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Current and future editing reagent delivery systems for plant genome editing

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Many genome editing tools have been developed and new ones are anticipated; some have been extensively applied in plant genetics, biotechnology and breeding, especially the CRISPR/Cas9 system. These technologies have opened up a new era for crop improvement due to their precise editing of user-specified sequences related to agronomic traits. In this review, we will focus on an update of recent developments in the methodologies of editing reagent delivery, and consider the pros and cons of current delivery systems. Finally, we will reflect on possible future directions.

genome editing, DNA, RNA, RNP and virus delivery, *Agrobacterium*-mediated transformation, biolistic method, protoplast transfection, ttransgene-free genome edited plant

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INTRODUCTION

Traditional plant breeding and genetic modification (GM) techniques have contributed to increasing crop yields. Traditional plant breeding techniques, including conventional intergeneric crosses, chemical and physical mutagenesis and other breeding methods are non-specific, either large segments of genome exchanged or random sites mutated, and time-consuming of backcross to segregate the unwanted changes in their offspring. Since the 1990s, traditional plant breeding techniques has been complemented by transgenic approaches (Hartung and Schiemann, 2014). These approaches have produced varieties with specific resistances against plant pests and diseases, such as Bt cotton and glyphosate-resistant maize, and with a large number of desirable nutritional properties (e.g. "purple" tomato, enriched for anthocyanin, or "Golden Rice" containing beta carotene). Transgenic approaches (also known as genetic modification or GM) provide great opportunities but also raise concerns about possible negative impacts such as gene flow to other plants, ecological damage, toxicity and allergenicity. Also GM technology lacks precision and is time-consuming.

Recent advances in genome editing have provided opportunities to address these shortcomings and allow scientists to mutate specific genes, reprogram epigenetic markers, and generate site-specific sequence modifications. This type of targeted genome engineering employs engineered nucleases that induce site-specific double strand breaks that enable efficient gene modifications by either non-homologous end joining (NHEJ) or homology directed repair (HDR)

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(Voytas and Gao, 2014; Bortesi and Fischer, 2015). Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided endonucleases all cleave at specific sites generating targeted genome modifications as well as the gene addition used for trait stacking (Petolino, 2015; Weeks et al., 2016; Rinaldo and Ayliffe, 2015). CRISPR/Cas system is the most popular toolkit currently used for plant genome editing due to its high efficiency, easy design and high flexibility (Paul and Qi, 2016; Schaeffer and Nakata, 2015). Plants modified by these approaches are indistinguishable from ones generated by conventional breeding or mutagenesis. Therefore, they might be classified as non-GM, and if so would avoid the costly and time consuming requirements of GMO legislation (Hartung and Schiemann, 2014).

Current plant genome editing is based on the techniques used for plant transformation (Altpeter et al., 2016), and can currently only be achieved in a limited number of species; thus the limiting factors in plant genome editing are the ability to "deliver" editing reagents and to identify and recover successfully targeted plants. Meanwhile the need to produce transgene-free edited plants for commercial production imposes certain restrictions on editing reagent delivery involving DNA-free reagents as well as methods of delivery.

The general strategy and procedure for genome editing in plants is shown in Figure 1. Editing reagents, including DNA, RNA or protein have been successfully used for genome editing. Delivery of these editing components into plant cells and recovery of the edited events are the key steps in the process. However, no delivery methods have been developed specifically for plant genome editing and most are based on established plant transformation systems.

Delivery of the genome engineering reagents into plant cells is still the major barrier to the use of these technologies for creating novel traits (Baltes et al., 2014). The delivery methods can be divided into two main groups: indirect and direct. The indirect methods are based on the introduction of a plasmid-carrying gene construct into the target cell by means of bacteria—*Agrobacterium tumefaciens* or *A. rhizogenes*, or viruses including tobacco rattle virus (TRV) and geminiviruses. The direct methods can deliver most forms of editing reagents and do not use other biological organisms as mediators. The most common direct methods are protoplast transfection and microprojectile bombardment. Currently, agroinfiltration and protoplast transfection are usually used for transient assays to test editing efficiency in plant cells. *Agrobacterium*-mediated delivery, biolistic delivery, protoplast transfection delivery and viral carrier delivery have been used for obtaining stable editing events. The following sections will summarize the delivery methods used in plant genome editing.

In this review, current editing reagents, transient validation methods, delivery methods for recovery mutants and their applications are compared. Future directions, especially the development of cell-based editing reagent delivery technology, are also considered.

FORMS OF GENOME EDITING REAGENT

The most common types of editing reagents include ZFNs, TALENs, sgRNA and Cas9, which are in the form of DNA plasmids. In addition to their roles in genome editing, these DNA plasmids can be randomly integrated into the plant genome, which places them under the same regulation as transgenic plants. In order to produce non-transgenic genome edited plants, one useful way is to switch from DNA to RNA or protein including CRISPR/Cas9 ribonucleoprotein (RNP) complex.

DNA

DNA plasmids are the most common editing reagents and can be delivered by direct and indirect methods. The typical construct used for *Agrobacterium*-mediated method is shown in Figure 2.

Single-stranded oligonucleotides (*ssODNs*). ssODNs have been used as repair templates in genome editing. Sauer et al. used an ssODN together with CRISPR/Cas9 to develop an herbicide tolerance trait in flax (*Linum usitatissimum*) by accurate editing of the 5-enolpyruvylshikimate-3-phosphate

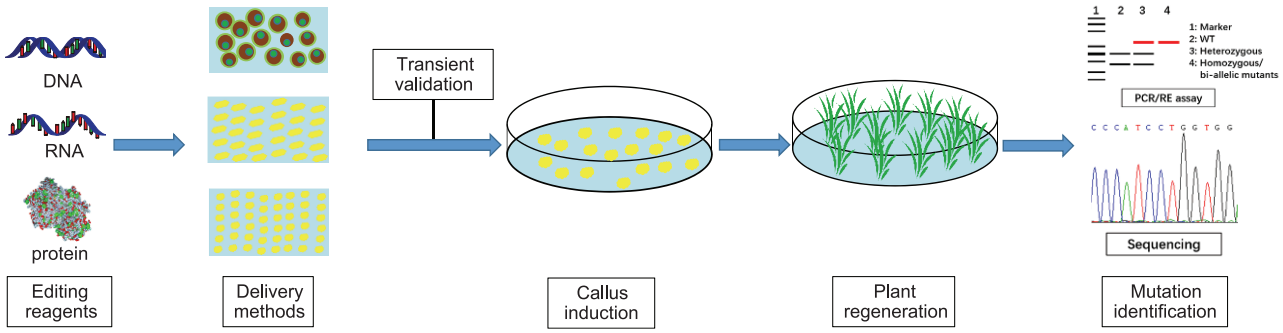

Figure 1 General procedure for genome editing in plants.

