BURLEIGH DODDS SERIES IN AGRICULTURAL SCIENCE

Viruses as vectors for the delivery of gene-editing reagents

Evan E. Ellison, James C. Chamness and Daniel F. Voytas,
University of Minnesota, USA

burleigh dodds
SCIENCE PUBLISHING

Viruses as vectors for the delivery of gene-editing reagents

Evan E. Ellison, James C. Chamness and Daniel F. Voytas, University of Minnesota, USA

1 Introduction

Viruses are natural vectors for nucleic acids, and both DNA and RNA plant viruses have been engineered to extend or replace conventional vectors for delivery of gene-editing reagents. This chapter reviews aspects of viral biology essential for engineering vectors, highlight landmark studies using viruses to overcome traditional limitations in gene editing, and outline important considerations for the use of viral vectors in new systems or for new targets.

Motivated by fundamental differences in both their infection modes and utility as vectors, DNA and RNA viruses are treated separately. DNA viruses are assessed as replicon vectors for efficient gene editing through homology-directed repair (HDR). The chapter then reviews RNA viruses as mobile vectors for gene-editing reagent delivery. The chapter includes key case studies as well as future trends in research.

http://dx.doi.org/10.19103/AS.2020.0082.28

Chapter taken from: Willmann, M. R. (ed.), *Genome editing for precision crop breeding*,
Burleigh Dodds Science Publishing, Cambridge, UK, 2021, (ISBN: 978 1 78676 447 8; www.bdspublishing.com)

2 DNA viruses: replicon vectors for efficient gene editing through homology-directed repair (HDR)

Since the first reports over 25 years ago, various plant DNA viruses have been sequenced, cloned, and modified to introduce exogenous sequences into plant cells. More recently, delivery of sequences encoding components for the major classes of site-specific nucleases (SSNs) has demonstrated the utility of DNA viral vectors for gene editing in a broad range of host species. Among both foundational studies in viral engineering and contemporary studies in gene editing, the vast majority describe work with *Geminiviridae*: single-stranded DNA (ssDNA) viruses that constitute the largest known family of DNA viruses in plants[1].

Engineered geminiviral clones may be delivered to plant cells by biolistics or, more commonly, by agroinfection. While some infectious clones retain mobility within the delivered tissue (and potentially to natural insect vectors)[2], most reports describe clones deconstructed into non-infectious, geminiviral replicons (GVRs): self-replicating, cell-autonomous DNA vectors, which transiently drive their genetic payload to high-copy number and overexpression in host cells, regardless of whether the source vectors integrate into the nuclear genome. Investigation of GVRs as overexpression vectors predates the widespread availability of gene-editing tools, but their utility for editing was quickly realized. In particular, GVRs offer key advantages for precision-editing strategies requiring homology-directed repair (HDR) from a donor template molecule to incorporate specific modifications into the genome.

When used to deliver SSNs and donor templates, GVRs dramatically increase the frequency of HDR in host cells[3]. Intuitively, stronger expression of SSNs may lead to more efficient double-stranded break induction. GVRs also increase the local concentration of donor template molecules available for recombination, and pleiotropic effects of viral replication proteins render the cell environment more amenable to HDR. In the following sections, we describe the basic structure and function of GVRs, consider vector design choices influencing their activity within plant cells, and review case studies using GVRs to drive efficient HDR in several species of both dicots and monocots.

3 Geminiviral replicons (GVRs): deconstructed geminiviruses that serve as replicating vectors in plants

3.1 Geminiviruses

Geminiviridae are a family of circular, ssDNA viruses, vectored by insects and infecting diverse host plants. The name derives from the twin-icosahedral shape of the encapsulated virion. The viral ssDNA genome is converted by host cell polymerases into a double-stranded DNA (dsDNA) intermediate,

Published by Burleigh Dodds Science Publishing Limited, 2021.

which serves as a template for rolling-circle replication and for transcription of viral gene products[1]. With small genomes, encoding just a few open-reading frames (ORFs), geminiviruses require host cell machinery to complete their life cycle; this reliance underscores complex interactions between viral and host factors[4], which are important to consider for the function of geminiviral vectors in plant cells. Geminiviruses encompass at least nine distinct taxa. Historically demarcated by host range or by variations in genome structure[1], more complex phylogenetic relationships are suggested due to viruses identified through high-throughput metagenomics[5,6], or isolates that challenge traditional structure-based classifications[7].

Despite the increasing availability of novel sequence isolates, the best-studied taxa remain *Begomoviruses* and *Mastreviruses*. The attention paid to these genera can be attributed to their status as significant pathogens of agricultural crops, including cereals, beans, cassava, and tomato. Most studies of the molecular biology of geminiviruses, and of their use as replicon vectors, have focused on three species of mastreviruses: *Maize Streak Virus* (MSV) and *Wheat Dwarf Virus* (WDV), infecting monocots (as do most mastreviruses), and *Bean Yellow Dwarf Virus* (BeYDV), from an outgroup infecting dicots. Begomoviruses may have monopartite or bipartite genomes; mastreviruses, with rare exceptions,[8] are monopartite. As nearly all GVRs used for gene editing are derived from WDV and BeYDV, this chapter focuses on mastreviruses.

3.2 Mastrevirus genome structure, function, and replication

The canonical mastrevirus genome is 2.6–2.8kb[1] (Fig. 1a). Two structural sequences termed the short (SIR) and long (LIR) intergenic regions separate reading frames in both orientations of the dsDNA intermediate, on the virion-sense (v-sense; +) and complementary-sense (c-sense; -) strands. A c-sense DNA primer molecule within the SIR, unique to mastreviruses, is thought to function as the origin for the first stage of viral replication: conversion of ssDNA to dsDNA by synthesis of the complementary strand. The origin for rolling-circle replication, conserved across nearly all geminiviruses, is a nonanucleotide stem loop sequence within the LIR that is nicked by the replication protein, Rep[9]. The LIR and SIR function as a bidirectional promoter and terminator, respectively, for the transcripts from both strands.

On the c-sense strand, alternative splicing of a 3′ intron gives rise to two protein-coding transcripts with overlapping reading frames: the unspliced 5′ transcript (RepA) and spliced transcript (Rep). Despite sharing much of their N-terminal sequence, studies in both monocot- and dicot-infecting mastrevirus species implicate these proteins in distinct and conserved functional roles. C-terminal deletion and splice-site mutants indicate the spliced transcript and minor product, Rep, is both necessary and sufficient for replication in host

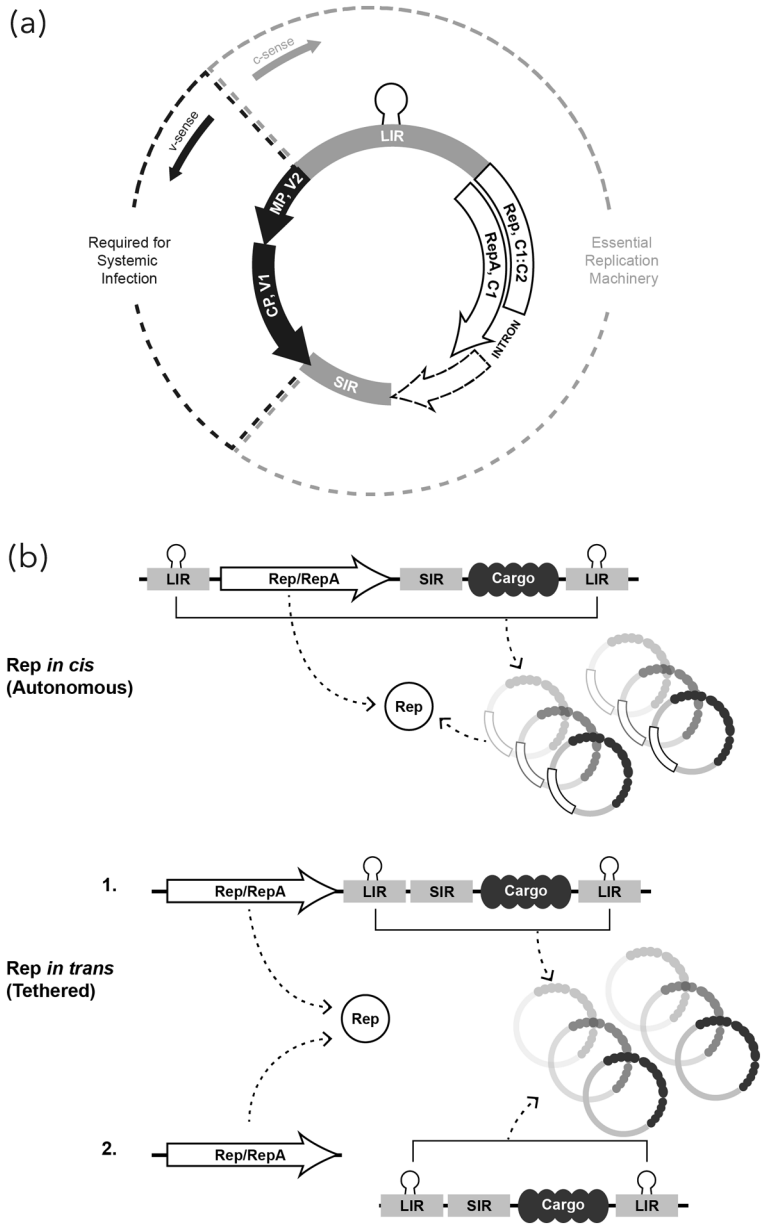

Figure 1 Structure and delivery of GVRs. (a) The dsDNA genome of a canonical mastrevirus. The double-stranded origin (DSO) for rolling circle replication is a nonanucleotide stem loop sequence, TAATATTAC, within the long intergenic region (LIR; ~0.4 kb). LIR-promoter activity drives transcription of the v-sense gene products (MP, ~0.3 kb; CP, ~0.8 kb) and c-sense gene products (Rep, ~1.1 kb; RepA, ~0.8 kb). The short intergenic region (SIR, ~0.2 kb) terminates transcription of both sets of transcripts. (b) Geminiviral

