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Review Article

Recent advances in DNA-free editing and precise base editing in plants

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Genome-editing technologies based on the CRISPR (clustered regularly interspaced short palindromic repeat) system have been widely used in plants to investigate gene function and improve crop traits. The recently developed DNA-free delivery methods and precise base-editing systems provide new opportunities for plant genome engineering. In this review, we describe the novel DNA-free genome-editing methods in plants. These methods reduce off-target effects and may alleviate regulatory concern about genetically modified plants. We also review applications of base-editing systems, which are highly effective in generating point mutations and are of great value for introducing agronomically valuable traits. Future perspectives for DNA-free editing and base editing are also discussed.

Introduction

CRISPR (clustered regularly interspaced short palindromic repeats) systems originated in bacteria and archaea, and are adaptive immune systems deployed against invading phage and plasmid DNA [1]. Nowadays, these systems, especially the type-II *Streptococcus pyogenes* CRISPR/Cas9 system, have been widely used in plants to introduce targeted mutations for studying gene function and providing new avenues for crop improvement [2]. Recently, the type-V CRISPR/Cpf1 system was also employed to make mutant plants [3–7]. To take advantage of CRISPR systems, the CRISPR expression cassettes are delivered into plant cells, where they usually integrate into the plant genome and are expressed; cuts at chromosomal target sites are then made, producing site-specific DNA double-strand breaks (DSBs). An endogenous repair mechanism then repairs the DSBs by error-prone NHEJ (non-homologous end joining) or high-fidelity HR (homologous recombination) and can generate targeted genome modifications [8]. The mutant cells can be grown into plants, and transgene-free derivatives can be generated by segregating the integrated CRISPR cassettes by selfing or crossing. However, two problems restrict the widespread application of CRISPR systems. The first is the fact that the transformation process introduces foreign DNA into the plants, which raises regulatory concerns [9]; and the second is that HR-mediated knock-in and gene replacement occur at a substantially lower frequency than NHEJ-mediated gene knockout. Fortunately, the newly developed DNA-free and precise genome-editing approaches can reduce these problems. Here, we review these novel approaches (Table 1).

DNA-free genome editing

Unlike overexpression and RNAi technologies, which usually depend on the sustained expression of foreign plasmids, genome editing only needs transient expression of the genome-editing constructs; in fact, prolonged expression increases the risk of off-target effects in plants [10–12], which is a major concern for the CRISPR system [13–15]. Given the regulatory concerns and the need for transient expression, one promising editing method is DNA-free genome editing. In animal experiments, researchers have achieved DNA-free genome editing by directly injecting CRISPR/Cas9 RNA or

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Table 1 Recent advances in DNA-free editing and precise base editing in plants

Species	Target genes	Delivery methods	CRISPR editing type	Off-target	References
Arabidopsis, tobacco, rice, lettuce	AtPHYB, NaAOC, OsP450, OsDWD1, AtBRI1, LsBIN2	PEG-mediated protoplast transformation	CRISPR/Cas9 RNP	Not detected	[22]
Soybean, wild tobacco	GmFAD2, NaAOC	PEG-mediated protoplast transformation	CRISPR/Cpf1 RNP	Not detected	[23]
Wheat	TaGASR7, TaDEP1, TaGW2, TaPIN1, TaNAC2	Particle bombardment-mediated transformation	CRISPR/Cas9 DNA, CRISPR/Cas9 RNA	Detected	[27]
Maize	ZmLIG, ZmALS2, MS26, MS45	Particle bombardment-mediated transformation	CRISPR/Cas9 RNP	Not detected	[28]
Wheat	TaGASR7, TaGW2	Particle bombardment-mediated transformation	CRISPR/Cas9 RNP	Not detected	[29]
Rice	OsNRT1.1B, OsSLR	*Agrobacterium*-mediated transformation	APOBEC1-XTEN-nCas9	N/A	[37]
Rice	OsPDS, OsSBEIIb	*Agrobacterium*-mediated transformation	APOBEC1-XTEN-nCas9-UGI	N/A	[38]
Arabidopsis	AtALS	*Agrobacterium*-mediated transformation	APOBEC1-XTEN-nCas9-UGI	N/A	[39]
Rice, maize, wheat	OsCDC48, OsNRT1.1B, OsSPL14, ZmCENH3, TaLOX2	*Agrobacterium*/particle bombardment-mediated transformation	APOBEC1-XTEN-nCas9/dCas9-UGI	Not detected	[40]
Rice, tomato	OsALS, OsFTIP1e, SlDELLA, SlETR1	*Agrobacterium*-mediated transformation	nCas9/dCas9-PmCDA1	Detected	[41]
Rice	OsCERK1, *ipa1*, *pi-ta*	*Agrobacterium*-mediated transformation	APOBEC1-XTEN-nCas9/nCas9(VQR)-UGI	N/A	[43]
Rice	OsALS	*Agrobacterium*-mediated transformation	HR	N/A	[44]
Rice	OsALS	Particle bombardment -mediated transformation	HR	N/A	[45]
Flax	LuEPSPS	PEG-mediated protoplast transformation	HR	Not detected	[47]
Rice	OsEPSPS	Particle bombardment -mediated transformation	NHEJ	Not detected	[48]

