation

Electroporation has also been used to deliver DNA directly through the cell wall, but with limited success. This method has been used to deliver a vector containing the hygromycin-resistance gene into maize immature embryos to recover stable transgenic events (D'Halluin et al., 1992). Recently, electroporation of wheat microspores resulted in both Ds-RED expression and CRISPR/Cas9-mediated mutagenesis of an endogenous gene (Bhowmik et al., 2018). Finally, electroporation of CRE recombinase protein has been used to facilitate site-specific recombination, excising GFP and

activating GUS expression in Arabidopsis suspension cells (Furuhata et al., 2019).

4.3 Agrobacterium

Since the early days of transformation in dicotyledonous species, *Agrobacterium* has been favored for its ability to transform an ever-increasing number of species and a range of plant tissues such as cotyledons (Nakajima et al., 2013), cotyledonary nodes (Paz et al., 2006), embryo axis (Krishnamurthy et al., 2000), stems (Moore et al., 1992), stem internodes (Maheshwari and Kovalchuk, 2016), apical meristems (Dutt et al., 2007), leaf tissue (De Block, 1988), and flower via floral-dip (Clough and Bent, 1998; Hu et al., 2019a). In contrast, for most cereals, the preferred explant is the immature embryo where the scutellar tissue is competent for both transformation and regeneration (Jones et al., 2009; Hiei et al., 2014).

Since the discovery of *Agrobacterium*-mediated plant transformation (Barton et al., 1983; Caplan et al., 1983), there has been continuous improvement in this system. Exploring different *Agrobacterium* strains led to the widespread use of such workhorse strains as LBA4404 (Ooms et al., 1981), AGL1 (Lazo et al., 1991), and the EHA strains 101 and 105 (Hood et al., 1993). Altering the expression of virulence genes (Hansen et al., 1994), or increasing the copy number of vir genes (Hiei et al., 1994; Ishida et al., 1996), resulted in further enhancements of T-DNA delivery and efficacy across an ever-increasing range of species. Such modifications to *Agrobacterium* have also helped broaden the range of genotypes accessible for genome modification (see Sardesai and Subramanyam, 2018, for review).

In its wild-type state, the virulence of *Agrobacterium* is managed through a large Ti plasmid which comprises both the T-DNA that is transferred to the host as well as virulence genes that provide the machinery to infect and deliver this T-DNA. Because of its large size (~200 kb), it is difficult to modify the Ti plasmid to reprogram the T-DNA cassette. This barrier led to the invention of a binary plasmid system in which the T-DNA component was separated from the virulence genes (Hoekema et al., 1983; Bevan et al., 1984). Further improving upon this design, Komari and colleagues introduced supplemental copies of several virulence genes in addition to the T-DNA plasmid to create what is known as the pSB1 super-binary vector (Komari et al., 1990), which has served as a system of choice for the transformation of numerous plant species (Hiei et al., 1994; Ishida et al., 1996).

Recently, Anand et al. (2018) described a ternary pVir system that simplified and improved upon pSB1 *Agrobacterium*-mediated transformation. The authors removed unnecessary sequences and split the supplemental virulence genes away from the T-DNA into a new accessory plasmid driven by a

smaller and higher copy pVS1 origin of replication. In addition, they corrected deficiencies of pSB1 including restoring a functional virC operon and a full length virD2 gene and identified novel combinations of supplemental vir genes that, when added to the accessory plasmid, further improved transformation. Finally, because pSB1 was known to spontaneously develop resistance to tetracycline, the authors utilized gentamicin as an improved selectable agent. The resulting accessory system (pVir) no longer conferred spontaneous TET resistance, was smaller, and was more virulent than pSB1 (Anand et al., 2018). These modifications simplified and improved stable plant-transformation among historically recalcitrant elite maize and sorghum varieties (Anand et al., 2018; Che et al., 2018), resulting in higher frequencies of transformation and single-copy integrations (Tables 2 and 3). Importantly, moving the vir genes to a separate plasmid significantly decreased complexity and further simplified the T-DNA vector assembly process.