cells[10–12]. While not strictly required for replication, the unspliced transcript and major product, RepA, likely contributes to regulation of replication through interactions with both host and viral factors. First, RepA interacts with the host cell retinoblastoma-related protein, a highly conserved regulator of the cell cycle. By driving mitotically inactive somatic cells into a pseudo S-phase, mastreviruses upregulate the expression of host factors required for viral replication[4]. Second, RepA appears to be an obligate trans-activator for transcription of v-sense gene products. By supporting production of the capsid protein to encapsulate newly synthesized genomes (thus sequestering them from further replication), RepA may contribute to the downregulation of genome replication in live viruses[13]. Additional interactions between Rep and RepA, and their mechanistic roles in replication, have been the subject of considerable study, but are beyond the scope of this review. For an in-depth treatment of geminiviral replication, see Gutierrez[9,14,15].

On the v-sense strand, sequential reading frames encode the viral movement protein (MP) and capsid or coat protein (CP). These proteins are critical for infection. In addition to directing nuclear import of the virion to begin the replication cycle, the virion capsid is required for insect transmission and efficient systemic movement. The movement protein, which localizes to the cell periphery, mediates interactions between plasmodesmata and the virion capsid to facilitate movement among plant host cells[13]. In one classic example of maize agroinoculation with MSV clones, deletions of the CP and MP, or CP alone, abolished systemic movement, insect transmission, and typical disease symptoms versus a wild-type control. Cell-autonomous replication of these deletion vectors, however, was still detectable[2].

3.3 Deconstructing geminiviruses into replicon vectors

Experiments with mutants to elucidate wild-type gene function were soon followed by reports describing the addition of exogenous sequences, typically by replacing the v-sense gene products with reporter or marker gene cassettes. By maintaining components essential for viral replication (the LIR, SIR, and replication proteins) and eliminating components required for transmission

Figure 1 (*Continued*)
replicons (GVRs) can be delivered to cells in different architectures. When Rep is in its native position *in cis* to the replicon, driven by c-sense promoter activity of the LIR, it can be expressed from both the source vector and the high-copy replicon (hundreds to thousands of dsDNA intermediates per cell), creating a positive feedback loop for replication. When placed *in trans* to the linear LSL sector of the source vector, continued Rep expression depends upon persistent expression (i.e. from stable integration) of the source vector.

and infection (MP, CP), these clones function as cell-autonomous replicons. To date, replicons have been derived from mastreviruses including WDV[16,17], MSV[18,19], and BeYDV[20,21]. Although not a focus of this review, it should be noted that replicons have also been derived from begomoviruses including *Tomato Golden Mosaic Virus* (TGMV)[22,23], *Bean Dwarf Mosaic Virus* (BDMV)[24], *Tomato Leaf Curl Virus* (ToLCV)[25,26], and *Cabbage Leaf Curl Virus* (CaLCuV)[3]. In the context of plant viral expression vectors, replicons represent a transition from a 'full virus' strategy to a 'deconstructed virus' strategy[27], which has become the archetype for DNA viral expression vectors.

In moving away from a wild-type viral architecture, synthetic clones open up new design possibilities (Fig. 1b). First, natural mastrevirus genomes (~2.7 kb) are tightly size-constrained by the need to fit within the capsid, and for the virions to traverse plasmodesmata for systemic movement within the host. Removal of the MP and CP sequences alone (~1 kb) reduces the size of the replicon, but removal of requirements for encapsulation and cell-cell movement effectively shifts the upper bound for replicon size to the limit imposed by processive replication. This has the effect of greatly increasing vector 'payload' capacity versus infectious, full-virus clones. While no studies have systematically explored an upper limit to replicon size, evidence suggests that the efficiency of replication (and thus copy number) decreases with the replicon size[28]. In our own group, we have transformed functional BeYDV and WDV replicon vectors of approximately 15 kb. Finally, even independent of encapsulation, the size of plasmodesmata exerts a significant selective pressure on viral genome size, such that cell-cell mobility of larger plasmids is compromised[24].

Another design choice in the 'deconstructed' strategy is the placement of the replication proteins. In the native virus, both ORFs are driven by the c-sense promoter of the LIR, and so are expressed *in cis* to the replicon. However, these proteins can also be supplied *in trans*, by placing the ORFs under a heterologous promoter on the source vector or a second vector. This separation provides additional degrees of freedom for control over replicon activity, by 'tethering' expression of the replication protein to the source vector. For example, replication may be constrained by a chemically inducible promoter,[21] or by obligate co-delivery of two vectors.[3] Finally, as noted earlier, only Rep is strictly required for replication. While some synthetic clones purposed solely for overexpression eschew RepA,[21] the pleiotropic benefits for replication and HDR in somatic cells conferred by RepA have motivated its inclusion in all reports of gene-editing vectors to date.

3.4 Delivery and expression of replicons in plant cells

Replication-competent geminiviral clones can be delivered into plant cells as plasmids or as linear dsDNA fragments by traditional means of transformation:

PEG-treated protoplasts[29], biolistics[20], or agroinfection. In early reports describing agroinfection of begomovirus[30] and mastrevirus[31,32] clones, source vectors included full tandem repeats of the viral genome. Such repeats might enable escape and replication of a unit-length viral genome from the source vector by either of two, non-exclusive mechanisms: homologous recombination between repeats or by a replicative intermediate copied from the dsDNA source vector. It was later shown that duplication of just the v-sense ORI within the LIR is sufficient, implicating a rolling-circle replication mediated 'replicational release' mechanism[33] for viral escape. Subsequent reports largely follow a LIR-SIR-LIR (LSL) architecture for delivery of viral genomes from a dsDNA source vector. A benefit of this architecture and delivery mechanism is that the population of replicating viral genomes is both homogenous and predictable, consisting of the sequence between the origins within the duplicated LIRs. By contrast, circular viral replicons produced by homologous recombination, while observed, are far more likely to be heterogenous populations. Importantly, recombination of T-DNA source vectors may be stimulated by Rep-mediated viral replication, whether due to the availability of replicated sequences, or to potential crosstalk between replication and recombination machinery[34].

Upon delivery to plant cells, replicon copy number and cargo expression typically peak within a few days to a week after infection[3,35]. While this transient activity does not strictly require stable integration of the source vector (especially for autonomous replicons: see Fig. 1b), integration can lead to persistent replicon activity over time through mitotic and meiotic cell lineages. Studies have detected circular and high-copy replicons in the somatic tissues of T_0 rice and tomato plants regenerated through tissue culture[28,36], and there is evidence for persistence of a BeYDV replicon among the transgenic progeny of *Arabidopsis* lines as far as the T_3 generation[37].

4 Replicon vectors for efficient homology-directed repair

Precise gene targeting (GT), encompassing site-specific sequence alterations and targeted insertions, is typically achieved by HDR with a donor template molecule. Since induction of double-stranded breaks (DSBs) greatly increases the frequency of HDR in plant cells[38], SSNs including zinc-finger nucleases (ZFNs)[39], TAL-effector nucleases (TALENs)[40], and CRISPR/Cas nucleases have been used to stimulate HDR and recover stable GT events. However, the most common outcome from SSN-mediated DSB induction is mutagenesis, whereas GT events occur typically an order of magnitude less frequently. This is attributed to the predominance of alternative DNA repair pathways, namely microhomology-mediated end joining and non-homologous end joining, and to the additional requirement for HDR that DSB induction be coordinated with availability of the donor molecule.