Abbreviations: N/A, not available.
EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase gene.

ribonucleoprotein (RNP) into cell nuclei. For example, co-injection of Cas9 mRNA and sgRNA into zygotes resulted in mice carrying multiple mutations [16], and local delivery of engineered CRISPR/Cas9 RNP complexes into mouse brains generated genome-edited mice [17]. CRISPR/Cas9 RNP complexes can be directly transfected into nuclei of human cells via electroporation [18,19], or by using cell-penetrating peptides [20] or cationic lipids [21]. However, the cell wall of plants makes all these methods impossible, and recent advances in DNA-free plant genome editing can be largely divided into two categories according to the method of plant transformation employed.

Plant protoplasts are plant cells that retain cell activity but lack a cell wall and can be obtained from plant cells by treatment with cellulase. Like animal cells, plant protoplasts are good material for RNP-mediated transformation (Figure 1), and plant protoplasts can be transfected mainly by polyethylene glycol (PEG),

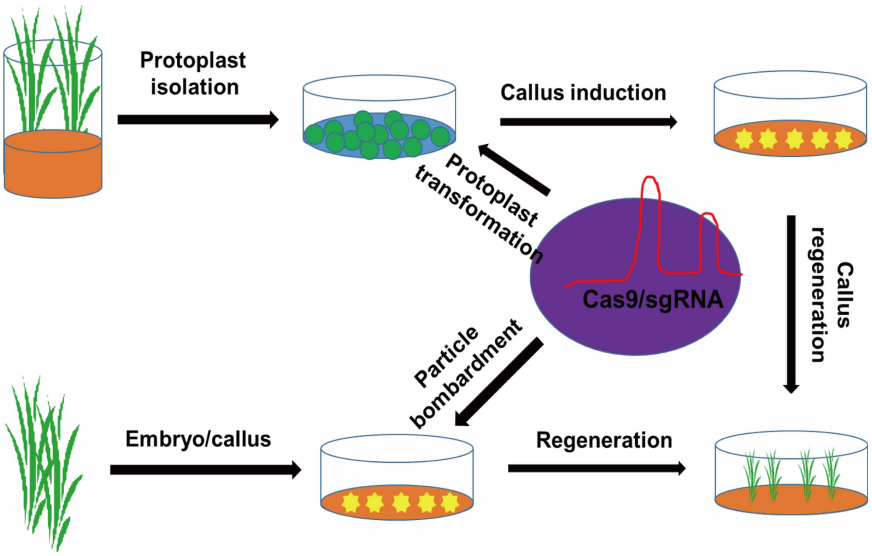

Figure 1. Schematic illustration of DNA-free genome editing in plants.