For both dicots and monocots, early studies focused on random DNA integration. As methods for genome modification became more sophisticated and precise, various modes of gene targeting were developed using a diverse set of tools, including CRE and FLP recombinase-mediated site-specific integration in soybean (Srivastava and Ow, 2002; Li et al., 2009) or maize (Anand et al., 2019), and homology-dependent integration using either homing endonucleases such as I-SceI (Puchta et al., 1996) or I-CreI (Gao et al., 2010), zinc-finger nucleases (Wright et al., 2005; Shukla et al., 2009), TALENs (Li et al., 2012; Haun et al., 2014), or CRISPR/Cas9 (Liang et al., 2014; Svitashev et al., 2015; Malzahn et al., 2017).

Table 2 Comparison of transformation and single-copy frequencies in the Pioneer maize inbred PH2RT, using either the helper plasmid pSB1 or the pVir helper plasmid PHP71539

Construct	Transformation frequency (%)	Single copy, no Backbone (%)
pSB1	13.7	5.1
pVir pPHP71529	31.1	9.1

Table 3 Transformation and single-copy frequencies for four different sorghum varieties using the pVIR helper plasmid PHP71539

Cultivar	Transformation frequency (%)	Single copy, no Backbone (%)
Tx430	27	16
Malisor 84-7	7.5	3.6
Tegemeo	1.5	NA
Macia	1	NA

NA, Not available.

4.4 Viral-based replicons

Plant virus-based replicons have recently become an attractive supplement to *Agrobacterium*-mediated delivery. A key advantage of a viral replicon delivery system is the prolific accumulation of nucleic acids, making the gene-editing process potentially more efficient. Thus, a transient viral platform could provide an ideal screening tool to quickly assess gRNA design and to measure desired effects. If gRNA delivery using traditional methods is a limiting factor to obtain efficient genome editing in plants, the abundant virus replication systems could potentially provide high gRNA yields for efficient, quick, and systemic editing (Cody and Scholthof, 2019).

In the past, focus on viral replicons centered mainly on production of recombinant proteins in plants (Ibrahim et al., 2019). When first attempted, plant cells were infected with a full-genome copy of the plant virus, resulting in transgene instability, limited cargo size, and raising biosafety concerns. This led to the design of a second generation of viral-based vectors that retained the replication function, supported gene expression, but lacked coat proteins and movement (Gleba et al., 2004). Removal of the mobility function necessitated delivery of the viral replicon vector to the plant cell by *Agrobacterium* or particle bombardment (Marillonnet et al., 2004). Once in the plant cell, replicon amplification increases the available template DNA, resulting in increased HDR-based editing efficiency (Baltes et al., 2014). The choice of RNA- or DNA-based viruses affects both efficacy and versatility of the expression system. RNA-based viruses have small genomes facilitating cloning and multiplexing of gRNA, and don't integrate into the plant genome. However, their cargo capacity is limited, they are unsuitable for HDR edits and have been reported to have issues with their stability and genomic integrity (Seaberg et al., 2012).

Early reports of CRISPR/Cas9-mediated gene knockouts in plants used either the RNA Tobacco Rattle Virus (TRV) (Ali et al., 2015b) or a DNA geminivirus (Baltes et al., 2014; Ali et al., 2015a). TRV has been most widely used for genome editing vector designs because of its high level of accumulation and associated gene expression in a variety of hosts. The use of TRV-based vectors has been demonstrated in *Nicotiana ssp.*, *Petunia hybrida*, and Arabidopsis (Marton et al., 2010; Honig et al., 2015; Ali et al., 2015a, 2018). Other RNA viruses used for genome editing include Tobacco Mosaic Virus (TMV) in *N. benthamiana* (Cody et al., 2017), the Pea Early Browning Virus in Arabidopsis (Ali et al., 2018), and the Beet Necrotic Yellow Vein Virus in *N. benthamiana* (Jiang et al., 2019). The TMV-based vector was shown to transiently deliver high concentrations of sgRNAs in *N. benthamiana* to obtain ~70% insertion-deletion mutations, most of which were observed within 2–3 days after *Agrobacterium* infection (Cody et al., 2017). In this manner, the authors successfully demonstrated targeting of two *Argonaute 1* (*AGO1*) paralogs with one gRNA, as well as codelivery of