GVRs contribute to higher frequencies of HDR in several ways. Perhaps the most significant is by greatly increasing availability of the donor molecule. With traditional T-DNA vectors, at most several copies of the donor are delivered to the cell. Biolistics can deliver a greater amount of donor, but the likelihood of multi-copy, random integration makes this approach less appealing for applications in breeding where segregation of integrated reagents is likely required. GVRs can be delivered to cells on T-DNA vectors, but increase the copy number of the donor molecule to hundreds or thousands within a single cell nucleus. Additionally, the activity of the replication proteins contributes to a more favorable cell environment for HDR. The prevalence of DNA repair pathways is highly influenced by the cell state, and HDR is particularly low in the mitotically inactive somatic cells which are primary targets for regeneration in many species. Even in the absence of DSB induction or a synthetic repair template, geminiviral infection was observed to enhance somatic HDR in a transgenic reporter line of *Arabidopsis*[41].

Besides the known influence of RepA on cell state, the replication proteins may also have evolved a role to promote recombination, due to an evolutionary selective pressure to generate new viral variants. For example, Rep and/or RepA may mediate recombination by their affinity for host factors. This could take the form of crosstalk between replication and recombination machinery[34], or even of more direct recruitment: one report from the begomovirus, *Mungbean yellow mosaic virus* (MYMIV), identified a protein-protein interaction between the MYMIV Rep and *Arabidopsis* RAD51, a highly conserved protein mediating homologous recombination in eukaryotes. While no such direct interactions have been described for mastreviruses, it is conceivable that analogous links between viral replication proteins and host recombination factors contribute to increasing the recombination potential of sequences carried on GVRs.

Regardless of mechanism, GVR vectors typically increase HDR frequencies by an order of magnitude. In the following case studies, we will highlight notable results and conclusions from reports establishing GVR-based editing strategies in dicots and monocots.

5 Case studies: GVR-based editing strategies in dicots and monocots

5.1 Baltes et al. (2014): DNA replicons for plant genome engineering[3]

Baltes et al. (2014) published the first report demonstrating the utility of GVRs for HDR. They used a previously established assay, leveraging a broken *nptII::gus* fusion gene present in a transgenic tobacco reporter line[42]. A ZFN pair targeting the middle of the broken fusion gene stimulates DSB induction, and templated repair completes the 5′ *nptII* and 3′ *gus* sequences, restoring the

function of both markers. Positive GUS staining in agroinfiltrated leaves from the reporter line thus indicates HDR, and the density of positive cells within sectors of an infiltrated leaf provides a proxy for gene-targeting frequency. Using this assay, Baltes and colleagues found that the GVR system outperformed the standard T-DNA by as much as two orders of magnitude. They also tested what specific benefit might be attributed to enhanced SSN expression (putatively, via greater DSB induction), or to replication of the repair template, and found that gene-targeting frequencies were boosted only when the repair template was replicated. Removing the ZFNs entirely attenuated GUS staining, but placement of the ZFN pair inside or outside the LSL segment of the same T-DNA made no significant difference. Taken together, these results suggest DSB induction is essential but not rate-limiting to higher gene-targeting frequencies versus repair template copy number. Next, possible contributions from pleiotropic activity of the replication proteins were assessed. The gene-targeting frequency in a non-replicating T-DNA control was significantly increased by co-delivery of a plasmid expressing Rep and RepA, consistent with a role for these proteins in enhancing HDR independent from GVR replication. Finally, using kanamycin selection to identify cells in which the *nptII* marker was repaired through HDR, Baltes and colleagues were able to regenerate tobacco plants with sequence-confirmed HDR events, demonstrating the utility of the GVR-based approach to generate heritable events.

5.2 Cermak et al.[26]: High-frequency, precision modification of the tomato genome

Cermak et al.[26] used a similar strategy to recover edited tomato plants, targeting knock-in of a selectable marker. Unlike Baltes et al., however, they targeted an endogenous locus: *ANT1*, a transcription factor controlling anthocyanin accumulation. The purple pigmentation arising from *ANT1* overexpression provides a phenotype that can be scored non-destructively, unlike GUS, and is thus useful for selection in vivo. To create an HDR reporter, Cermak and colleagues targeted TALENs or CRISPR/Cas9 reagents to the *ANT1* promoter. Their repair template contained, in sequence, a *nptII* cassette, and an additional strong promoter to drive overexpression of the native *ANT1* gene. Vectors were delivered to tomato cotyledons by agroinfection. The fraction of cotyledons showing purple spots, normalized for transformation efficiency, provided an estimate for gene-targeting efficiency. The best treatment among their experiments was a single-vector GVR with Cas9 and sgRNA, which achieved a normalized gene-targeting frequency of 11.66%. This was an order of magnitude better than the conventional T-DNA treatment at 1.27%. Importantly, these experiments were carried out using kanamycin selection in the growth media; when cultured on non-selective media, no purple spots were observed,

even with the best GVR treatment. While kanamycin was used in this initial phase of tissue culture to recover growing purple calli, visual selection alone proved sufficient to then regenerate plants with gene-targeting events; over two dozen plants were regenerated from several purple calli in the absence of kanamycin. Subsequent molecular analyses revealed events including perfect repair at both junctions, and one-sided targeting events that still gave rise to the phenotype. Most surprisingly, both PCR and Southern assays failed to detect the presence of the T-DNA or extrachromosomal replicon among any of the regenerated plants. This suggested that transient expression of the gene-editing reagents was sufficient, and nuclear integration was not required, to recover events. This is a notable result for some breeding systems where transgenesis may be undesirable, either for regulatory concerns, or due to difficulty in segregating integrated reagents from the desired edit (e.g. in clonally propagated species). Finally, Cermak and colleagues showed their gene-targeting events were heritable. T_1 progeny scored visually and genotyped by PCR approximated Mendelian expectation. A fitness penalty incurred from anthocyanin overproduction might explain the slight segregation bias against the edited allele, and might also have contributed to their failure to recover purple growths from transformed cotyledons in the absence of kanamycin selection.

5.3 Dahan-Meier et al.[36]: Efficient in planta *gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system*

Both of the previous studies used HDR to activate chemical and phenotypic selectable markers, a requirement which limits utility in many breeding applications. Dahan-Meier et al.[36] developed a new strategy, demonstrating efficient and heritable gene targeting for a 'neutral' trait in tomato. Instead of selecting for their targeted knock-in, they created transgenic plants that carried a T-DNA with the editing reagents, combining an *in planta* approach to gene targeting[43] with a GVR vector. Their target was a mutant allele of the *CRTISO* locus, which encodes an enzyme in the carotenoid biosynthesis pathway. Whereas wild-type *CRTISO* plants have red fruits, their target line featured a 281 bp deletion in *CRTISO*. This recessive, loss-of-function mutation gives rise to orange fruits in homozygotes, which can be restored to red fruit through HDR-mediated correction of a single allele. While providing a scorable phenotype, the fruit color trait is only visible in mature plants; this contrasts to the anthocyanin marker used in Cermak et al., which permitted enrichment of events during the primary tissue culture step. The experimental T-DNA contained a *nptII* marker for transgenic selection, Cas9 and a sgRNA targeting the mutant allele, and a BeYDV GVR (with Rep *in trans*) to amplify

the donor sequence. Among 36 regenerated T_0 plants, nine had entirely red fruit. Molecular evidence from junction PCR and Southern blots confirmed HDR in sampled leaf tissue. Of these nine T_0, eight fertile plants showed Mendelian segregation for the fruit color phenotype among their T_1 progeny, demonstrating heritability of these gene-targeting events. In contrast to the GVR treatment, only one, chimeric plant, with both red and orange fruits, was recovered among the 12 T_0 plants regenerated with a control construct that lacked the Rep cassette (thus disabling GVR replication). T_1 progeny from these red fruits segregated for the fruit color trait, indicative of a heterozygous knock-in, whereas no red fruits came from the orange T_0 fruits from the same chimeric plant.

The above results underscore a crucial point: in order to recover heritable gene-targeting events, the edit must occur in a germline cell, or germinal cell linage. The targeted *ANT1* tomato lines in the Cermak study were edited soon after transformation, as indicated by the purple phenotype already visible at the callus stage. In the Dahan-Meier study, the low rate of chimerism evidenced by uniform fruit color and Mendelian segregation of T_1 progeny in the replicon treatment similarly indicated that most of these events occurred early in development. By contrast, Shan et al.[37] failed to recover heritable gene-targeting events in *Arabidopsis* using a BeYDV GVR, despite a high frequency of somatic editing among transformed plants. In the previous studies, the transgenic tobacco or tomato plants were regenerated through tissue culture of somatic explants, involving organogenesis and establishment of a new germline. In *Arabidopsis*, the floral dip protocols used directly transformed existing germline cells. It is possible that established germline cells restrict viral activity more stringently than somatic cells. Alternatively, it may be that additional unknown barriers prevent efficient GVR-mediated HDR in *Arabidopsis*; another report failed to observe even efficient somatic gene targeting, again using a BeYDV replicon in Arabidopsis[44]. However, these results stand in contrast to the majority of published studies in other dicot species.