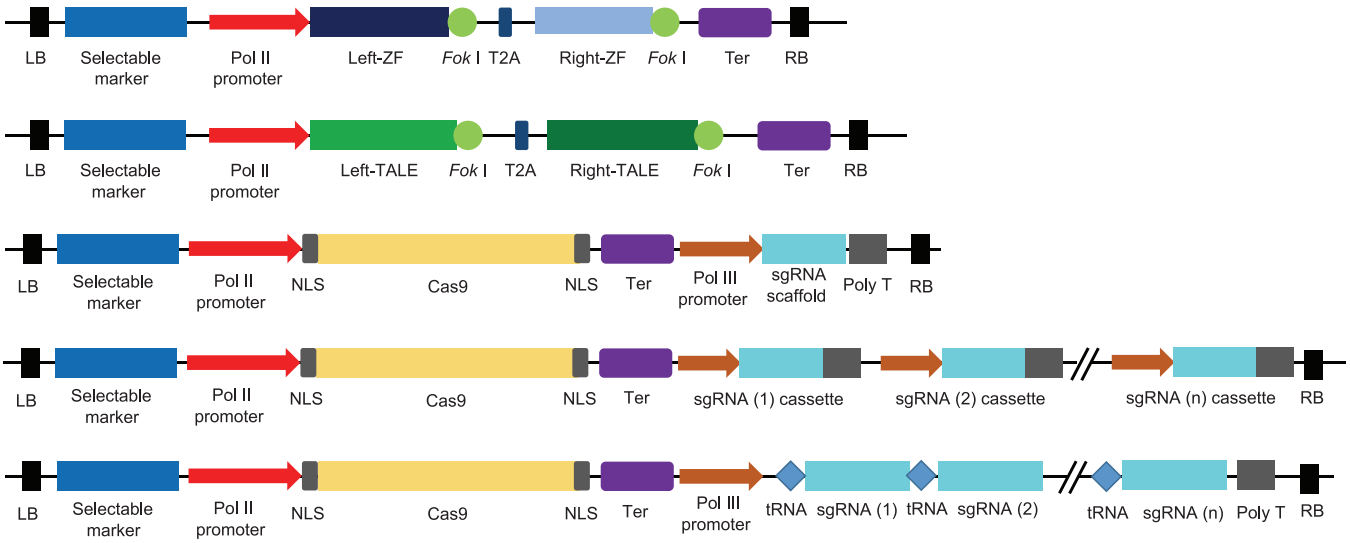

Figure 2 Construct design for ZFNs, TALENs and CRISPR/Cas9 when *Agrobacterium* is used as the delivery method.

synthase (*EPSPS*) genes (Sauer et al., 2016). EPSPS editing events were sufficiently frequent that whole plants could be regenerated from edited protoplasts without selection. These plants were subsequently shown to be tolerant of the glyphosate in greenhouse spray tests, and inheritance of the tolerance was confirmed.

Chimeric RNA/DNA oligonucleotides (*COs*). COs are short self-complementary chimeras consisting of RNA and DNA residues, capped at both ends by sequences that fold into a hairpin and render the COs resistant to cellular helicases and exonucleases. Furthermore, the RNA residues are 2′-O-methylated rendering the oligonucleotides resistant to the RNase H activity of mammalian cells (Cole-Strauss et al., 1996; Yoon et al., 1996). The low molecular weight (generally 68 nucleotides) of COs compared to conventional plasmid donors' makes it possible to introduce many copies into cells by biolistics. COs have been used for site-directed mutagenesis in maize (Zhu et al., 1999; Zhu et al., 2000), tobacco (Beetham et al., 1999), rice (Okuzaki and Toriyama, 2004) and wheat (Dong et al., 2006). Wang et al. co-delivered TALENs and a donor template for the *OsEPSPS* gene into rice and demonstrated that homozygous gene-edited mutants free from foreign DNA could be obtained in one generation (Wang et al., 2015).

RNA

RNA as a plant virus vector. Editing reagents transcribed as RNA via virus expression systems were introduced by Marton et al. (Marton et al., 2010), who demonstrated ZFN-mediated genome editing in tobacco and petunia using a TRV expression system. The TRV system (Ali et al., 2015) have been extensively used for plant genome editing using CRISPR/Cas9.

RNA only. Cas9 and sgRNA with target sequences plasmid can be *in vitro* transcribed and purified RNAs were ready to be delivered. Zhang et al. demonstrated the transiently expressed RNA of CRISPR/Cas9 was delivered into wheat immature embryos and transgene-free homozygous wheat mutants were obtained in the T_0 generation (Zhang et al., 2016).

Proteins and RNPs

Protein or ribonucleoprotein complex used as genome editing reagents can avoid transgene integration and has also been applied to many species. For example, Luo et al. delivered the TALEN proteins, ALS2T1L and ALS2T1R, targeting a sequence 306 bp downstream of the acetolactate synthase two genes (*NbALS2*) of *N. benthamiana* into protoplasts and demonstrated a 1.4% mutation frequency (Luo et al., 2015). Woo et al. transfected preassembled complexes of purified Cas9 protein and guide RNA (RNP) into plant protoplasts of *Arabidopsis thaliana*, tobacco, lettuce and rice and demonstrated targeted mutagenesis in regenerated plants (Woo et al., 2015). Recently, CRISPR/Cas9 RNP complexes have also been successfully applied in DNA-free genome editing in bread wheat (Liang et al., 2017) and maize (Svitashev et al., 2016).

METHODS FOR VALIDATION OF GENOME EDITING TRANSIENT EVENTS

Transient events here refer to genome editing events that are identified in cells or tissues that have not been regenerated into whole plants. Transient assays for such events are frequently used for preliminary experiments to test quickly the efficiency of editing reagents or specific editing mechanisms such as NHEJ or HDR pathways *in vivo*.

Protoplast transfection

Protoplast transfection is used for transient assays to test editing efficiency and measure editing events. In many

cases enzymatically digested leaf mesophyll protoplasts are isolated and DNA constructs or other biomaterials are introduced by electroporation or PEG-mediated transfection. Multiple DNA constructs with circular or linearized plasmids, or DNA expression cassettes, can be co-delivered into the same protoplasts. Successful genome editing using protoplast transfection was reported with ZFNs in tobacco, with TALENs in *Arabdopsis*, tobacco, *Brachypodium*, rice, maize and wheat and with CRISPR/CAS9 in *Arabdopsis*, *N. benthamiana*, rice, wheat, and maize (Table 1). Transient assays play a very important role when genome editing technologies are applied in plant research and crop improvement. Different gene targeting results, including indels, large deletions and homology-recombination mediated gene targeting can be quickly validated by transient protoplast assay. For example, Wright et al. transferred ZFNs and a donor DNA template into tobacco protoplasts by electroporation, and obtained 10% homologous recombination for editing the targeted gene (Wright et al., 2005). Shan et al. delivered TALENs targeted to four rice genes into rice protoplasts and TALENs targeted to eight *Brachypodium* genes into *Brachypodium* protoplasts by PEG-mediated transfection and all the desired mutations were generated (Shan et al., 2013a). Zhou et al. also demonstrated deletion of a large DNA fragment between two loci targeted by a combination of different sgRNAs, and deletion of a cluster of 10 labdane-related diterpenoid synthetic genes (about 245 kb) in rice chromosome 2 (Zhou et al., 2014). Protoplast-based plant genome editing has been examined using most editing methods including gene interruption (deletion and insertion) and gene replacement through NHEJ or HDR mechanisms.

