Plant viral vectors: Expanding the Possibilities of Precise Gene Editing in Plant Genomes

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Research Article

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Abstract

The lack of a highly efficient method for delivering reagents for genome engineering to plant cells remains a bottleneck in achieving efficient gene-editing in plant genomes. A suite of recent reports uncovers the newly emerged roles of viral vectors, which can introduce gene-edits in plants with high mutation frequencies through *in planta* delivery. Here, we focus on the emerging protocols that utilized different approaches for virus-mediated genome editing in model plants. Testing of these protocols and the newly identified hypercompact Cas systems is needed to broaden the scope of genome-editing in most plant species, including crops, with minimized reliance on conventional plant transformation methods in the future.

Key Message

We highlight how viral vector-based delivery of gene-editing reagents has contributed to genome editing in plants and would expand it further, especially after identifying new hypercompact Cas systems.

Introduction

The lack of a highly efficient method for delivering reagents for genome engineering to plant cells remains a bottleneck in achieving efficient gene-editing in plant genomes. A suite of recent reports uncovers the newly emerged roles of viral vectors, which can introduce gene-edits in plants with high mutation frequencies through *in planta* delivery. Here, we focus on the emerging protocols that utilized different approaches for virus-mediated genome editing in model plants. Testing of these protocols and the newly identified hypercompact Cas systems is needed to broaden the scope of genome-editing in most plant species, including crops, with minimized reliance on conventional plant transformation methods in the future.

Conventionally, plant genome editing is performed by agroinfiltration of reagents such as Cas9 and single-guide RNAs (sgRNAs) to explants. The infected cells are then allowed to differentiate using tissue culture procedures (Fig. 1A). The technical challenges associated with plant transformation and regeneration, such as the long recovery process for a gene-edited plant through tissue culture or low mutation frequencies in many instances, obstruct its rapid implementation for gene-editing in most crops. *In vitro* plant recalcitrance and the undesired somaclonal variations in the regenerated cells further contribute to the overall technical limitations (Fossi et al. 2019). Physical methods such as biolistics are not suitable either as they offer little control over reagent delivery and regeneration of the bombarded cells. Altogether, the current situation demands further optimization of plant transformation technologies for gene-editing, especially for crops (Altpeter et al. 2016). Due to their routine application in fundamental gene function studies and transient expression of foreign genes in plants, viral vectors could offer an alternative approach to overcome some of these challenges (Senthil-Kumar and Mysore 2011). Indeed, both DNA and RNA viral vectors have been successfully used to deliver gene-editing reagents in plants. In one of such earliest reports, Marton et al. (2010) used a tobacco rattle virus (TRV) based system for Zinc

Finger Nuclease (ZFN) expression in tobacco and petunia. They successfully demonstrated the induction of heritable site-specific mutations, through tissue regeneration, in the infected plants.

The DNA viruses belonging to the geminivirus class have been used to deliver gene-editing reagents in plants. The advantage of these viruses is that their single-stranded genome is converted to a double-stranded intermediate by host DNA polymerases once they are inside a host cell's nucleus. Alternatively, these may also get encapsidated by coat protein (CP) to produce virions, which may move to adjacent cells through the plasmodesmata in plants. Baltes et al. (2014) adopted the deconstructed virus strategy in the bean yellow dwarf virus by replacing the movement protein (MP) and CP with sequence-specific nucleases (SSN). Expression of ZFN using these geminivirus replicons (GVR) induced mutations in six out of eight sequenced samples from the infiltrated leaves of transgenic tobacco expressing reporter gene with ZFN target site. The authors further used GVRs to simultaneously introduce ZFNs and repair templates and achieved gene targeting with 81% efficiency. The advantage of GVRs in gene targeting is their ability to efficiently replicate donor and own template DNA, thereby ensuring sufficiently available homology-directed repair templates. However, the dependence of geminiviruses on replication initiator protein (Rep) limits their application in gene-editing as Reps are known to interact with several core proteins of host cells involved in the cell cycle, DNA replication and repair, and DNA methylation machinery.