5.4 Gil-Humanes et al.[35] and Wang et al.[28]: Gene targeting in cereals with WDV

Soon after the first gene-editing reports with BeYDV, two studies described knock-ins in cereals using WDV. Gil-Humanes et al.[35] targeted fluorescent reporters to the wheat genome. Cas9 and a sgRNA targeted the endogenous ubiquitin locus, while a repair template included the coding sequence for GFP behind a T2A self-cleaving peptide linker. HDR of the break site placed the GFP gene in-frame with the native ubiquitin gene, leading to reporter expression driven by the native promoter. In protoplast transfections, green fluorescing

cells were identified only in treatments placing all of the editing reagents on the WDV replicon; in the best case, with a strong promoter for Cas9, 3.8% of cells underwent the targeting event. Additional protoplast experiments demonstrated multiplexed gene targeting using a BFP tag at a second locus, and molecularly confirmed targeting at all three of the wheat ubiquitin homeoalleles. Despite these promising results, Gil-Humanes and colleagues were unable to recover plants with the gene-targeting event. They did observe reporter positives in biolistic transfection of embryonic scutella, a typical system for tissue culture of wheat. However, these cells could not be regenerated even under chemical selection for the biolistic vector or for an alternative knock-in of the *bar* selectable marker gene (Gil Humanes et al., unpublished).

Wang et al.[28] described gene targeting in rice using selectable and screenable markers. Similar to Gil-Humanes et al., they used Cas9 and a sgRNA to knock in markers behind the promoter of either of two highly expressed native loci. In this case, a *nptII::GFP* fusion gene provided means to select for HDR events in agroinfected-rice calli. Geneticin-resistant plantlets were recovered through tissue culture, and screened by PCR for correct repair at the repair template junctions. Two different rice lines were transformed with test constructs: a transgenic line constitutively expressing Cas9, and a wild-type line, for which Cas9 was included on the T-DNA vector but outside the replicon. Double-junction PCR positives were recovered among the T_0 plants of both lines transformed with WDV GVRs, at frequencies ranging from 4.7% (in the wild type line) to 19.4% (in the transgenic line). Positives were also recovered from the non-GVR control treatment in the Cas9 line at 6.8%. No non-GVR control treatment was reported for the wild-type line. T_1 progeny or other analyses demonstrating germinal transmission were also not reported.

Together, these results show that GVRs can enhance gene targeting in monocots, but do not yet present a convincing case that WDV vectors are simple to use for the recovery of targeted plants. Our own group and several collaborators have been unable to regenerate rice plants with knock-ins using WDV, even while targeting several different loci and applying selection for the T-DNA. We suspect Wang et al. succeeded in recovering transgenic plantlets largely by virtue of having imposed selection for the HDR event. The absence of additional reports reporting success suggests continued research is required to generalize the GVR strategy to cereals and other monocots.

6 GVR-based editing strategies: summary and future trends

In case studies described above and several additional reports, GVRs have been used to mediate HDR across species including tobacco, tomato, potato, cassava, wheat, and rice[3,26,28,35–37,45,46]. High-frequency, germline edits have been

achieved in dicots with BeYDV, whereas WDV has shown promise for HDR in rice and in wheat cell culture. For these and other plant systems, additional research will be required to optimize GVR-based editing strategies. For example, the current BeYDV-and WDV-derived replicons may not function in all target crops: GVRs from other viruses have failed to replicate in some species, likely indicative of virus-specific host range requirements[25]. In monocots, more work is needed to expand the utility of GVRs within extant tissue culture regimes to enable heritable edits to be recovered. These challenges may be addressed by leveraging increasing viral sequence resources made available through metagenomics[5], or by more advanced engineering strategies for the delivery and control of GVRs.

7 RNA viruses: mobile vectors broadly applicable for gene-editing reagent delivery

RNA viral vectors are rapidly emerging as effective means for delivering genome engineering reagents. RNA viruses have small genomes with only a few essential genes required for replication, movement, and infection[47–50]. These viruses have been used as vectors for protein production, viral-induced gene silencing, and more recently, gene editing[51–53]. The earliest reports of their use for gene editing involved using them to express a ZFN to edit an integrated reporter[54]. Vectors then quickly adopted the CRISPR/Cas9 technology by expressing sgRNAs for editing in a transgenic plant that expresses Cas9[51]. This was first demonstrated in *Nicotiana benthamiana* and then expanded into several other species[55,56]. More recently, the technology was further improved to enable high frequencies of heritable editing in *N. benthamiana*[57]. Future work is required to expand heritable editing to other species and to develop vectors that no longer require transgenic plants for gene editing. Further advancement of the technology could allow transgene and tissue-culture-free gene editing of a variety of plant species. This would be a major advance for plant gene editing.

RNA viruses, or viruses that do not have a DNA intermediate in their replication cycle, have many advantageous properties for use as vectors, including compact genomes, systemic plant movement, non-host integrating replication cycle, and well-studied interactions with host defense systems. Regardless of whether RNA viral genomes are mono or multipartite, they are relatively simple, containing only a few coding regions essential to their replication, movement, and infection (Fig. 2). Replication of the viral genome and expression of sub-genomic RNAs, which are translated into proteins, is dependent on the RNA-dependent RNA polymerase (RdRP)[50,58]. Cell-to-cell and systemic movement of RNA viruses relies on the movement protein (MP). RNA-viral MPs guide the viral genome to the plasmodesmata[47] and often

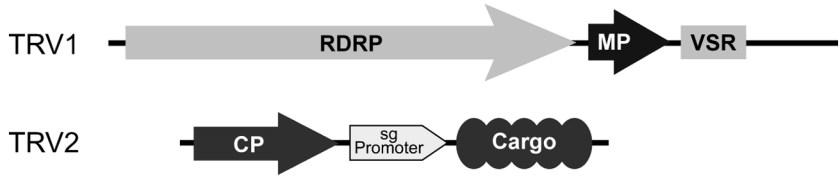

Figure 2 Tobacco Rattle Virus (TRV) vector. TRV is a bipartite positive-sense single-stranded RNA virus. TRV1 contains the RNA-dependent RNA polymerase (RdRP) which replicates both TRV1 and TRV2 and expresses subgenomic RNAs of other coding regions. TRV1 also encodes the movement protein (MP), responsible for enabling cell-to-cell movement, and the viral suppressor of RNA silencing (VSR), responsible for suppression of the host immune response. TRV2 contains the coat protein (CP) and can be modified for expression of heterologous sequences. The native TRV2 contains coding sequences to enable transmission between plants, which are removed in the vector. Heterologous sequences are expressed from a subgenomic promoter, either duplicated from the same virus or from a closely related virus.

increase the plasmodesmata size exclusion limit[48], permitting the full viral genome to pass from cell-to-cell. Systemic infection occurs by transport of the virus through the phloem, with unloading of the virus at distal sites, leading to systemic infection[49]. Some viruses require encapsidation, or coating of the viral genome with a viral encoded coat protein (CP), for systemic infection[59]. Encapsidation is also an important factor in vector transmission between plants by insects. Different plant RNA viruses may encode other factors involved in infection, transmission between plants, or other host interactions such as combating host defense.

The well-studied genomes of several RNA viruses make them ideal for engineering and repurposing for use as vectors. Indeed, shortly after some of the first infectious cDNA clones of RNA viruses were created, efforts were undertaken to use them as vectors for expression of heterologous proteins[53,60]. Early approaches involved expression of reporter genes[61], but vectors were quickly adapted for the expression of proteins of value for pharmaceutical[62] or agricultural applications[63].

7.1 Modifications of RNA viruses into RNA viral vectors

One of the first steps in creating a plant RNA viral vector is to generate a cDNA of the viral transcript and adapt it for compatibility with various molecular cloning techniques. The cDNA vectors are often placed behind standard plant promoters. When transferred to plant cells, a transcript is produced that serves as the RNA virus genome; the transcript is replicated and enables systemic infection throughout the plant. A second modification commonly used with viral vectors is to incorporate them into a backbone that enables replication in *Agrobacterium* and T-DNA transfer into plant cells. Finally, the viral genome

itself is modified to express heterologous sequences (Fig. 2). This can involve viral gene replacement or gene insertion behind a subgenomic promoter[53]. For further reading on the construction of plant viral vectors we recommend Pasin et al.[64].