two adjacent gRNAs using a single construct. Recently, Barley Stripe Mosaic Virus has been reported to enable targeted genome editing in both wheat and maize (Hu et al., 2019b) expanding the use of RNA virus-derived vectors beyond model plants. DNA virus-derived vectors overcome the cargo limitations and instability associated with RNA virus-derived counterparts. DNA vectors derived from geminiviruses are most developed and implemented. The viruses in this family are characterized by a single-stranded circular DNA genome, which is converted to double-stranded DNA by plant DNA polymerases after amplification to high copy number via rolling circle replication (Hanley-Bowdoin et al., 1999). The Bean Yellow Dwarf Virus replicon system has been efficiently deployed for delivery of ZFNs, TALENs, and CRISPR/Cas9 reagents resulting in the production of calli and plants with precise DNA sequence changes (Baltes et al., 2014; Čermák et al., 2015). Similar vectors used for genome modification have been derived from other geminiviruses such as Wheat Dwarf Virus (Gil-Humanes et al., 2017; Wang et al., 2017) and Cabbage Leaf Curl Virus (Yin et al., 2015).

4.5 Nanoparticles

Nanoparticles have been explored for many years, with the earliest studies demonstrating successful delivery of either DNA (Torney et al., 2007) or proteins (Martin-Ortigosa et al., 2012) using gold-functionalized mesoporous silica delivered by particle bombardment into plant cells. Recently, the use of carbon nanotubules for delivery of siRNA (Demirer et al., 2019a) and DNA (Demirer et al., 2019b) into a range of plant species has been demonstrated for gene silencing and transgene expression, respectively. In the case of RNA delivery, the nanotubules appeared to provide protection against degradation, and for DNA they permit transient expression while precluding DNA integration into the plant genome. Interestingly, the chemistry used to pre-treat the carbon nanotubules may impact sub-cellular delivery. For example, for siRNA and DNA delivery, the carbon nanotubules were treated with polyethyleneimine which results in both nuclear and chloroplast localization. In contrast, treating with chitosan appeared to favor chloroplast delivery (Kwak et al., 2019). Irrespective of the final subcellular localization, both chemistries appeared to support transient gene expression while protecting the DNA against degradation. Efficacy of nanoparticle delivery has been demonstrated for CRISPR/Cas9-mediated editing in mammalian cells (Durfee et al., 2016). Based on the encouraging observations that nanotubule-delivered siRNA and DNA are transiently functional in plant cells, Wang et al. (2019) suggested that nanotubule delivery could potentially be developed for CRISPR/Cas9-mediated production of transgene-free genome edited plants, an outcome similar to that already demonstrated using particle delivery of RNPs (Svitashev et al., 2016).

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5 Alternatives to DNA delivery

In most genome editing experiments, the gRNA as well as the Cas9 and selectable marker genes, have been delivered into plant cells using either T-DNA (*Agrobacterium* infection) or plasmid DNA (particle bombardment). In both cases, the delivered DNA can integrate into the genome leading to various side effects such as gene disruption, plant mosaicism, and potential off-site cutting (Svitashev et al., 2015; Kanchiswamy, 2016).

Direct DNA-free genome editing can be accomplished through biolistic delivery of gRNA and Cas9 either in the form of *in vitro* transcribed RNA molecules (IVTs) or as *in vitro* assembled ribonucleoprotein (RNP) complexes. Guide RNA and Cas9 mRNA have been successfully delivered to wheat immature embryos resulting in approximately 3-fold lower frequency of targeted mutagenesis in comparison to conventional DNA-based genome editing methods (Zhang et al., 2016). Lower efficiency of this approach is likely related to incomplete IVTs, partial degradation of RNA molecules during gold particles preparation, and/or RNA degradation upon delivery to the plant cells (before RNP complexes are formed).