Agroinfiltration methods

Agrobacterium spp. are soil-dwelling plant pathogens that cause tumorous growths on the roots of infected plants. Agrobacteria have the ability to transfer a particular DNA segment (T-DNA) into the nucleus of infected plant cells where it can be stably integrated into the host genome and transcribed. Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crown gall and hairy root, respectively. These strains contain a large megaplasmid (more than 200 kb) that plays a key role in tumour induction and is therefore named the Ti plasmid, or Ri in the case of *A. rhizogenes*. These two bacteria, modified to have both-disarmed Ti plasmid and binary vector, have been used as foreign DNA delivery tools for transgenic plant production (the detailed mechanisms are reviewed by Gelvin (Gelvin, 2003).

Agroinfiltration is an *A. tumefaciens*-mediated transient expression assay mainly used for dicot plant. *Agrobacterium* is infiltrated into plant leaves as a liquid culture, and mediates transfer of transgenes from the T-DNA region of the bacterial Ti plasmid into plant cells. Most of the plant cells in the infiltrated region express the transgene. It can be used with long DNA fragments (>2 kb) and can deliver several transgenes into the same cell (Kapila et al., 1997). The transgenes to be co-expressed can be present in different *Agrobacterium* cultures, which are mixed prior to infiltration. Alternatively, one *Agrobacterium* strain can contain multiple binary vectors or multiple genes in one binary vector. This method has been extensively deployed to test *in vivo* mutagenesis by genome editing technologies (Li et al., 2013; Belhaj et al., 2013; Nekrasov et al., 2013; Jia and Wang, 2014a; Piatek et al., 2015; Peer et al., 2015). For example, Peer et al. reconstituted GUS activity in apple and fig leaves by co-delivery of 35S::mGUS and 35S::QQR-ZFN (Peer et al., 2015). Mahfouz et al. using agroinfiltration, confirmed that a Hax3 TALE-based hybrid nuclease generated double strand breaks in its target sequence in *N. benthamiana* leaves (Mahfouz et al., 2011). Li et al. co-expressed pcoCAS9 and *AtPDS3* or *NbPDS* gRNA in a single binary plasmid in *N. benthamiana* and *Arabidopsis* leaves and the expected mutations were identified in the two target sequences of both species (Li et al., 2013).

Validation in hair roots

A. rhizogenes can also be used to test editing efficiency for a specific editing factor in plant root hairs. So far, this method has only been used in soybean genome editing (Curtin et al., 2011; Jacobs et al., 2015; Cai et al., 2015; Du et al., 2016). For example, Curtin et al. used the root hair transformation method to evaluate ZFN mutagenesis of the nine endogenous soybean genes (*DCL1a/DCL1b*, *DCL4a/DCL4b*, *DCL2a*, *DCL2b*, *RDR6a*, *RDR6b*, and *HEN*) and demonstrated mutations in all but two targets, *DCL2a* and *2b* (Curtin et al., 2011). Du et al. compared the efficiencies of TALEN and CRISPR/Cas technologies targeted to two genes—*GmPDS11* and *GmPDS18* in root hairs of soybean (Du et al., 2016).

DELIVERY VEHICLES FOR GENERATING STABLE PRECISELY MUTATED OR EDITED PLANTS

Indirect methods

The indirect methods are based on the introduction of a plasmid-carrying gene construct into the target cell by means of *Agrobacterium*, or plant virus systems including TRV and geminiviruses.

Agrobacterium-mediated DNA delivery. Agrobacterium-mediated DNA delivery accounts for about 80% of the mutations and editing events that have been reported in the public domain (Table 1), covering almost all model plant species including *Arabidopsis*, *N. benthamiana*, tobacco, petunia; main

Table 1 Summary of reports of plant genome editing using ZFNs, TALENs and Crispr/Cas9 technology[a)]

Plant species/Editor/Targeted gene(s)	Targeted outcome	Delivery method for transient assay or stable edited cells	Delivery method for stable events	Reference
At/C/PDS3, FLS2, RACK1b, 1c	Deletion, replacement and insertion (Multiplex)	Protoplast transfection, Agroinfiltration		Li et al., 2013
At/C/-GFP	Deletion and insertion	Agroinfiltration		Jiang et al., 2013
At/C/-GFP	Deletion and insertion		Floral dipping	Jiang et al., 2014
At/C/-BRI1, *JAZ1*, *GAI*, *YFP*	Deletion and insertion	Protoplast transfection	*A. tumefaciens*	Feng et al., 2013
At/C/-BRI1, JAZ1, GAI, CHL1, AP1, TT4, GUUS	Deletion and insertion		*A. tumefaciens*	Feng et al., 2014
At/C/-CHL1, CHL2, TT4i	Deletion, replacement (HDR, NHEJ) and insertion (Multiplex)	Protoplast transfection	*A. tumefaciens*	Mao et al., 2013
At/C/-ADH1	Replacement (HDR)		Floral dipping	Schiml et al., 2014
At/C/-TRY, CPC, *ETC2*	Deletion and insertion		Floral dipping	Xing et al., 2014
At/C/-5g55580 with 3 targets sets	Deletion and insertion		Floral dipping	Ma et al., 2015
At/C/-ADH1, TT4, *RTEL1, Guus*	Deletion and insertion. (HR-*GUS* gene)		Floral dipping	Fauser et al., 2014
At/C/-ETC2, TRY, *CPC*	Deletion and insertion (Multiplex)		Floral dipping	Wang et al., 2015
At/C/-BRI1	Deletion and insertion		Floral dipping	Yan et al., 2015
Nb/C/-PDS3	Deletion, replacement (HDR, NHEJ) and insertion	Protoplast transfection, Agroinfiltration		Li et al., 2013
Nb/C/-PDS	Deletion	Agroinfiltration		Belhaj et al., 2013
Nb/C/-PDS	Deletion	Agroinfiltration	*A. tumefaciens*	Nekrasov et al., 2013
Nb/C/-PDS	Deletion	Agroinfiltration		Upadhyay et al., 2013
Nb/C/-Transcriptional activation-EDLL domain, dHax3 TAD of phytopathogenic *Xanthomonas* spp. *Repression*-SRDX repression domain	Regulation	Agroinfiltration		Piatek et al., 2015
Nb/C/-PCNA, PDS	Deletion and insertion		TRV-mediated transformation	Ali et al., 2015
GFP	Deletion and insertion (NHEJ)	Agroinfiltration		Jiang et al., 2013
Nb/C/-PDS, IspH, fsGUS	Deletion and insertion		Agro-geminivirus	Yin et al., 2015
Nt/C/-PDS, PDR6	Deletion and insertion	Protoplast transfection		Gao et al., 2015
Nt/C/-SurA, *SurB*	Deletion and insertion	Agro-geminivirus		Baltes et al., 2014
Os/C/-ROC5, SPP, YSA	Deletion and insertion		*A. tumefaciens*	Feng et al., 2013
Os/C/-SWEET11, SWEET14, dsRED	Deletion and insertion (NHEJ)	Protoplast transfection		Jiang et al., 2013
Os/C/-NbPDS	Deletion and insertion	Agoinfiltration		Belhaj et al., 2013
Os/C/-PDS-SP1, BADH2, 02g23823, MPK2	Deletion, replacement (HDR, NHEJ) and insertion	Protoplast transfection	Biolistic delivery	Shan et al., 2013b
Os/C/-MYB1	Deletion and insertion	Protoplast transfection	*A. tumefaciens*	Mao et al., 2013
Os/C/-MPK5	Deletion and insertion	Protoplast transfection		Xie and Yang, 2013
Os/C/-CAO, LAZY1	Deletion		*A. tumefaciens*	Miao et al., 2013
Os/C/-PTG1, 2, 3, 4, 5, 6, 7, 8, 9	Deletion and insertion (individual and multiplex)		*A. tumefaciens*	Xie et al., 2015
Os/C/-BEL	Replacement		*A. tumefaciens*	Xu et al., 2014
Os/C/-11 *FTL* genes, *GSTU*, *MRP15*, *AnP Waxy*	Deletion, substitution (HDR, NHEJ) and insertion		*A. tumefaciens*	Ma et al., 2015
Os/C/-SWEET1a, 1b, 11 and 13; P450; 10 diterpenoid genes	Deletion and insertion -large deletion (245 kb)	Protoplast transfection	*A. tumefaciens*	Zhou et al., 2014
Os/C/-PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP, YSA	Deletion, substitution (HDR, NHEJ) and insertion		*A. tumefaciens*	Zhang et al., 2014