7.2 RNA viral vectors for virus-induced gene silencing

Perhaps one of the best-known applications of RNA viral vectors is for viral-induced gene silencing (VIGS). VIGS functions by taking advantage of the native plant post-transcriptional RNA silencing (PTGS) mechanism. By placing a small fragment of a gene of interest into an RNA viral vector, host PTGS, in an attempt to silence the virus, will also silence the native gene[52,65]. This has proven a valuable approach for reverse genetics to better understand gene function, and VIGS vectors have been developed for a large number of plant species[66]. A disadvantage of viral vectors for heterologous protein production or VIGS is that the effects of the assay are transient. Approaches to use viral vectors to create permanent genetic changes are outlined in the following sections.

8 Case studies: use viral vectors to create permanent genetic changes

8.1 Marton et al. 2010: Nontransgenic genome modification in plant cells[54]

With the advent of SSNs, RNA viral vectors were soon tested to determine if they could be used to express SSNs to create genetic modifications in infected plant tissues. One of the earliest examples of this is the use of Tobacco Rattle Virus (TRV) to express ZFNs for plant gene editing[54]. Traditionally, genome-editing reagents are delivered to plant cells through stable transformation, wherein the SSN is introduced into plant cells by *Agrobacterium* or biolistics. Transgenic cells are then regenerated into whole plants through tissue culture, and progeny are screened for the presence of the targeted genome modification. Marton and colleagues described a method for non-transgenic gene editing of plant cells using a TRV expression system. TRV is a bipartite virus with two RNAs: TRV1 and TRV2. TRV2 was modified to contain the Pea Early Browning Virus (PEBV) coat protein subgenomic promoter upstream of a multiple cloning site. This expression system was tested by first adding fluorescent reporter genes into the multiple cloning site. When plants were infected with these viruses, expression of the report (i.e., fluorescence) was observed in developing tissues throughout the plant. This indicated that TRV2 could be used to carry and express cargo in infected and growing portions of the plant. Next a ZFN monomer was added

to TRV2 that targets a defective *gus* reporter integrated into the genomes of *N. benthamiana* and petunia. Mutagenesis by the ZFN should create a functional ***GUS*** gene. ***GUS*** expression was observed in infected tissue in both species, indicating ZFN-mediated editing of the reporter. Finally, *N. benthamiana* and petunia tissue infected with TRV2 expressing the ZFN was placed onto tissue-culture medium to regenerate plants from the infected tissue. ***GUS*** activity was observed in the regenerated tissue, and the edited target was stably inherited. The seedlings were also confirmed to be free of virus, verifying the method as a non-integrating delivery means of generating stably maintained, site-specific gene edits.

A longstanding goal for the use of RNA viral vectors for gene editing is to infect and edit endogenous genes in wild-type plants. This initial report of gene editing using RNA viruses was significant because it demonstrated editing in a plant by an RNA viral vector that expresses all the reagents required for gene editing. This was only made possible due to the relatively small size of a ZFN monomer. ZFN dimers are typically required to edit genes; however, the *gus* reporter had been engineered to carry two recognition sites for the ZFN monomer, thereby enabling gene editing by the monomer. Editing of endogenous genes is prohibited by the requirement of ZFN dimers for DNA cleavage and low-editing efficiencies[67]. Viral vectors for genome editing need improvements to effectively target endogenous loci.

Shortly after the previous case study was published, CRISPR/Cas9 technology was developed[68] which promised significantly improved ease of gene editing. One disadvantage of the CRISPR/Cas9 system is that while the targeting component of the site-specific nuclease is relatively small, namely the sgRNA, Cas9 is a large protein and well beyond the carrying capacity of RNA viral vectors developed to date. RNA viral vectors have a limited carrying capacity due to the error-prone RdRP, which often truncates non-essential viral components; shorter, wild-type viruses quickly outcompete modified vectors. Further, vectors modified to express large cargo are often unable to be encapsidated[53]. Ali and colleagues proposed a solution to this problem: they created a transgenic plant that expresses the Cas9 protein, and used the viral vector to express the sgRNA[51]. Their results are highlighted in the following case study.

8.2 Ali et al. 2015: Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system[51]

Recognizing delivery of genome engineering reagents to plant cells is a significant limitation to achieve gene editing, Ali and colleagues sought to provide a means to efficiently and quickly deliver CRISPR/Cas9 reagents using RNA viruses. They created transgenic *N. benthamiana* plants that express Cas9, and used TRV to

deliver sgRNAs that target unique sequences in the genome. TRV2 was modified in a similar manner to Marton et al. 2010 by expressing the sgRNA from a PeBV-subgenomic promoter. To test the effectiveness of this vector in gene editing, a sgRNA-targeting *N. benthamiana* phytoene d*esaturase* (PDS) was cloned into the TRV2 vector and subsequently agroinfected along with a TRV1 vector into the transgenic *N. benthamiana* plants that express Cas9. Gene editing was observed in the inoculated and systemically infected leaves. The method was further validated by targeting a second locus, PCNA, and editing was again observed in the inoculated and systemically infected leaves. Next, multiple TRV2 vectors, each expressing a unique sgRNA, were simultaneously tested to determine if they could achieve multiplexed editing. TRV2 vectors expressing sgRNAs targeting PDS or PCNA were co-infected into *N. benthamiana*, and editing was observed at both loci, although at a lower frequency than when inoculated separately.

The applicability of RNA viral vectors for gene editing would be greatly improved if the targeted mutation occurred in the plant germline. This would allow seeds containing the desired modification to be collected from the infected plant, thereby eliminating the need for tissue-culture in many gene-editing experiments and increasing ease and throughput. Ali and colleagues reported evidence of germinal transmission of a targeted mutation, although at a very low frequency. This indicated that future work was needed to increase germline edits to a frequency that is practical. Also, the approach needed to be expanded to species other than the *N. benthamiana* model.

8.3 Expanding RNA viral vector gene editing to other species

After Ali et al. demonstrated the ability of viral vectors to perform efficient gene editing in somatic cells of Cas9 expressing *N. benthamiana*, the same group and several others tested this approach for gene editing in other species and with other viral vectors. PEBV and Beet Necrotic Yellow Vein Virus (BNYVV) were demonstrated to perform gene editing in Cas9-expressing somatic cells of *N. benthamiana*[69], and Tobacco Mosaic Virus (TMV) was used for multiplexed gene editing[70]. TRV was also used to demonstrate gene editing in Cas9-expressing *Arabidopsis*[55], highlighting the wide host range and utility of this virus. In order to expand to monocot species, Foxtail Mosaic Virus (FoMV) was modified to contain a duplicated coat protein subgenomic promoter for expression of sgRNAs. This vector was able to perform gene editing in Cas9 expressing *N. benthamiana*, *Setaria viridis*, and maize[56]. Finally, a Barley Yellow Striate Mosaic Virus (BYSMV) vector was modified to express proteins in wheat, barley, foxtail millet, and maize. BYSMV is a negative-sense RNA virus, whereas all other virus vectors discussed are positive-sense RNA viruses. Negative-sense RNA viruses could provide a larger carrying capacity than the positive-sense RNA viruses, as the authors of this study demonstrated expression of both the sgRNA and

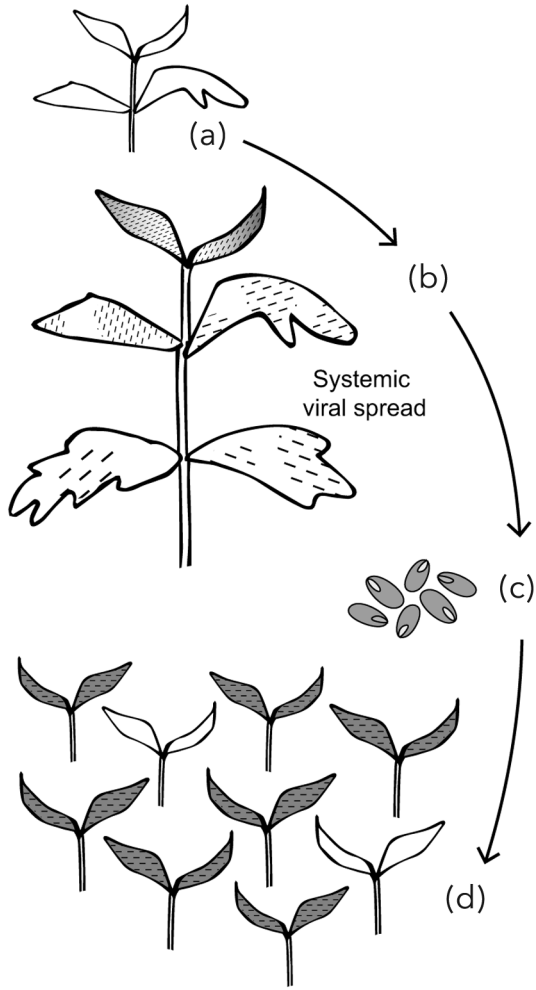

Figure 3 A method for infecting plants with RNA viral vectors and generating heritable genome edits. (a) Transgenic plants expressing Cas9 are infected with an RNA viral vector expressing a mobile sgRNA to increase genome-editing frequencies. (b) This vector systemically infects the plant, interacting with endogenously expressed Cas9 for high-frequency genome editing (future methods may express all the genome-editing reagents from the virus, allowing editing of wild-type plants). Dashed lines and shading indicate genome editing. (c) Seeds are collected from infected plants and progeny are screened for editing of the targeted site. (d) High percentages of the progeny contain the desired edit; shaded seedlings indicate the presence of targeted mutations.