Delivery of Cas9 and gRNA in the form of RNP complexes not only mitigates many issues associated with DNA vectors but also has several additional advantages over RNA delivery. First, the preassembled complexes are considerably more stable and not subjected to the same level of degradation as RNA molecules. We have recently demonstrated that replacement of *in vitro* transcribed gRNA with chemically synthesized molecules further increased activity of RNPs resulting in a 2–4-fold increase in targeted mutagenesis (Svitashev et al., unpublished data). Second, particle bombardment allows simultaneous delivery of thousands of RNP molecules into a single cell resulting in highly efficient target site cleavage. Third, the complex is active immediately upon delivery, eliminating the time required for transcription (i.e. for DNA) and translation of Cas9 protein (both DNA and RNA). The last two considerations are especially important for gene-editing applications relying on homology-based repair of DSB using oligonucleotides as donor templates, which are subjected to rapid degradation by cellular nucleases.

Efficient genome editing using RNP was first demonstrated in cultured human cells using electroporation-mediated delivery (Kim et al., 2014; Lin et al., 2014). Later, plant protoplasts were successfully used for RNP delivery in a variety of plants such as tobacco, Arabidopsis, lettuce, rice, and Petunia (Woo et al., 2015; Subburaj et al., 2016; Murovec et al., 2018; Park et al., 2019). However, for most monocot species, including major crops such as maize, wheat, rice, barley, and sorghum, regeneration of plants from protoplasts remains either unattainable or inefficient (Davey et al., 2005).

Delivery of RNP complexes and efficient gene editing has been recently demonstrated in maize and wheat using particle bombardment of immature embryo cells (Svitashev et al., 2016; Liang et al., 2017). The regenerated plants contained specifically targeted gene mutations at frequencies comparable to conventional DNA-based delivery methods. Editing efficiency using this approach has allowed for recovery of completely transgene-free plants with mutated alleles at high frequencies without selection. This approach has also been used to edit the maize *Acetolactate Synthase (ALS)* gene conferring resistance to the herbicide chlorsulfuron (Svitashev et al., 2016).

Considering the current limitations in transformation and regeneration for many plant species (especially those that are asexually propagated), the ability to deliver RNP complexes significantly broadens options to modify plant genomes while eliminating unwanted transgene integrations. This provides new opportunities to advance agricultural breeding practices for any plant species amenable to biolistic delivery.

6 Morphogenic genes increase transformation efficiency and extend genotype range

Genes that control the organized spatiotemporal development of plant embryos, meristems, tissue, and organs (the process of morphogenesis) have been cloned and characterized since the late 1990s. In short order, genes that regulate embryo formation and meristem maintenance began to intrigue plant transformation researchers, as a steady stream of reports demonstrated that mis-expression could result in ectopic formation of embryo- and/or meristem-like structures, with early examples including genes such as *LEC1* (Lotan et al., 1998), *LEC2* (Stone et al., 2001), *ESR1* (Banno et al., 2001), *WUS* (Zuo et al., 2002), and *BBM* (Boutilier et al., 2002). Since these early reports in Arabidopsis, new observations using orthologous genes, or newly characterized genes such as *SERK1* (Pérez-Pascual et al., 2018), *AGL15* (Harding et al., 2003; Thakare et al., 2008; Yang et al., 2014), or *STM* (Elhiti et al., 2010) were reported to either enhance preexisting somatic embryogenesis in culture, or to elicit ectopic formation of somatic embryos or meristems from differentiated tissues (for review, see Gordon-Kamm et al., 2019). While such reports were exciting, these early observations relied on constitutive expression of these morphogenic genes, which typically prevented the regeneration of normal fertile plants. Thus, use of pleiotropic genes for improved transformation has relied on recombinase-mediated excision to facilitate recovery of normal, fertile T0 plants after using either hormone biosynthesis or morphogenic genes (Ebinuma et al., 1997; or Lowe et al., 2016, respectively).

More recently, such studies have been refined, with controlled expression of morphogenic genes being used to restrict growth stimulation, enabling the

subsequent recovery of transgenic plants in such diverse species as *Capsicum annuum* (Heidmann et al., 2011), *Arabidopsis thaliana* (Lutz et al., 2015; Wang et al., 2009), *Theobroma cacao* (Shires et al., 2017), *Nicotiana tabacum* (Kyo et al., 2018), *Populus tomentosa* (Deng et al., 2009), *Zea mays* (Lowe et al., 2016, 2018; Mookkan et al., 2017; Du et al., 2019), *Sorghum bicolor* (Lowe et al., 2016; Mookkan et al., 2017), and *Oryza sativa* ssp Indica (Lowe et al., 2016).