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Plant species/Editor/Targeted gene(s)	Targeted outcome	Delivery method for transient assay or stable edited cells	Delivery method for stable events	Reference
Pre-integrated DsRED	Deletion and insertion		*A. tumefaciens*	Mikami et al., 2015
Os/C/-DMC1A	Deletion and insertion			Mikami et al., 2016
Ta/C/-MLO	Deletion and insertion (NHEJ)	Protoplast transfection		Shan et al., 2013b; Wang et al., 2014
Ta/C/-GW2(RNP)	Deletion and insertion	Protoplast transfection	Biolistic delivery	Liang et al., 2017
Ta/C/Inox, PDS	Deletion	Agroinfiltration		Upadhyay et al., 2013
Hv/C/-PM19	Deletion and replacement		*A. tumefaciens*	Lawrenson et al., 2015
Ds/C/-RED2	Deletion and insertion	*A. tumefaciens*		Jiang et al., 2013
Mp/C/-ARF1	Deletion		*A. tumefaciens*	Sugano et al., 2014
Zm/C/-IPK	Deletion and insertion	Protoplast transfection		Liang et al., 2014
Zm/C/-HKT1	Deletion and insertion (multiplex)	Protoplast transfection	*A. tumefaciens*	Xing et al., 2014
Zm/C/LIG1, *MS26*, *MS45*, *ALS1*, *ALS2*	Deletion, replacement and gene insertion		Biolistic delivery	Svitashev et al., 2015
Zm/C/-LIG, *MS26*, *MS45*, *ALS2*	Deletion and insertion		Biolistic delivery	Svitashev et al., 2016
Zm/C/-ARGOS8	Deletion , insertion and swap		Biolistic delivery	Shi et al., 2017
Gm/C/-preintegrated *gfp5a', gfp3a' 07g14530, 01gDDM1, 11gDDM1, 01g+11gDDM1-Chr-1,01g+11gDDM1-Chr11, Met1-04g, Met1-06g, miR1514, miR1509*	Deletion, insertion and replacement		*A. rhizogenes*	Jacobs et al., 2015
Gm/C/-DD20, *DD43*, *ALS1*	Deletion, insertion, replacement (HDR) and editing		Biolistic delivery	Li et al., 2015
Gm/C/-06g14180, *08g02290, Glyma12g37050*	Deletion and insertion		*A. rhizogenes*	Sun et al., 2015
Gm/C/-Transgene *BAR, FEI, FEI2, SHR*	Deletion and insertion		*A. rhizogenes*	Cai et al., 2015
Gm/C/-PDS11, GlymaPDS18	Deletion and insertion	*A. rhizogenes*	*A. tumefaciens*	Du et al., 2016
Cs/C/PDS	Deletion and replacement	Agroinfiltration		Jia and Wang, 2014a
Cp/C/CsPDS	Deletion and replacement	Agroinfiltration	*A. tumefaciens*	Jia and Wang, 2014b
Cp/C/CsLOB1 promoter	Regulation			Jia et al., 2016
Sl/C/-AGO7	Deletion and replacement		*A. tumefaciens*	Brooks et al., 2014
Sl/C/-Ant1	Insertion		Agro-germinivirus	Čermák et al., 2015
Sl/C/-RIN	Deletion and insertion		*A. tumefaciens*	Ito et al., 2015
St/C/-IAA2	Deletion and replacement		*A. tumefaciens*	Wang et al., 2015
St/C/-ALS1	Deletion and insertion		*A. tumefaciens*	Butler et al., 2015
St/C/-GBSS	Deletion and insertion		Potoplast transfection	Andersson et al., 2017
Pt/C/-PDS	Deletion and replacement		*A. tumefaciens*	Fan et al., 2015
Bo/C/-C.GA4.a	Deletion and replacement		*A. tumefaciens*	Lawrenson et al., 2015
Ps/C/-4'OMT2	Deletion	Agroinfiltration TRV-mediated		Alagoz et al., 2016
Cs/C/-eIF4E	Deletion and insertion		*A. tumefaciens*	Chandrasekaran et al., 2016
Vv/C/-IdnDH	Deletion and insertion		*A. tumefaciens*	Ren et al., 2016
Vv/C/-MLO-7	Deletion and insertion	Protoplast transfection		Malnoy et al., 2016
Md/C/-DIPM-1, DIPM-2, DIPM-4	Deletion and insertion	Protoplast transfection		Malnoy et al., 2016
At/Z/-Pre-integrated QQR	Deletion and insertion		Floral dipping	Lloyd et al., 2005
At/Z/-Incomplete *GUS* gene	Deletion and insertion		*A. tumefaciens*	Tovkach et al., 2009