Among the RNA viruses, both positive-strand viruses (PSV) and negative-strand viruses (NSV) have been used for genome-editing. In general, sgRNAs expressed in PSV vectors are co-infiltrated with Cas9 expressing *Agrobacterium* cultures (Fig. 1B-C). The widely used PSVs include TRV, tobacco mosaic virus (TMV), barley stripe mosaic virus, beet necrotic yellow vein virus, and foxtail mosaic virus with reported mutation frequencies of 30-56%, 70%, 48-78%, 85%, and 3-91% in the target tissues, respectively (Wang et al. 2020 and reference cited therein). One of the major drawbacks of most PSVs is their narrow cargo range, typically <1 kb. Due to the larger size of CRISPR/Cas9 nucleases, which are generally 3 to 4.2 kb (~160 kDa), PSVs are often unable to accommodate oversized cargo. For overcoming this issue, constitutively expressed Cas9 transgenic plants have been used in most of the studies. In a recent study, Ariga et al. (2020) demonstrated the potential of potato virus X (PVX) vector for overcoming the cargo limitations. The presence of a flexible filamentous genome wrapped by a single CP made PVX is an ideal vector system for the simultaneous delivery of Cas9 and sgRNA to the tobacco leaf. They reported the presence of the mutation in 62% of the regenerants, which were heritable through the seeds of the regenerated plants. The authors also used a fused base editor cytidine deaminase and nickase SpCas9 (*Streptococcus pyogenes* Cas9) to induce sequence-specific C-to-T substitution in agroinfiltrated tobacco leaves and reported mutation in 61% of the regenerated shoots. Despite its advantages, the major drawbacks of PVX are its restricted host range (Solanaceae species) and dependence on aseptic tissue regeneration for obtaining mutated progenies.

To further subjugate the low cargo capacity-related issue, rhabdoviruses have been used in plants. Gao et al. (2019) recovered a recombinant plant cytorhabdovirus, barley yellow striate mosaic virus (BYSMV), and successfully used it to express Cas9 proteins targeting the green fluorescent protein in leaves of

transgenic tobacco line 16c. Ma et al. (2020) utilized a negative-strand Sonchus yellow net rhabdovirus (SYNV) based vector system on achieving DNA-free *in planta* delivery of gene-editing reagents (Fig. 1D). An advantage of NSV genomes is that they are always encapsulated to form RNAse-resistant nucleocapsids. Hence, (anti)genomic RNAs of SYNV would remain inaccessible to cleavage. The authors designed an SYNV tgtRNA (tRNA-gRNA-tRNA)-Cas9 cassette to preserve Cas9 and sgRNA activity. Endogenous tRNA-processing enzymes ensured the precise processing of this transcript to release authentic sgRNA. All constructs efficiently infected tobacco plants systematically, and a high frequency of mutagenesis, ranging from 40% to 90% for different genes, was reported in somatic cells. Using this approach, they also successfully demonstrated multiplex editing capability by cloning multiple sgRNAs in the same tgtRNA cassette. Approximately 93% of the plants regenerated through tissue culture from the virus-infected tissues contained targeted mutations. The progeny of these tissue culture regenerated plants also inherited the edited genes. As rhabdoviruses are known not to invade meristem or germline cells, seeds of the viral vector-infected plants produced offspring lacking the gene-edits. One of the disadvantages of SYNV is the narrow host range, which limits its potential use to diverse crop species. The difficulty of identifying and creating rhabdovirus infectious clones poses another challenge to their use as viral vectors.