A third alternative for using morphogenic genes has been co-transformation with two *Agrobacterium* strains, one delivering a T-DNA for strong *Wus2* expression in one cell, and the second strain delivering a trait-containing T-DNA into a neighboring cell. Through either transient expression of *Wus2* (with no integration) and/or strong expression to produce WUS protein that can move to the trait gene-containing cells and stimulate embryogenesis, the trait cells form somatic embryos that regenerate without any integration of the *Wus2* T-DNA (Hoerster et al., 2020).

New morphogenic and/or growth-stimulating tools will continue to add new options to this toolbox. For example, Debernardi et al. (2020) recently report that delivery of a constitutively expressed fusion-protein composed of *Growth-Stimulating Factor4* (*Grf4*) and *Grf-Interacting Factor1* (*Gif1*) improve transformation in wheat and citrus, and naturally occurring miR396 down-regulation of *Grf4* transcript in the resultant T0 plants appears to mitigate pleiotropic problems. Similarly, *Grf5* has been used to increase transformation efficiency in sugar beet and maize (Kong et al., 2020).

In maize, the use of *Wus2* and *Bbm* has improved transformation frequency in recalcitrant inbreds by 10-fold or greater (Lowe et al., 2016), which has played an important role in rapid implementation of CRISPR/Cas9 genome editing applications at Corteva Agriscience (formerly DuPont Pioneer). In addition to simply increase the number of recovered transgenic events, there may be an equally important, although less obvious, benefit of using *Wus2/Bbm* for genome editing. Although WUS2 and BBM proteins don't directly interact with cell cycle components, cell division is stimulated (including replication during S-phase) and may be providing an HDR-conducive cellular environment. Likewise, it has been suggested that pleiotropic effects of Rep/RepA expression in plant cells harboring viral replicons may be favorable to DSB-initiated HDR-based genome editing (Baltes et al., 2014).

The regeneration bottleneck for many species can be alleviated using morphogenic regulators, facilitating genome editing. *Wus2* and *Bbm* have been used for transient CRISPR/Cas9-mediated genome editing through particle bombardment (Svitashev et al., 2015), for RNP-mediated editing (Svitashev et al., 2016), and for FLP-mediated site-specific recombination (Bett et al., 2019). This has facilitated a range of genome modifications, from simple mutagenesis to oligo-based edits and targeted gene insertion (Svitashev et al., 2015, 2016).

Additionally, they have been deployed to facilitate a wide range of CRISPR/Cas9-mediated changes in difficult maize inbreds (Chilcoat et al., 2017; Zhang et al., 2019). Similarly, *Wus2* in conjunction with other morphogenic genes has been demonstrated to be effective for genome editing in *N. benthamiana* (Maher et al., 2020).

Two examples in maize highlight the benefit of using *Wus2/Bbm* for genome editing using immature embryos as the target explant. The first focused on using CRISPR/Cas9-mediated excision of the endogenous *WAXY* allele in 11 commercially important Pioneer inbreds that were otherwise recalcitrant to transformation (Gao et al., 2020). Both Stiff-Stalk and Non-Stiff-Stalk inbreds, which hadn't been previously transformed, were chosen based on their genetics as parents in commercial hybrids. When these inbreds were transformed using *Wus2/Bbm* plus Cas9 and gRNA expression cassettes, transformation frequencies (based on numbers of regenerated T0 plants relative to the number of starting embryos) for the 11 inbreds ranged from approximately 1% to 50% (Fig. 1a). Successful *WAXY* deletions were recovered in all genotypes at frequencies ranging from 6% to 16% (Fig. 1b), relative to the number of T0 plants analyzed. These results illustrate two important points. First, the use of *Wus2/Bbm* permitted the recovery of transgenic events in all 11 inbreds, although a 50-fold range of transformation frequencies was observed. Second, the Cas9/gRNA-mediated deletions were observed to span a much narrower range of frequencies (from 6% to 16%) independent of the transformation rate. For example, PH25KM and PH1VST were at opposite ends of the transformation spectrum (49.7% and 1.7%) but had similar deletion frequencies of 11% and 12.3%, respectively. This illustrates that there was no correlation between transformation efficiency and edit frequency (Pearson's Correlation Coefficient, r = −0.21). Importantly, optimization of media