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Plant species/Editor/Targeted gene(s)	Targeted outcome	Delivery method for transient assay or stable edited cells	Delivery method for stable events	Reference
At/C/-ADH1, TT4	Deletion and insertion	Protoplast transfection	Floral dipping	Zhang et al., 2010
At/Z/-ABI4	Deletion and substitution		Floral dipping	Osakabe et al., 2010
At/Z/-Pre-integrated target sequence	Deletion and insertion		Floral dipping	de Pater et al., 2009
At/Z/-PPO	Replacement (HDR)		Floral dipping	de Pater et al., 2013
At/Z/-Pre-integrated *GUS* gene	Deletions and substitutions	viral vectors		Vainstein et al., 2011
At/Z/-Pre-integrated *GFP*	Replacement with hph (HDR)		*A. tumefaciens*	Weinthal et al., 2010
At/Z/-ADH1	Replacement (HDR) (in the absence of DNA repair proteins KU70 and LIG4)		Floral dipping	Qi et al., 2013a
At/Z/-3 *RLK* gene clusters, 1 large R gene cluster	Deletion, inversion and duplications		Floral dipping	Qi et al., 2013b
At/Z/-ADH1	Replacement		Biolistic delivery -geminivirus	Baltes et al., 2014
Petunia/Z/-Pre-integrated target *GUS* sequence	Deletion and insertion		*A. tumefaciens*	Marton et al., 2010
Gm/Z/-DCL1(DCL1a/DCL1b), DCL4 (DCL4a/DCL4b), DCL2a, DCL2b, RDR6a, RDR6b, HEN1a	Deletion and insertion	*A. rhizogenes*	*A . rhizogenes*	Curtin et al., 2011
Nt/Z/-gus:nptII	Replacement (HDR)	Agro-geminivirus		Baltes et al., 2014
Nb/Z/-NtSuR, NtSuRB	Deletion, replacement (HDR, NHEJ) and insertion	Protoplast electroporation		Townsend et al., 2009
Nb/Z/-Preintegrated GUS:NPTII	Replacement (HDR)	Protoplast electroporation		Wright et al., 2005
Nb/Z/-Pre-integrated target sequence *GFP*	Replacement (HDR)		*A. tumefaciens*	Cai et al., 2009
Nt/Z/-CHN50	Insertion of PAT gene		*A. tumefaciens*	Cai et al., 2009
Nb/Z/-GFP and *GUS*	Deletion and replacement (editing)	Agro-geminivirus	Agro-geminivirus	Baltes et al., 2014
Nb/Z/-Pre-integrated target sequence *GFP*	Replacement with hph (HDR)		*A. tumefaciens*	Weinthal et al., 2010
Nb/Z/-Pre-integrated target *GUS* sequence	Deletion and insertion		*A. tumefaciens*	Marton et al., 2010
Nb/Z/-Incomplete *GUS* gene	Deletion and insertion		*A. tumefaciens*	Tovkach et al., 2009
Zm/Z/-IPK	Replacement and gene insertion (HDR)		WHISKERS™	Shukla et al., 2009
Zm/Z/-Pre-integrated target sequence *PAT*	AAD1 gene insertion		Biolistic method	Ainley et al., 2013
Bn/Z/-A ZFP-TF for *KASII* expression	Gene regulation		*A. tumefaciens*	Gupta et al., 2012
Md/Z/-Pre-integrated target sequence *QQR–ZFN*	Deletion and insertion	Agroinfiltration	*A. tumefaciens*	Peer et al., 2015
Fc/Z/-Pre-integrated target sequence *QQR–ZFN*	Deletion and insertion	Agroinfiltration	*A. tumefaciens*	Peer et al., 2015
At/T/-CLV3	Deletion and insertion		Floral dipping	Forner et al., 2015
Nt/T/-ALS (SurA, and SurB)	Deletion, insertion and replacement (HDR)	Protoplast transfection		Zhang et al., 2013
Nt/T/-SurA and SurB	Deletion or insertion	Agro-geminivirus		Baltes et al., 2014
Nb/T/-Effector binding element (EBE)	Deletion or insertion	*A. tumefaciens*		Mahfouz et al., 2011
Nb/T/-ALS	Deletion	Agro-geminivirus		Baltes et al., 2014

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Plant species/Editor/Targeted gene(s)	Targeted outcome	Delivery method for transient assay or stable edited cells	Delivery method for stable events	Reference
Nb/T/-ALS2	Deletion	Protoplast transfection-mRNA		Stoddard et al., 2016
Nb/T/-FucT and XylT	Multiple deletion		Protoplast transfection	Li et al., 2016
Os/T/-EPSPS	Deletion and substitution		Biolistic transformation	Wang et al., 2015
Os/T/-11N3 (also called *SWEET14*)	Deletion and insertion		*A. tumefaciens*	Li et al., 2012
Os/T/-DEP1, BADH2, CKX2, SD1	Deletion, substitution and insertion	Protoplast transfection	Biolistic delivery	Shan et al., 2013a
Os/T/-BADH2, CKX2, DEP1	Deletion and insertion		*A. tumefaciens*	Shan et al., 2015
Os/T/-ALS	Homologous recombination		Biolistic delivery	Li et al., 2016
Os/T/-CSA, PMS3, DERF1, GN1a, TAD1, MST7, MST8	Deletion, substitution and insertion		*A. tumefaciens*	Zhang et al., 2016
Bd/T/-ABA1, *CKX2, SMC6, SPL, SBP*, *COI, RHT, HTA1*	Deletion, substitution and insertion	Protoplast transfection	Biolistic delivery	Shan et al., 2013a
Hv/T/-Promoter of *HvPAPhy*_a	Deletion		*A. tumefaciens*	Wendt et al., 2013
Hv/T/-Pre-integrated target sequence *GFP*	Deletion and insertion		*A. tumefaciens* (Pollen)	Gurushidze et al., 2014
Ta/T/-MLO	Deletion and insertion	Protoplast transfection	Biolistic delivery	Wang et al., 2014
Zm/T/PDS, IPK1A, IPK, MRP4	Deletion	Protoplast transfection	*A. tumefaciens*	Liang et al., 2014
Zm/T/-glossy2	Deletion	Biolistic delivery	*A. tumefaciens*	Char et al., 2015
Gm/T/-FAD2-1A, *FAD2-1B*	Deletion and insertion	*A. rhizogenes*	*A. rhizogenes*	Haun et al., 2014
Gm/T/-PDS11, *PDS18*	Deletion and insertion	*A. rhizogenes*	*A. tumefaciens*	Du et al., 2016
Sl/T/-PRO	Deletion and insertion		*A. tumefaciens*	Lor et al., 2014
Sl/T/-Ant1	Insertion		Agro-germinivirus	Čermák et al., 2015
St/T/-VInv	Deletion and insertion	Protoplast transfection	Protoplast transfection	Clasen et al., 2016
St/T/-ALS	Deletion and insertion	Protoplast transfection	Protoplast transfection	Nicolia et al., 2015
St/T/-Ubi7	Insertion of a herbicide resisitsnt gene *ASL*	Agroinfiltration	*A. tumefaciens*	Forsyth et al., 2016
Ss/T/-COMT	Deletion and insertion		*A. tumefaciens*	Jung and Altpeter, 2016

a) At, *A. thaliana* (*Arabidopsis*); Nt, *N. benthamiana*; Nc, *N. tobaccum* (Tobacco); Os, *O. sativa* (Rice); Hv, *H. vulgare* (barley); Sb, *S. bicolor* (sorghum); Mp, *M. polymorphal* (liverwort); Zm, *Z. mays* (maize); Gm, *G. max* (soybean); Cs, *C. sinensis* (sweet orange); Cp, *C. paradisi* (grapefruit); Sl, *S. lycopersicum* (tomato); St, *S. tuberosum* (potato); Pt, *P. tomentosa* (populous); Bo, *B. oleracea* (oil rape); Ps, *P. somniferum* (opium poppy); Ca, *C. sativus* (cucumber); Vv, *V. vinifera* (grape); Md, *M. domestica* (Apple); Bn, *B. napus* (canola); Fc, *F. carica* (Fig), Bd, *Brachypodium distachyon*; Ss, *Saccharum* spp. Hybrids (sugarcane); C, Crispr/CAS 9; Z, Zinc-finger nucleases; T, Talen; Agro-germinivirus, *Agrobacterium*-mediated germinivirus delivery.

crop species including rice, maize, *Brassica*, barley and soybean; vegetable and fruit crops including tomato, apple and fig and forest crops including *populus*. *A. rhizogenes* has also been used for genome editing, but almost exclusively for editing soybean (Curtin et al., 2011; Jacobs et al., 2015; Du et al., 2016).