Published by Burleigh Dodds Science Publishing Limited, 2021.

Cas9 from the virus and editing at the infiltrated site in *N. benthamiana*; they did not report any systemic editing[71]. None of the previously mentioned studies demonstrated heritable editing without using tissue culture, the exception being Ali et al. 2015, in which, as mentioned above, heritable editing was observed at a very low frequency: only 1 in 438 seedlings tested. In order for viral vectors to be widely adopted as vectors for site-specific editing, the heritable editing frequency needs to be improved. Ellison and colleagues show improved heritable editing frequency in *N. benthamiana* by adding motifs that promote mobility of the sgRNAs expressed from TRV vectors (Fig. 3). Their results are described below.

8.4 Ellison et al. 2020: Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs[57]

Ellison and colleagues sought to improve the frequencies of heritable gene editing with RNA viral vectors, recognizing that editing through infection could eliminate or reduce the need for tissue-culture – a major bottleneck in applying biotechnology to plant science research. The authors hypothesized that by enabling viral or sgRNA sequences better access to the germline, gene edits would be transmitted to the next generation at higher frequencies. To enable mobility, a *Flowering Locus T* (FT) motif was added to the 3′ end of sgRNA sequences. The FT motif was previously shown to promote mobility of RNAs into the meristem[72]. sgRNA sequences targeting *N. benthamiana PDS* and augmented with this mobility sequence were cloned into the same TRV2 vector described in Ali et al. 2015. These vectors were agroinfected into Cas9-expressing *N. benthamiana* along with TRV1. Shortly after infection, phenotypes suggesting PDS mutagenesis emerged, and this was substantiated by determining that somatic editing frequencies were greater than 80%. Germinal transmission of these edits was observed by phenotyping (Fig. 4) and genotyping seedlings: up to 65% contained a mutation in at least one PDS allele. This high frequency of mutagenesis was demonstrated at a second locus, *AGAMOUS* (AG). In this case, up to 100% of seedlings contained a mutation in at least one AG allele. The mobility motif was not limited to FT: tRNA-like sequences have previously been shown to promote cell-to-cell mobility[73], and such tRNA-like sequences were also demonstrated to enhance frequencies of heritable mutagenesis when fused to sgRNAs. In addition, heritable multiplexed editing of native *N. benthamiana* genes was achieved. Multiple sgRNAs could be expressed from a single TRV2 vector to achieve heritable mutations in all targets. This report establishes the ability of RNA viral vectors to perform high efficiency, heritable gene editing. Further work needs to be performed to expand this method to other species and to develop RNA vectors that are not reliant on a transgenic plant expressing Cas9.

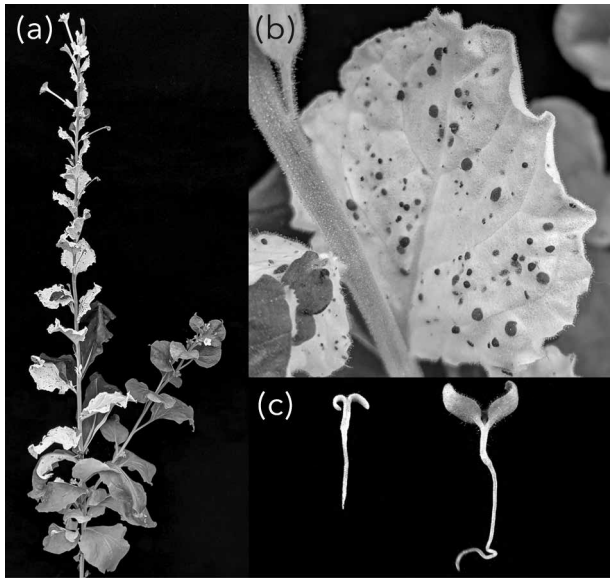

Figure 4 *Phytoene* D*esaturase* (PDS) phenotypes in Cas9-expressing *N. benthamiana* plants infected with Tobacco Rattle Virus (TRV) vectors. TRV expresses mobile sgRNAs targeting the PDS coding sequence, which results in a bleached phenotype due to chlorophyll photo-oxidation. (a) Infected plants show an increase in the PDS-knockout phenotype as the plant matures. In some cases, the entire upper portion of the plant is bleached, indicating a PDS mutation occurring in meristematic cells. (b) Systemic plant leaves contain high frequencies of editing from RNA viral vectors expressing mobile sgRNAs. The leaf shown here contains PDS mutations in nearly every cell, causing an almost completely bleached phenotype. (c) Seeds are collected from plants infected with the TRV vector expressing mobile *PDS* sgRNAs. Several of the resulting seedlings are entirely bleached, indicating germline mutagenesis of the *PDS* locus.

9 RNA viral vectors: conclusion and future trends

All three of the case studies described in this chapter illustrate the growing interest in the use of RNA viral vectors for plant gene editing. While there are considerable milestones yet to be achieved, these vectors provide considerable promise for tissue-culture-free gene editing. Future work to improve the technology should focus on expanding the species range of heritable gene editing. This will require optimization of vectors for each species along with the establishment of transgenic plant lines that express Cas9 at high levels and in the appropriate tissues. In order to eliminate the need for transgenesis, efforts should be made to test whether smaller Cas or other nucleases can be systemically transported by RNA viral vectors. Alternatively, it might be possible to increase the carrying capacity of virus vectors. Recently, Ma and colleagues developed vectors based on Sonchus Yellow Net Nucleorhabdovirus (SYNV), a negative-sense RNA virus.

Remarkably, these vectors could deliver both Cas9 and sgRNAs[74]. Gene-edited plants were recovered by regenerating infected plant tissue without selection, and over 90% of regenerated plants carried mutations. If SYNV could access the germline, perhaps through addition of RNA motifs that promote cell-to-cell mobility as described by Ellison et al.[57], it may be possible to recover heritable mutations solely through infection and without tissue culture and transgenesis.

RNA viral vector utility can also be expanded by using different Cas variants with different PAM requirements to increase the targeting range or to create unique editing outcomes. It should also be possible to move beyond non-homologous end joining or microhomology-mediated repair of the target locus to achieve more precise modifications through base editing[75–77] or prime editing[78]. Background work on RNA genome structure and function, followed by the development of vectors for protein expression, VIGS, and genome editing, will facilitate improvements and increase the scope, specificity, and broad applicability of RNA viral vectors.

10 Where to look for further information

Many viral vectors, including all of our group's published vectors, are available at Addgene, a non-profit plasmid repository. BeYDV and WDV replicons are among the available reagents, and they are compatible with the plant genome engineering toolkit described in Cermak et al.[79]. Lastly, viral sequences can sometimes be challenging to assemble and clone in bacterial hosts, due to structural features or toxicity of viral gene products; for a review of strategies for viral clone assembly, see Pasin et al.[64].

11 References

1. Zerbini, F. M., Briddon, R. W., Idris, A., Martin, D. P., Moriones, E., Navas-Castillo, J., Rivera-Bustamante, R., Roumagnac, P., Varsani, A. and Ictv Report Consortium. ICTV virus taxonomy profile: geminiviridae. *Journal of General Virology* 98(2), 131–133 (2017).
2. Lazarowitz, S. G., Pinder, A. J., Damsteegt, V. D. and Rogers, S. G. Maize streak virus genes essential for systemic spread and symptom development. *The EMBO Journal* 8(4), 1023–1032 (1989).
3. Baltes, N. J., Gil-Humanes, J., Cermak, T., Atkins, P. A. and Voytas, D. F. D. N. A. DNA replicons for plant genome engineering. *The Plant Cell* 26(1), 151–163 (2014).
4. Hanley-Bowdoin, L., Bejarano, E. R., Robertson, D. and Mansoor, S. Geminiviruses: masters at redirecting and reprogramming plant processes. *Nature Reviews. Microbiology* 11(11), 777–788 (2013).
5. Roossinck, M. J., Martin, D. P. and Roumagnac, P. Plant virus metagenomics: advances in virus discovery. *Phytopathology* 105(6), 716–727 (2015).
6. Fontenele, R. S., Alves-Freitas, D. M. T., Silva, P. I. T., Foresti, J., Silva, P. R., Godinho, M. T., Varsani, A. and Ribeiro, S. G. Discovery of the first maize-infecting Mastrevirus in