In conclusion, modifying viral vectors for their role in simultaneously delivering gene-editing reagents has opened up new avenues for boosting gene-editing in plant genomes. The successful use of the virus-induced gene silencing and virus-induced overexpression approaches in the weed black-grass displays the growing potential of viral vectors beyond model species (Mellado-Sánchez et al. 2020). Augmentation of sgRNAs with endogenous mobile RNA sequences from multiple genes, including *Arabidopsis Flowering Locus T*, using TRV vectors, have further resulted in an enormous increase in the gene-editing frequencies (90-100%) in the somatic tissue (Fig. 1C). Moreover, 57–100% of the progenies obtained from infected plants contained heritable mutations (Ellison et al. 2020). While the SYNV system described by Ma et al. (2020) does not have a prerequisite of a CRISPR/Cas9 transgenic line, it is to be seen if this virus's inability to invade plant germline cells could be modified by incorporating mobile RNA sequences into sgRNAs, as demonstrated by Ellison et al. (2020) (Fig. 1E). The recent discovery of hypercompact CRISPR-Cas systems (~70 kDa) in huge bacteriophages by Pausch et al. (2020) is exciting. The Cas systems require a single active site for both CRISPR RNA (crRNA) maturation and crRNA-guided DNA cleavage. With the requirement of minimal T-rich protospacer adjacent motif, the hypercompact system is expected to expand target recognition capabilities relative to other CRISPR-Cas proteins. Its successful use in editing *Phytoene desaturase* gene in *Arabidopsis* further shows the application of this system in genome editing in plants. Overall, a smaller nuclease would offer more possibilities for overcoming the cargo barrier with multiple viral vector systems, including PSVs, for efficient gene-editing in plants (Fig. 1F). Combining developmental regulators (DRs) of meristem identity, as demonstrated by Maher et al. (2020) for *de novo* induction of meristems, with gene-editing reagents, successful integration of induced mutations in progenitor cells is also possible, that too bypassing aseptic culture. However, whether DRs can be combined with other emerging protocols to induce gene-edits, even in soil-grown plants, warrant further investigations. Identification of BYSMV system for SSN-mediated gene targeting in more species

and a PVX-like system for non-solanaceous plants would further expand the scope of gene-editing in plants. Although the rapid improvements in gene-editing research and concomitantly evolving virus vector systems have opened new exciting avenues for plant scientists, their combined potential is yet to be fully understood. Only further research in this area would unravel if editing a gene of interest in nearly any plant belonging to model or non-model species, in laboratory or field conditions, is possible with minimal or no tissue culture.

Declarations

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Conflicts of interest/Competing interests

Authors declare no conflict of interest or financial conflicts to disclose.

Availability of data and material

The authors declare that no new data was generated in this article.

Authors' contributions

RK conceived the idea. RK and SK wrote the original draft, and MSK edited it. The final draft was read and approved by all authors.