Delivery to single targets. When a genome editing system is being used to target a single gene locus including the multiple genes of a gene family with conserved sequences, all the components of the system, whether ZFNs, TALENs or CRISPR/Cas9, are assembled into a construct in one binary vector together with a selectable marker cassette, as shown in Figure 2. This binary vector is then introduced into an agrobacterium strain. Most mutagenesis in plants so far has been aimed at single gene targets, particularly in TALEN and ZFN systems (Table 1).

Delivery to multiple targets (multiplex targeting). Theoretically, editors can be constructed as single gene targeting cassettes or multiple gene targeting cassettes in binary constructs. Multiple binary vectors can be delivered into *Agrobacterium* and so co-transformed into plant cells (Gelvin, 2003). Two *Agrobacterium* strains each containing a binary vector with editors for different target sites can also be mixed together to infect the same plant cells, or one *Agrobacterium* strain harboring a binary vector containing editors for several different target sites can be constructed. Multiplex genome editing by ZFNs and TALENs has been limited by the large gene cassette of the editing reagents for each target. CRISPR/Cas9 system targeting multiple loci can share the Cas9 protein and only the transcription of sgRNA for each

target is needed. The sgRNAs can be transcribed separately by multi-promoters or by one promoter with tRNA-processing system (summarized in Raitskin and Patron, 2016). For example, Ma et al. prepared two constructs with eight and three sgRNA expression cassettes, respectively, targeting 11 genes of an FT-like (FTL) gene family with 13 members and generated rice plants that were mutant for all the genes, with a phenotype of premature leaf senescence (Ma et al., 2015). They also demonstrated simultaneous targeting of three sites in an *OsWaxy* gene, which functions in the synthesis of amylose in rice, resulting in loss of up to 14% of amylose content. Xie et al. developed a polycistronic tRNA-gRNA (PTG) system in rice, for which each sgRNA is flanked by tRNA and can be precisely processed into single sgRNAs and multiple genome editing and large deletions are obtained in T0 generation (Xie et al., 2015).

Agrobacterium-mediated delivery is the most popular method of plant transformation; it is convenient, cheap and easy to set up in the laboratory and available in most plant biology laboratories. However, it has some drawbacks: (i) there is limited opportunity to construct independent editing reagents in different binary vectors; (ii) it cannot deliver small DNA fragments, RNA or protein; (iii) the use of this method still depends on the recipient genotype, especially for monocot plants; (iv) it is hard to avoid the Ti plasmid backbone being integrated into the plant genome, thus producing transgenic plants.

Virus-mediated editing reagent delivery. It has not been as common to use virus vectors to deliver genetic material into plants as in mammalian cells, where they were first used. In plants, the first viral vector employed was tobacco mosaic virus (TMV*),* resulting in virus-induced gene silencing (VIGS) of an endogenous gene in *N. benthamiana* (Kumagai et al., 1995). From then on, various plant viruses have also been exploited for VIGS. Since the great majority of plant virus genomes consist of single-stranded RNA (ssRNA), gene delivery for VIGS is often performed by inoculating viral vector transcripts synthesized *in vitro*. With DNA viruses such as geminiviruses, inoculation is simplified since it requires only the viral DNA. Alternatively, the virus genome can be inserted as a cDNA fragment into a binary vector and introduced into the plant cell via agroinfection, so facilitating the delivery process. Recently TRVs have been shown to be efficient vectors for gene delivery of RNA into plant cells for genome editing (Marton et al., 2010; Ali et al., 2015). Geminiviruses have also been modified to introduce genome editing reagents and achieve genome modifications in plants, especially for homologous directed recombination mediated gene targeting (Baltes et al., 2014; Čermák et al., 2015; Gil-Humanes et al., 2017).

TRV is an efficient vector for plant virus-induced gene silencing, facilitating functional genomics in diverse plant species. TRV is an ssRNA virus with a bipartite genome consisting of two positive-sense single-stranded RNAs, designated RNA1 and RNA2. The RNA2 genome can be modified to carry exonic gene fragments for post-transcriptional gene silencing (Dinesh-Kumar et al., 2003), the target gene fragment for silencing being inserted into the RNA2 element. Inoculation, either mechanical or via agroinfiltration, requires the presence of both genome components. In the case of agroinfiltration, two different *Agrobacterium* clones, one carrying the RNA1 genome and the other with the RNA2 containing the target gene fragment, are mixed together and co-infiltrated into leaf tissue (English et al., 1997). Since the virus RNA genome does not integrate into the plant genome, the edited products are not transgenic.

TRV has also shown promise as a vector for genome engineering: when RNA2 was replaced with an RNA for the Zif268:FokI ZFN, targeted genome modifications were recovered in a pre-integrated reporter gene in somatic tobacco and petunia cells (Marton et al., 2010). TRV has also been used to deliver components of CRSIPR/Cas9. Ali et al. modified the *PDS* gene in a Cas9 transgenic *N. benthamiana* by delivering a vector derived from TRV RNA2 containing gRNA for *PDS*, and introduced the TRV into leaves of *N. benthamiana* overexpressing Cas9 (Cas9-OE) via agroinfection of mixed *Agrobacterium* cultures harbouring the RNA1 genome (Ali et al., 2015). They also demonstrated that multiple targets (both *PDS* and *PCNA*) cold be simultaneously edited by mixing RNA2 cultures that conferred sequence specificities for multiple targets with RNA1 cultures.