the Americas using a vector-enabled metagenomics approach. *Archives of Virology* 163(1), 263–267 (2018).
7. Varsani, A., Shepherd, D. N., Dent, K., Monjane, A. L., Rybicki, E. P. and Martin, D. P. A highly divergent South African geminivirus species illuminates the ancient evolutionary history of this family. *Virology Journal* 6, 36 (2009).
8. Kumar, J., Kumar, J., Singh, S. P. and Tuli, R. Association of satellites with a Mastrevirus in natural infection: complexity of wheat Dwarf India virus disease. *Journal of Virology* 88(12), 7093–7104 (2014).
9. Gutierrez, C. Geminivirus DNA replication. *Cellular and Molecular Life Sciences: CMLS* 56(3–4), 313–329 (1999).
10. Schalk, H. J., Matzeit, V., Schiller, B., Schell, J. and Gronenborn, B. Wheat dwarf virus, a geminivirus of graminaceous plants needs splicing for replication. *The EMBO Journal* 8(2), 359–364 (1989).
11. Wright, E. A., Heckel, T., Groenendijk, J., Davies, J. W. and Boulton, M. I. Splicing features in maize streak virus virion- and complementary-sense gene expression. *The Plant Journal: For Cell and Molecular Biology* 12(6), 1285–1297 (1997).
12. Liu, L., Davies, J. W. and Stanley, J. Mutational analysis of bean yellow dwarf virus, a geminivirus of the genus Mastrevirus that is adapted to dicotyledonous plants. *Journal of General Virology* 79(9), 2265–2274 (1998).
13. Boulton, M. I. Functions and interactions of Mastrevirus gene products. *Physiological and Molecular Plant Pathology* 60(5), 243–255 (2002).
14. Gutierrez, C. D. N. A.. DNA replication and cell cycle in plants: learning from geminiviruses. *The EMBO Journal* 19(5), 792–799 (2000).
15. Gutierrez, C. Strategies for geminivirus DNA replication and cell cycle interference. *Physiological and Molecular Plant Pathology* 60(5), 219–230 (2002).
16. Ugaki, M., Ueda, T., Timmermans, M. C., Vieira, J., Elliston, K. O. and Messing, J. Replication of a geminivirus derived shuttle vector in maize endosperm cells. *Nucleic Acids Research* 19(2), 371–377 (1991).
17. Matzeit, V., Schaefer, S., Kammann, M. and Gronenborna, B. Wheat dwarf virus vectors replicate and express foreign genes in cells of monocotyledonous plants *The Plant Cell* 3, 247–258 (1991).
18. Shen, W.-H. and Hohn, B. Amplification and expression of the B-glucuronidase gene in maize plants by vectors based on maize streak virus. *The Plant Journal* 5(2), 227–236 (1994).
19. Shen, W. H. and Hohn, B. Vectors based on maize streak virus can replicate to high copy numbers in maize plants. *Journal of General Virology* 76(4), 965–969 (1995).
20. Mor, T. S., Moon, Y. S., Palmer, K. E. and Mason, H. S. Geminivirus vectors for high-level expression of foreign proteins in plant cells. *Biotechnology and Bioengineering* 81(4), 430–437 (2003).
21. Zhang, X. and Mason, H. Bean Yellow Dwarf Virus replicons for high-level transgene expression in transgenic plants and cell cultures. *Biotechnology and Bioengineering* 93(2), 271–279 (2006).
22. Hayes, R. J., Petty, I. T. D., Coutts, R. H. A. and Buck, K. W. Gene amplification and expression in plants by a replicating geminivirus vector. *Nature* 334(6178), 179–182 (1988).
23. Hayes, R. J., Coutts, R. H. and Buck, K. W. Stability and expression of bacterial genes in replicating geminivirus vectors in plants. *Nucleic Acids Research* 17(7), 2391–2403 (1989).

24. Gilbertson, R. L., Sudarshana, M., Jiang, H., Rojas, M. R. and Lucas, W. J. Limitations on geminivirus genome size imposed by plasmodesmata and virus-encoded movement protein: insights into DNA trafficking. *The Plant Cell* 15(11), 2578–2591 (2003).
25. Pandey, P., Choudhury, N. R. and Mukherjee, S. K. A geminiviral amplicon (VA) derived from Tomato leaf curl virus (ToLCV) can replicate in a wide variety of plant species and also acts as a VIGS vector. *Virology Journal* 6, 152 (2009).
26. Čermák, T., Baltes, N. J., Čegan, R., Zhang, Y. and Voytas, D. F. High-frequency, precise modification of the tomato genome. *Genome Biology* 16, 232 (2015).
27. Gleba, Y., Marillonnet, S. and Klimyuk, V. Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Current Opinion in Plant Biology* 7(2), 182–188 (2004).
28. Wang, M., Lu, Y., Botella, J. R., Mao, Y., Hua, K. and Zhu, J. K. Gene targeting by homology-directed repair in rice using a geminivirus-based CRISPR/Cas9 system. *Molecular Plant* 10(7), 1007–1010 (2017).
29. Woolston, C. J., Reynolds, H. V., Stacey, N. J. and Mullineaux, P. M. Replication of wheat dwarf virus DNA in protoplasts and analysis of coat protein mutants in protoplasts and plants. *Nucleic Acids Research* 17(15), 6029–6041 (1989).
30. Rogers, S. G., Bisaro, D. M., Horsch, R. B., Fraley, R. T., Hoffmann, N. L., Brand, L., Scott Elmer, J. and Lloyd, A. M. Tomato golden mosaic virus A component D replicates autonomously in transgenic plants. Cell 45(4), 593–600 (1986).
31. Grimsley, N., Hohn, B., Hohn, T. and Walden, R. "Agroinfection," an alternative route for viral infection of plants by using the Ti plasmid. *Proceedings of the National Academy of Sciences of the United States of America* 83(10), 3282–3286 (1986).
32. Woolston, C. J., Barker, R., Gunn, H., Boulton, M. I. and Mullineaux, P. M. Agroinfection and nucleotide sequence of cloned wheat dwarf virus DNA. *Plant Molecular Biology* 11(1), 35–43 (1988).
33. Stenger, D. C., Revington, G. N., Stevenson, M. C. and Bisaro, D. M. Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. *Proceedings of the National Academy of Sciences of the United States of America* 88(18), 8029–8033 (1991).
34. Zhao, X., Coats, I., Fu, P., Gordon-Kamm, B. and Lyznik, L. A. T-DNA recombination and replication in maize cells. *The Plant Journal: For Cell and Molecular Biology* 33(1), 149–159 (2003).
35. Gil-Humanes, J., Wang, Y., Liang, Z., Shan, Q., Ozuna, C. V., Sánchez-León, S., Baltes, N. J., Starker, C., Barro, F., Gao, C. and Voytas, D. F. High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. *The Plant Journal: For Cell and Molecular Biology* 89(6), 1251–1262 (2017).
36. Dahan-Meir, T., Filler-Hayut, S., Melamed-Bessudo, C., Bocobza, S., Czosnek, H., Aharoni, A. and Levy, A. A. Efficient *in planta* gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. *The Plant Journal: For Cell and Molecular Biology* 95(1), 5–16 (2018).
37. Shan, Q., Baltes, N. J., Atkins, P., Kirkland, E. R., Zhang, Y., Baller, J. A., Lowder, L. G., Malzahn, A. A., Haugner, J. C., Seelig, B., Voytas, D. F. and Qi, Y. ZFN, TALEN and CRISPR-Cas9 mediated homology directed gene insertion in Arabidopsis: a disconnect between somatic and germinal cells. *Journal of Genetics and Genomics = Yi Chuan Xue Bao* 45(12), 681–684 (2018).