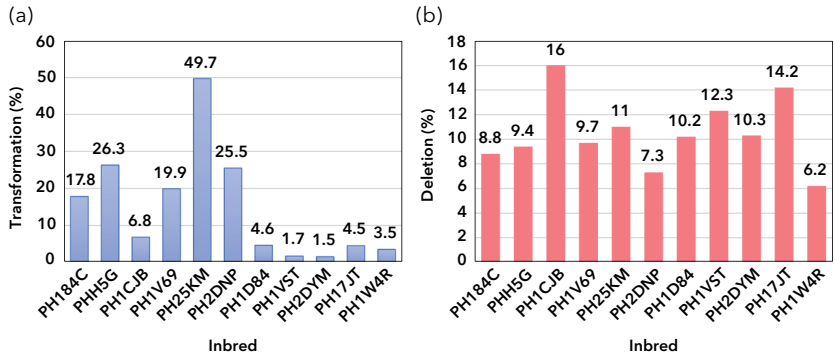

Figure 1 Transformation frequency (a) and *WAXY* deletion frequency (b) for plants recovered after delivery of a Ubiquitin-driven Cas9 and two U6-driven gRNAs, Axig1::WUS2, and PLTP::BBM (Gao et al., 2020).

composition and selection (while still using the same *Wus2/Bbm* expression cassettes) has continued to improve transformation frequencies for inbred PH1VST from 1.7% to greater than 70% (Gordon-Kamm et al., unpublished result).

The second example used CRISPR/Cas9 and *Wus2/Bbm* along with a donor template to replace the endogenous ARGOS8 promoter with a constitutive maize promoter (GOS2) in a Pioneer maize inbred. As predicted from earlier transgenic studies (Habben et al., 2014; Shi et al., 2015), precise exchange of the promoter resulted in enhanced grain yield under drought conditions (Shi et al., 2017). As with the deletion of an endogenous gene in 11 inbreds, this cisgenic edit was successful because of the optimization and combination of two complementary technologies (CRISPR/Cas9-mediated editing and *Wus2/Bbm*-assisted transformation).

7 Morphogenic genes permit transformation in non-traditional explants

For many years, the requirement of using immature embryos has rendered maize transformation unattainable for most academic labs (Altpeter et al., 2016), because maintaining a consistent supply of immature embryos is both expensive and labor intensive (Que et al., 2014). Recently a viable alternative has been developed, since Lowe et al. (2016) demonstrated that *Agrobacterium*-mediated delivery of constitutively-expressed *Wus2* and *Bbm* allows transformation of both mature embryo slices and seedling-derived leaf segments to efficiently produce fertile transgenic events.

These alternative explants can also be used for genome editing. For example, a transgenic Pioneer Stiff-Stalk inbred that contained inducible *Wus2/Bbm* expression cassettes has been generated. When *Wus2* and *Bbm* were induced by the addition of ethametsulfuron, somatic embryogenesis was stimulated in leaf tissue. Using this inducible *Wus2/Bbm* germplasm as the starting point for a new experiment, seedling-derived leaf tissue was then used as the target explant for particle bombardment. To further enhance morphogenesis (beyond that provided by inducible expression), plasmids containing constitutive *Wus2* and *Bbm* expression cassettes were co-delivered with Cas9 and gRNA, as well as the template DNA (containing a *NPTII* expression cassette). It should be noted that due to high levels of *Wus2* and *Bbm* expression (inducible plus constitutive), selection using *NPTII* and G418 became less efficient, resulting in escape (wild type) plants being regenerated. However, three integration events were recovered from a total of 142 T0 plants that were regenerated and analyzed (Table 4). This result clearly shows that when *Wus2/Bbm* are used to aid the process, CRISPR/Cas9-mediated genome editing can be accomplished via leaf transformation.