Geminiviruses, with their DNA-based genomes, enable direct infection with plasmid DNA by mechanical inoculation instead of requiring *in vitro* transcription. Geminiviruses are a large family of plant viruses with single stranded, circular DNA genomes of 2.5 to 3.0 kb. Once inside a host cell's nucleus, their single-stranded genome is converted to a double-stranded intermediate by host DNA polymerases. This double-stranded genome is then used as a template for transcription of virus genes and for rolling-circle replication. The only geminivirus protein necessary for replication is the replication initiator protein (Rep). Rep initiates rolling-circle replication by binding to a site within the large intergenic region (LIR; mastreviruses), intergenic region (curtoviruses and topocuviruses), or common region (begomoviruses with bipartite genomes). There, it creates a single-strand nick within an invariant 9-nucleotide sequence (5′-TAATATTAC-3′) located at the apex of a conserved hairpin structure. Following rolling-circle replication, single-stranded genomes are either converted back to a double-stranded intermediates or encapsidated by coat protein to produce virions. These virions are then transported to adjacent cells through the plant's endogenous plasmodesmata pathways. Geminiviruses were developed as vectors for delivering DNA for plant genome editing and first used for transient expression of sequence-specific nucleases (ZFNs,

TALENs and CRSPR/Cas9 system) and delivery of DNA repair templates via bean yellow dwarf virus (BeYDV) by Baltes et al. (Baltes et al., 2014). In tobacco (*N. tabacum*), replicons based on the bean yellow dwarf virus enhanced gene gave targeting frequencies for the acetolactate synthase (*ALS*) gene one to two orders of magnitude higher than conventional *Agrobacterium tumefaciens* T-DNA. In addition to nuclease-mediated targeted mutagenesis, gene targeting via homologous recombination was also promoted by replication of the repair template in plant cells. This approach was further applied for repairing a non-functional *gus:nptII* gene through homologous recombination. Similar editing work with different genes in tobacco (*NbPDS3* and *NbIspH*) using a modified cabbage leaf curl virus (CaLCuV) vector was carried out by (Yin et al., 2015) and in tomato with BeYDV to insert a constitutive 35S promoter into the promoter region of anthocyanin mutant 1 gene (*ANT1*) at frequencies tenfold higher than traditional methods of DNA delivery (i.e., *Agrobacterium*) by Čermák et al. (Čermák et al., 2015) and others (Table 1). Recently, Gil-Humanes et al. has also applied this approach in wheat and gene targeting efficiency was enhanced by optimizing the wheat dwarf virus (WDV) system (Gil-Humanes et al., 2017).

Plant viruses can systemically infect a broad range of plant species, both naturally and under laboratory conditions. The virus vector is easily introduced into growing points of the plant via *Agrobacterium* and this can be followed by transient expression of sequence-specific nucleases that do not integrate into the genome. Furthermore, repair templates for genome editing can also multiply using the virus replication system. This delivery system may be an ideal method for plant gene targeting in the future.

Direct methods

Direct methods deliver genome editing components or reagents directly to the plant cell by physical or chemical means. The most common methods used for plant genome editing are biolistic delivery and protoplast transfection. Several other methods have been developed and have potential to be used for genome editing. Among them, the most frequently used for transgenic plant production are silicon carbide fibre (whiskers)-mediated transformation, electroporation of cells and tissues, electrophoresis of embryos, microinjection, transformation via the pollen tube pathway and liposome-mediated transformation (Rakoczy-Trojanowska, 2002). These direct delivery methods can deliver multiple DNA constructs simultaneously, including the reagents for gene targeting and the repair templates, and various forms of editing reagents including DNA fragments, RNA or proteins, and the direct methods do not rely on the genotype either as long as a regeneration system is available. Many successful plant genome editing systems have used protoplast transfection and biolistic methods (Table 1).

Protoplast transfection. Plant protoplasts can be obtained by enzymatically digesting cell walls, and genetic material including DNA, proteins and other reagents can be delivered into the protoplasts by electroporation or by polyethylene glycol (PEG) treatment. Mesophyll protoplasts are frequently used to regenerate dicot plants. Embryogenic callus-derived protoplasts are used for monocot species. Protoplast transfection has been the ideal delivery method for editing reagents due to the ability to deliver multiple components and the large number of transfectable cells. It is especially suitable for modifying genes with donor repair templates since a large number of cells can be transfected and regenerated, and gene editing events dependent on donor template repair, i.e. HR, can be recovered. The main disadvantages of direct protoplast transfection are problems with plant regeneration, especially in monocotyledonous plants.

Plant genome editing can be achieved using protoplast transfection and culture at the level of cell, tissue or whole plant depending on the regeneration capacity of the host. Wright et al. introduced both ZFN and target repair template DNA by electroporation into tobacco protoplasts of a transgenic line with a defective *GUS:NPTII* reporter gene and obtained plants with the reporter gene repaired by homologous recombination (Wright et al., 2005). Since then, protoplast transfection and regeneration have been used to demonstrate genome editing with TALENs in potato (Nicolia et al., 2015; Clasen et al., 2016), and with CRISPR/Cas9 ribonucleoproteins (RNPs) in potato (Andersson et al., 2017), *Arabidopsis*, tobacco (*N. attenuata*), and lettuce (*Lactuca sativa*) (Woo et al., 2015) and DNA-free genome editing plants were obtained in lettuce. For example, Clasen et al. reported that knockouts of the vacuolar invertase gene (*VInv*) in potato plants could be regenerated from protoplasts transfected with TALENs and their tubers had undetectable levels of reducing sugars (Clasen et al., 2016); processed chips made from these potatoes contained reduced levels of acrylamide and were lightly coloured. However, most regenerated plants have been limited to certain species, using the established protoplast regeneration system (Table 1).

Biolistic delivery. Biological ballistics, commonly known as biolistics, is a method of transfecting cells by bombarding them with microprojectiles coated with DNA. DNA is delivered using a gene gun with sufficient physical force to penetrate the plant cell wall, and the DNA is randomly integrated into the plant genome. This is the most common method for delivering DNA to transform plants; it is reasonably efficient, multiple DNA constructs can be delivered simultaneously and it is not restricted to particular plant species. The most distinctive factor limiting the use of this method is the presence of multiple copies of the introduced genes in the transfectants, which can lead to various undesirable effects such as gene suppression and/or altered gene expression in the recovered transgenic plants.

Plant genome editing has been achieved using biolistic DNA delivery (summarised in Table 1). For example, the precise gene disruption in *Brachypodium*, rice and wheat using either the TALEN or CRISPR/Cas system by Shan et al. (Shan et al., 2013a; Shan et al., 2013b), insertion of a herbicide resistance gene (*aad1*) into a ZFN targeted site by Ainley et al. (Ainley et al., 2013) and sequence replacement in the *HPT* gene and an endogenous *ALS1* gene via precise editing by HDR recombination in soybean using the CRISPR/Cas system (Li et al., 2015). Svitashev et al. obtained HDR-mediated specific target gene editing (*ALS*) via biolistic delivery of all reagents and templates in maize (Svitashev et al., 2015). They obtained some target gene insertion events via biolistic delivery that they failed to obtain by the agrobacterium method, which was used for comparison.

Recently, particle bombardment has been used for DNA-free genome editing in crops to bypass the GMO regulation. CRISPR/Cas9 reagents delivered as *in vitro* transcripts (IVTs) or ribonucleoproteins (RNPs) have been successfully mediated genome editing in hexaploid wheat (Liang et al., 2017). Also, transgene-free genome editing in maize by CRISPR/Cas9 RNPs has been demonstrated by Svitashev et al. (Svitashev et al., 2016).