38. Puchta, H., Dujon, B. and Hohn, B. Homologous recombination in plant cells is enhanced by *in vivo* induction of double strand breaks into DNA by a site-specific endonuclease. *Nucleic Acids Research* 21(22), 5034–5040 (1993).
39. Townsend, J. A., Wright, D. A., Winfrey, R. J., Fu, F., Maeder, M. L., Joung, J. K. and Voytas, D. F. High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature* 459(7245), 442–445 (2009).
40. Zhang, Y., Zhang, F., Li, X., Baller, J. A., Qi, Y., Starker, C. G., Bogdanove, A. J. and Voytas, D. F. Transcription activator-like effector nucleases enable efficient plant genome engineering. *Plant Physiology* 161(1), 20–27 (2013).
41. Richter, K. S., Kleinow, T. and Jeske, H. Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner. *Virology* 452–453, 287–296 (2014).
42. Wright, D. A., Townsend, J. A., Winfrey, R. J., Irwin, P. A., Rajagopal, J., Lonosky, P. M., Hall, B. D., Jondle, M. D. and Voytas, D. F. High-frequency homologous recombination in plants mediated by zinc-finger nucleases. *The Plant Journal: For Cell and Molecular Biology* 44(4), 693–705 (2005).
43. Fauser, F., Roth, N., Pacher, M., Ilg, G., Sánchez-Fernández, R., Biesgen, C. and Puchta, H. In planta gene targeting. *Proceedings of the National Academy of Sciences of the United States of America* 109(19), 7535–7540 (2012).
44. Hahn, F., Eisenhut, M., Mantegazza, O. and Weber, A. P. M. Homology-directed repair of a defective glabrous gene in Arabidopsis With Cas9-based gene targeting. *Frontiers in Plant Science* 9, 424 (2018).
45. Butler, N. M., Baltes, N. J., Voytas, D. F. and Douches, D. S. Geminivirus-mediated genome editing in potato (Solanum tuberosum L.) using sequence-specific nucleases. *Frontiers in Plant Science* 7, 1045 (2016).
46. Hummel, A. W., Chauhan, R. D., Cermak, T., Mutka, A. M., Vijayaraghavan, A., Boyher, A., Starker, C. G., Bart, R., Voytas, D. F. and Taylor, N. J. Allele exchange at the EPSPS locus confers glyphosate tolerance in cassava. *Plant Biotechnology Journal* 16(7), 1275–1282 (2018).
47. Atabekov, J. G. and Taliansky, M. E. Expression of a plant virus-coded transport function by different viral genomes. *Advances in Virus Research* 38, 201–248 (1990).
48. Atabekov, J. G. and Dorokhov, Y. L. Plant virus-specific transport function and resistance of plants to viruses. *Advances in Virus Research* 29, 313–364 (1984).
49. Carrington, J. C., Kasschau, K. D., Mahajan, S. K. and Schaad, M. C. Cell-to-cell and long-distance transport of viruses in plants. *The Plant Cell* 8(10), 1669–1681 (1996).
50. Ortín, J. and Martín-Benito, J. The RNA synthesis machinery of negative-stranded RNA viruses. *Virology* 479–480, 532–544 (2015).
51. 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. and Mahfouz, M. M. Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. *Molecular Plant* 8(8), 1288–1291 (2015).
52. Burch-Smith, T. M., Anderson, J. C., Martin, G. B. and Dinesh-Kumar, S. P. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal: For Cell and Molecular Biology* 39(5), 734–746 (2004).
53. Scholthof, H. B., Scholthof, K. B. and Jackson, A. O. Plant virus gene vectors for transient expression of foreign proteins in plants. *Annual Review of Phytopathology* 34, 299–323 (1996).

54. Marton, I., Zuker, A., Shklarman, E., Zeevi, V., Tovkach, A., Roffe, S., Ovadis, M., Tzfira, T. and Vainstein, A. Nontransgenic genome modification in plant cells. *Plant Physiology* 154(3), 1079–1087 (2010).
55. Ali, Z., Eid, A., Ali, S. and Mahfouz, M. M. Pea early-browning virus-mediated genome editing via the CRISPR/Cas9 system in Nicotiana benthamiana and Arabidopsis. *Virus Research* 244, 333–337 (2018).
56. Mei, Y., Beernink, B. M., Ellison, E. E., Konečná, E., Neelakandan, A. K., Voytas, D. F. and Whitham, S. A. Protein expression and gene editing in monocots using foxtail mosaic virus vectors. *Plant Direct* 3(11), e00181 (2019).
57. Ellison, E. E., Nagalakshmi, U., Gamo, M. E., Huang, P. J., Dinesh-Kumar, S. and Voytas, D. F. Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nature Plants* 6(6), 620–624 (2020).
58. Ahlquist, P., Noueiry, A. O., Lee, W. M., Kushner, D. B. and Dye, B. T. (Host). Host Factors in positive-strand RNA virus genome replication. *Journal of Virology* 77(15), 8181–8186 (2003).
59. Dall'Ara, M., Ratti, C., Bouzoubaa, S. E. and Gilmer, D. Ins and outs of multipartite positive-strand RNA plant viruses: packaging versus systemic spread. *Viruses* 8(8), 228 (2016).
60. Siegel, A. Plant-virus-based vectors for gene transfer may be of considerable use despite a presumed high error frequency during RNA synthesis. *Plant Molecular Biology* 4(5), 327–329 (1985).
61. Chapman, S., Kavanagh, T. and Baulcombe, D. Potato virus X as a vector for gene expression in plants. *The Plant Journal: For Cell and Molecular Biology* 2(4), 549–557 (1992).
62. Czapar, A. E. and Steinmetz, N. F. Plant viruses and bacteriophages for drug delivery in medicine and biotechnology. *Current Opinion in Chemical Biology* 38, 108–116 (2017).
63. McGarry, R. C., Klocko, A. L., Pang, M., Strauss, S. H. and Ayre, B. G. Virus-induced flowering: an application of reproductive biology to benefit plant research and breeding. *Plant Physiology* 173(1), 47–55 (2017).
64. Pasin, F., Menzel, W. and Daròs, J. A. Harnessed viruses in the age of metagenomics and synthetic biology: an update on infectious clone assembly and biotechnologies of plant viruses. *Plant Biotechnology Journal* 17(6), 1010–1026 (2019).
65. Ruiz, M. T., Voinnet, O. and Baulcombe, D. C. Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10(6), 937–946 (1998).
66. Lacomme, C. Strategies for altering plant traits using virus-induced gene silencing technologies. *Methods in Molecular Biology* 1287, 25–41. doi:10.1007/978-1-4939-2453-0_2 (2015).
67. Gaj, T., Gersbach, C. A., Barbas, C. F. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology* 31(7), 397–405 (2013).
68. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E. A programmable dual-RNAGuided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096), 816–821 (2012).
69. Jiang, N., Zhang, C., Liu, J. Y., Guo, Z. H., Zhang, Z. Y., Han, C. G. and Wang, Y. Development of Beet necrotic yellow vein virus-based vectors for multiple-gene expression and guide RNA delivery in plant genome editing. *Plant Biotechnology Journal* 17(7), 1302–1315 (2019).

70. Cody, W. B., Scholthof, H. B. and Mirkov, T. E. Multiplexed gene editing and protein overexpression using a *tobacco mosaic virus* viral vector. *Plant Physiology* 175(1), 23–35 (2017).
71. Gao, Q., Xu, W. Y., Yan, T., Fang, X. D., Cao, Q., Zhang, Z. J., Ding, Z. H., Wang, Y. and Wang, X. B. Rescue of a plant Cytorhabdovirus as versatile expression platforms for planthopper and cereal genomic studies. *New Phytologist* 223(4), 2120–2133 (2019).
72. Li, C., Zhang, K., Zeng, X., Jackson, S., Zhou, Y. and Hong, Y. A cis element within flowering locus T mRNA determines its mobility and facilitates trafficking of heterologous viral RNA. *Journal of Virology* 83(8), 3540–3548 (2009).
73. Zhang, W., Thieme, C. J., Kollwig, G., Apelt, F., Yang, L., Winter, N., Andresen, N., Walther, D. and Kragler, F. tRNA-related sequences trigger systemic mRNA transport in plants. *The Plant Cell* 28(6), 1237–1249 (2016).
74. Ma, X., Zhang, X., Liu, H. and Li, Z. Highly efficient DNA-free plant genome editing using virally delivered CRISPRCas9. *Nature Plants* 6(7), 773–779 (2020).
75. Kang, B. C., Yun, J. Y., Kim, S. T., Shin, Y., Ryu, J., Choi, M., Woo, J. W. and Kim, J. S. Precision genome engineering through adenine base editing in plants. *Nature Plants* 4(7), 427–431 (2018).
76. Shimatani, Z., Kashojiya, S., Takayama, M., Terada, R., Arazoe, T., Ishii, H., Teramura, H., Yamamoto, T., Komatsu, H., Miura, K., Ezura, H., Nishida, K., Ariizumi, T. and Kondo, A. Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nature Biotechnology* 35(5), 441–443 (2017).
77. Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., Qiu, J. L., Wang, D. and Gao, C. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nature Biotechnology* 35(5), 438–440 (2017).
78. Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A. and Liu, D. R. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576(7785), 149–157 (2019).
79. Čermák, T., Curtin, S. J., Gil-Humanes, J., Čegan, R., Kono, T. J. Y., Konečná, E., Belanto, J. J., Starker, C. G., Mathre, J. W., Greenstein, R. L. and Voytas, D. F. A multipurpose toolkit to enable advanced genome engineering in plants. *The Plant Cell* 29(6), 1196–1217 (2017).

Published by Burleigh Dodds Science Publishing Limited, 2021.