Table 4 Recovery of G418-resistant T0 plants using four different levels of antibiotic selection. After PCR analysis of the total number of T0 plants for each treatment, the number of Homology-Dependent Repair (HDR) events was determined first by a positive PCR result across both the upstream and downstream flanking recombination junctions (No. HDR), and subsequently using long-PCR across the entire insertion (No. Perfect HDR)

No. seedlings	G418 (mg/l)	No. T0 Plants	No. HDR (PCR)	No. Perfect HDR
8	150	46	1	0
8	200	34	0	0
9	250	38	**4**	**3**
9	300	24	0	0

A recent variation on using morphogenic genes to assist in gene editing has also been reported by Maher et al. (2020). In this study, the authors introduced a geminiviral-based replicon directly into the wounded leaf axil after removal of the petiole in *N. benthamiana*. Replicons containing both *Wus2* and *Ipt* (isopentenyl transferase from *Agrobacterium*) expression cassettes stimulated growth of new shoots from the treated axils. The introduction of combinations of *Wus2*, *STM* (*Shootmeristemless*) and *Ipt* into the axillary region also stimulated the formation of transgenic meristem-like structures in tomato, and transgenic shoots arose from aseptically grown potato and grape cuttings. Furthermore, delivery of Cas9 and a gRNA targeting the phytoene desaturase gene (*PDS*) along with *Wus2* and *Ipt*, generated uniformly white shoots characteristic of *PDS* mutagenesis, which in one case was transmitted to progeny. While still early in development, this study clearly shows the potential of combining morphogenic genes with CRISPR/Cas9 for further development of *in planta* genome editing. This is particularly encouraging, considering that axillary meristems have been an attractive transformation target (even when morphogenic genes are not used) for such diverse species as pear (Matsuda et al., 2005), grape (Fujita et al., 2009), crambe (Qi et al., 2014), soybean (Olhoft et al., 2007), and sugarcane (Mayavan et al., 2015).

8 Future trends

Progress in plant genome editing can currently only be described as explosive, fueled by advances in many contributing areas. New CRISPR/Cas nucleases such as Cas12-based proteins with different PAM solutions are greatly increasing the potential flexibility of the system (e.g. Fonfara et al., 2014). Cas12-based type V and the Cas13-based type VI systems may also add to this versatility (Murugan et al., 2017). Recent development of the prime editing system, which relies on a Cas9 fusion with reverse transcriptase and a priming gRNA appears to be a potentially versatile and efficient genome modification system (Anzalone et al., 2019). In addition to genome modification *per se*, both

type II and type I systems continue to be optimized as transcriptional activators or repressors (Cheng et al., 2013; Gilbert et al., 2013; Farzadfard et al., 2013; Rath et al., 2015; Pickar-Oliver et al., 2019).

Robust transformation/regeneration methods remain a major bottleneck in genome editing processes, not only for agronomic crops in general (Altpeter et al., 2016), but also for forestry (Chang et al., 2018), temperate fruit/nut crops (Song et al., 2019), and tropical fruits (Gómez-Lim and Litz, 2004). Difficulties in genome editing applications can also be exacerbated in crops with added genomic complexity, whether through self-incompatibility and genomic heterozygosity (e.g., in pineapple, grape and apple), or due to polyploidy as in wheat, canola, and as an extreme example, sugarcane (Botella, 2019). Rapid advances in genomics such as Next-Generation Sequencing and BioNano (Mak et al., 2016) continue to help resolve the complexity of the genomes, making targeted genome modifications more feasible.

Across species, *Agrobacterium* and physical delivery via particle bombardment are the current mainstays for crop genome editing, depending on the cargo and intended outcome. *Agrobacterium* is widely used for crops in which stably integrated T-DNA can later be segregated away. However, in cases of asexually or vegetatively propagated species where no transgene introduction is desired, particle gun delivery of RNPs has been successfully used in diverse species (Metje-Sprink et al., 2019). A resurgent interest in nanoparticle delivery may promise yet another rapid change in this landscape, permitting transient delivery of CRISPR/Cas9 components to recover non-transgenic genome-edited plants (Wang et al., 2019). Using *Agrobacterium*-mediated co-transformation for supplying morphogenic gene activity adds another tool for genome modification without integration of genes such as *Wus2* (Hoerster et al., 2020).