Whisker-mediated delivery. This method has been demonstrated to generate targeted mutagenesis and gene insertion events at the *IPK1* locus mediated by ZFNs using embryogenic cell cultures derived from maize Hi-II (Shukla et al., 2009).

Other methods. Cell-penetrating peptides (CPPs), which are nonviral carrier systems, are capable of delivering nucleic acids and nanomaterials into cells. CPPs have become increasingly popular and are used as carriers of DNA, RNA, protein, nanomaterials, and pharmaceuticals. Typical CPPs are (i) made up of less than 30 amino acids, (ii) rich in arginine and lysine, (iii) positively charged or amphipathic, (iv) easy to prepare, and (v) nontoxic (Huang et al., 2015). They have been used to deliver DNA and protein into plant cells including gametophytic cells, as reviewed by Chugh et al. (Chugh et al., 2010). CPPs with a 12-amino-acid membrane translocation sequence (MTS) have also been used for intracellular delivery of the Cre recombinase into rice callus tissues by incubated calli in medium containing a His6-NLS-Cre-MTS protein construct; with this procedure site-specific DNA excision in rice (Cao et al., 2006) and transformation in citrus plants (Jensen et al., 2014) have been achieved.

Nano-biotechnology offers attractive vehicles for delivering editing reagents since nanoparticles can be precisely tailored to deliver a particular biomolecule to the cell, tissue, or organism of interest (Du et al., 2012). Mesoporous silica nanoparticles (MSNs) are used for this purpose. These porous nanoparticles are formed of a matrix of well-ordered pores with a high loading capacity for molecules like proteins (Popat et al., 2011). The pioneer work using MSNs to deliver biomaterials into plant cells was done in the laboratory of Dr Kan Wang (Martin-Ortigosa et al., 2012). MSNs have been used to co-deliver DNA and chemicals (Torney et al., 2007) as well as DNA and proteins (Martin-Ortigosa et al., 2012) to plant cells via biolistics. Martin-Ortigosa et al. used MSNs as carriers to deliver Cre recombinase into maize (*Zea mays*) cells (Martin-Ortigosa et al., 2014). Cre was loaded into the pores of gold-plated MSNs, which were biolistically delivered to plant cells harbouring loxP sites flanking a selectable gene, the glyphosate acetyltransferase gene (*gat*), and a reporter gene, the *Anemonia majano* cyan fluorescent protein gene (*AmCyan1*). Cre protein was released inside the cells, leading to recombination of the loxP sites, DsRed2 expression and elimination of both genes. Both indirect and direct editing reagent delivery methods are extensively used.

The direct method including protoplast transfection and the biolistic method facilitates multiple reagent delivery in various forms and may play an important role in future due to its high efficiency. The multiple random integration events with unwanted DNA fragments occurring by this method can be avoided and use of the non-DNA reagents may open up transgene-free gene targeting plants for commercial crop production. The indirect methods such as viral infection also provide opportunities to deliver different forms of reagent and are easy to use and cheap to set up. As pointed out in the review by Altpeter et al. (Altpeter et al., 2016), most editing reagent delivery and edited event recovery systems do not meet the need for applications in many crops due to the long tissue culture periods required to recover edited plants from engineered cells and tissues, the low frequency of stable events, the low DNA titres delivered by *Agrobacterium*-mediated gene transfer for HR events, and the low precision of bombardment-mediated gene transfer. Therefore, all these technologies need to be improved.

FUTURE PROSPECTS

Genomic scissors including engineered homing endonucleases/meganucleases (MENs), ZFNs, TALENs, especially CRISPR/Cas system are now used widely in various crop species and have been used to obtain targeted mutations, deletions, homologous recombination, as well as for making *cis* transgene stacks and transcriptional reprogramming of endogenous genes (Table 1). New crop varieties, with improved traits including disease resistance, improved food quality, and high yields, derived through these technologies will be developed in the near future, and the first commercial crop generated by genome editing has been approved for use in Canada (Pratt, 2014) despite the fact that it has not yet been decided whether these precision-engineered crops will be classified as GM or non-GM for regulatory purposes (Hartung and Schiemann, 2014; Voytas and Gao, 2014).

Recently, the USDA has indicated that the CRISPR-edited crops, including mushroom and waxy corn can enter into market without oversight because they do not contain foreign DNA materials.

Now, the most prominent issue for plant genome editing is the lack of an optimal delivery tool and recovery system for edited plant cells as well as a need for further understanding of editing mechanisms. Delivery methods are not available for all species and the currently available methods are limited to specific genotypes, tissues and types of culture. According to the literature, most genome editing is still focused on creating DNA breaks which result in gene disruption. However, there have been only a limited number of reports of modification of endogenous genes by sequence correction, replacement and addition via homologous recombination. These procedures have been impeded by lack of knowledge of molecular mechanisms and efficient methods for delivering multiple editing reagents. Ideally (i) The delivery method could be used to efficiently deliver editing reagents to particular genotypes of crop species. The explants would consist of meristematic tissue of any species that could be regenerated without limitation of genotype. Recent development on plant regeneration demonstrated in maize with transgene Baby boom (Bbm) and maize Wuschel2 (Wus2) genes (Lowe et al., 2016) showed potential to overcome tissue and genotype limitation. Delivery could be by virus so that it could spread systemically and would edit the plant germ line. (ii) Delivery would be via the plant germline i.e. pollination or artificial hybridization which would avoid any limitation of species, genotype or regeneration system. (iii) There would be an efficient regeneration system giving rise to a sufficient number of independent edited plants, i.e. a large number of cells would be transfected and regenerated. The protoplast regeneration system is a typical example, where millions of cells can be transfected and potentially thousands of plants can be regenerated. This would permit the regeneration of a sufficient number of independent plants edited by HDR or gene addition despite the low success rate. The challenge is to develop a protoplast regeneration system for all plant species. (iv) Genome editing nucleases including ZFNs, TALENs and CRISPR/Cas9 could be transiently expressed in the form of protein or RNA. This DNA-free system can remove the variability caused by choice of promoters selected to drive expression of vector-based CRISPR/Cas9 systems because not all promoters are functional in every cell or cell type. This approach would also generate transgene-free plants, thus increasing the acceptability of plants modified by these gene editors. (v) High efficient delivery methods are expected. Delivery methods using new technologies have being developed in medical research field, which showed high efficiency in mammalian cells. Except for the viral vectors, non-viral vectors containing lipid nanoparticles (LNPs), liposome (e.g., 1,2-dioleoyl-3-trimethylammonium-propane—DOTAP, cholesterol), polymers (e.g., Polyethylenimine-PEI, poly (L-lysine)-PLL)), cell-penetrating peptides (CPPs), and conjugates, as well as some novel ones such as cell-derived membrane vesicles (CMVs) have been applied to encapsulate the plasmid or mRNA of these programmable nucleases or nuclease proteins, and carry them into target tissues or cells without degradation (Kelley et al., 2016; Wang et al., 2016). Those methods can be used for gene delivery in plant, at least for protoplast transfection.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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