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Figures

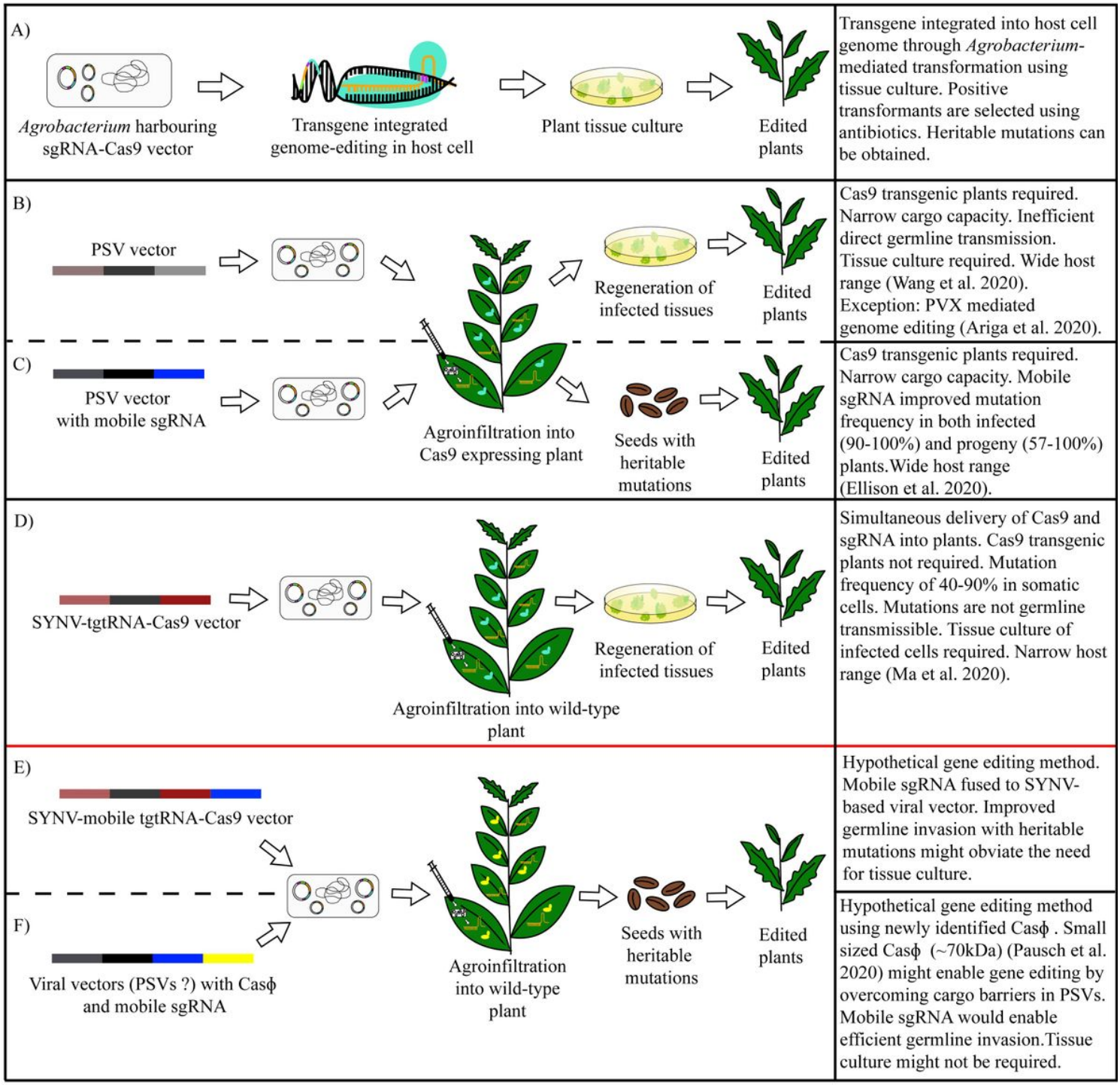

Figure 1

Schematic representation of the genome-editing methods in plants A) Conventional Agrobacterium-mediated transformation method: Targeted mutation is induced using Agrobacterium-mediated transfer of transgene (sgRNA/Cas9 T-DNA construct) to the host genome. Infected tissues are regenerated and selected using tissue culture in the presence of an antibiotic. B) Positive-strand viral vectors (PSVs) mediated genome editing: sgRNA is cloned into PSVs and introduced into stable Cas9 expressing plants using agroinfiltration. Targeted mutations are produced in the infiltrated plants but are not transmitted to

the germline cells. Regeneration of infected tissues by tissue culture produces mutated edited plants. PVX vector-mediated genome editing, which can accommodate both Cas9 and sgRNA in a single construct and overcome the narrow cargo limitation of most PSVs, remains an exception. C) Mobile sgRNA-mediated genome editing: Mobile RNAs are fused with sgRNA in PSVs to induce their systemic spread and facilitate invasion into germline cells. Agroinfiltrated Cas9 expressing plants contain targeted mutations which are also transmissible through seeds. D) Negative strand viral vector (NSV) mediated genome editing: Sonchus yellow mosaic virus (SYNV) vector is modified to carry Cas9 and tRNA-gRNA-tRNA (tgtRNA) sequences. Agroinfiltration of the SYNV-tgtRNA-Cas9 construct induces mutations in the infiltrated plants. Mutations are not germline transmissible. Regeneration of the infected tissues is required to obtain mutated progenies. E) Hypothetical SYNV-mobile tgtRNA-Cas9-mediated genome editing: SYNV-tgtRNA-Cas9 vector can be modified by replacing normal sgRNA with mobile sgRNA (sgRNA augmented mobile RNA sequence, as in Fig. 1C) to improve germline invasion and facilitate heritable mutations through seeds in the agroinfiltrated plants. F) Hypothetical viral vector with hypercompact Cas□ (~70 kDa which is half of the size of Cas9 nuclease) and mobile sgRNA-mediated genome editing: Cas9 in viral vectors can be replaced by the newly identified hypercompact Cas□ to overcome narrow cargo barrier of PSVs. The fusion of sgRNA with mobile RNAs would enable efficient germline invasion.