Changes in plant transformation and regeneration methods are also accelerating. For an increasing number of historically recalcitrant dicots, the successful use of the mature embryonic axis in soybean has been emulated in other crops, for example in grape (Dutt et al., 2007), pigeon pea (Rao et al., 2008), Camelia (Sitther et al., 2018), chickpea (Das Bhowmik et al., 2019), and cowpea (Che et al., 2019). For crops where the apical dome remains difficult to work with, the use of morphogenic genes is receiving increased attention and will continue to broaden the spectrum of plants that can successfully be edited. Using hormone biosynthesis or morphogenic genes to facilitate transformation has been reported by many groups (see Gordon-Kamm et al., 2019 for review). Recently, morphogenic genes have been seeing a resurgence of interest, and in addition to their use in producing transgenic plants (Lowe et al., 2016; Mookkan et al., 2017; Lowe et al., 2018), they are facilitating genome editing in both monocots (Svitashev et al., 2015, 2016; Zhang et al., 2019) and dicots (Maher et al., 2020).

In addition, the use of morphogenic genes can contribute to extending transformation/editing methods to an increasing range of difficult plant species. However, looking at how different species respond to morphogenic genes such as *Wus2*, *Kn1*, and *Bbm* (Gordon-Kamm et al., 2019) it becomes clear that there will probably be no universal single solution across crops. Instead, when trying to extend the use of morphogenic genes to a new crop, we would advise screening a handful of genes proven most promising in the literature (i.e. *Wus2*, *Bbm*, *Kn1*/*STM*, and *Ipt*), paying attention to select the most closely related ortholog possible (for plant genes), while also using optimal codon usage and promoter choices for the crop of interest. Once the optimal T-DNA has been constructed containing morphogenic gene(s) along with both a fluorescent and a selectable marker gene, this T-DNA can be used to optimize *Agrobacterium* infection and co-cultivation parameters (OD, temperature, media components, and duration) based on relative transient fluorescence levels, and then finally used to test different selection levels and timing.

As an alternative to reduce the complexity that accompanies *in vitro* organogenic or embryogenic culture methods, combining *in planta Agrobacterium*-mediated T-DNA delivery with morphogenic gene growth stimulation has recently been demonstrated for successful genome editing in tobacco (Maher et al., 2020) and has been proposed for more difficult species such as forestry crops (Nagle et al., 2019). Expanding on the concept of *in planta* transformation (or editing), combining this strategy with a non-integrating *Wus2* method (Hoerster et al., 2020) may prove crucial to *in planta* genome modification in long-life-cycle or asexually propagated crops. Continued development of such *in planta* strategies have great potential for simplifying and accelerating genome editing and may prove useful to a wide range of crops.

Plant genome editing is complex, requiring the effective coordination of interdependent processes and mechanisms such as delivery method, cargo, target explant, and, if appropriate, the use of morphogenic genes to aid in the recovery of fertile plants. Recent progress across all technologies is providing exciting new options. How these options fit together in a successful protocol ultimately depends on the requirements of the crop species.

9 Where to look for further information

CRISPR/Cas9-mediated genome editing in plants has become a widely publicized field, with far-ranging potential for the future of agriculture. However, genome editing *per se* represents only the tip of the technological iceberg. Below the surface, gene editing relies on the fundamentals and techniques of plant transformation, which is true for all plants. The component methods that have been combined to realize successful maize transformation thus

represent the often-underappreciated foundation of current successes. These component areas include establishing reliable culture systems, optimizing DNA delivery, selecting transgenic events, and finally regenerating plants that contain the desired edits. Some of the seminal supporting technologies have been developed across a range of species but nonetheless have ended up contributing to the current state of maize transformation. Understanding these foundational technologies can be very helpful when trying to comprehend how the field has developed and its current status.

For further reading, see the following articles on using *Agrobacterium* for DNA delivery (Gelvin, 2017), old and new alternatives for delivering DNA (Demirer and Landry, 2017), development of chemical and/or visible markers for selection (or screening) of transgenic events (Rosellini, 2012), technological advances that have contributed to maize transformation (Yadava et al., 2017), utilizing CRISPR/Cas9 in maize (Chilcoat et al., 2017), and development of commercial agronomic traits in maize (Que et al., 2014).

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