The upper part of the figure illustrates the process of protoplast-mediated DNA-free genome editing. First, protoplasts are isolated and cultured and then CRISPR/Cas9 RNP is transfected into the protoplasts; thereafter, edited calli are induced and mutant plants regenerated. The lower part of the figure illustrates particle bombardment-mediated DNA-free genome editing. In this method, embryos or calli are subjected to RNP bombardment, and mutant plants are regenerated. Both strategies are free of herbicide/antibiotic selection.

electroporation, lipidosome-mediated transformation, and *Agrobacterium*-mediated co-transformation. For example, using PEG-mediated transformation, Woo et al. [22] transfected CRISPR/Cas9 RNPs into protoplasts of *Arabidopsis thaliana*, tobacco, lettuce, and rice; targeted modifications were introduced in all four species, and genome-edited lettuce plants were regenerated from the transfected protoplasts. Similarly, the CRISPR/Cpf1 system was transformed into the protoplast of soybean and wild tobacco in the form of RNP [23]. Cpf1 proteins with *in vitro* transcribed or chemically synthesized target-specific recombinant crRNAs were delivered into protoplasts by PEG, and targeted mutations, especially various nucleotide deletions, were induced in both soybean and wild tobacco [23]. Though this protoplast-mediated DNA-free genome-editing method is simple and efficient, for most plant species the isolation and culture of protoplasts is cumbersome, and in most monocotyledons, regeneration of plants from cultured protoplasts is still not feasible.

Particle bombardment-mediated transformation is one of the most common transformation methods; it is usually highly efficient and genotype independent [24]. Previous studies have shown that RNA and protein molecules can be transferred to plant cells by particle bombardment [25,26]. Recently, CRISPR/Cas9 RNA was delivered into wheat embryos by this method, and genome-edited wheat plants were obtained [27]. In addition, CRISPR/Cas9 RNP has been transferred into maize and wheat embryos, and mutant plants were generated with rather high efficiency [28,29] (Figure 1). Moreover, compared with DNA delivery methods, the use of RNP reduced off-target effects to a hardly detectable level, in agreement with previous reports in human cells [19–21]. The editing efficiency of RNP was similar to that obtained with the transient DNA delivery method, though bi-allelic mutations in maize and multiple allelic mutations in wheat were much less frequent [28,29].

In addition to reducing off-target effects and alleviating regulatory concerns related to genetically modified organisms, DNA-free genome editing can also simplify the lengthy and costly tissue culture step involved in growing transgenic plants under herbicide/antibiotic selection. It also avoids the need to segregate integrated CRISPR cassettes, which is usually time consuming, and even impossible for perennial and vegetatively propagated plants.

Precise base editing

When CRISPR systems introduce DSBs in targeted sites, DNA repair pathways are stimulated. NHEJ is error-prone; it simply rejoins broken DNA ends, creating small insertions and/or deletions (indels) at breakpoints.

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This usually produces frame-shift mutations, leading to gene knockout [30]. On the other hand, HR is a high-fidelity process that can generate precise gene replacements and insertions through sequence-specific recombination with the help of homologous donor DNA [30]. Until now, many gene knockouts have been produced in plants [2], but only a few precise gene modifications, such as point mutations. The reason for this is that HR occurs only during the S and G2 phases, whereas NHEJ occurs throughout the cell cycle [31]; hence, generating precise modifications in plants remains a challenge.

Base editing is a new genome-editing approach that directly converts one target DNA base into another in a controlled manner [32]. In principle, conjugation of Cas9 with an enzymatic or chemical catalyst that mediates the direct conversion of one base to another could enable RNA-programmed DNA base editing. Cytidine deaminases [human activation-induced cytidine deaminase (AID), human APOBEC3G, rat APOBEC1, and lamprey CDA1] can catalyze the deamination of cytosine (C) to uracil (U), which has the base-pairing properties of thymine (T). When it is introduced with CRISPR/Cas9, cytidine deaminase can bring about the direct conversion of cytidine to uridine, thereby creating a C → T (or G → A) substitution (Figure 2) [32,33]. This CRISPR/Cas9-mediated base-editing method makes use of Cas9 nickase (nCas9) or dead Cas9 (dCas9), and as a result, base editing does not require dsDNA backbone cleavage, and is a precise genome-editing method. Komor et al. [32] fused CRISPR/Cas9 with rat APOBEC1 and produced a highly efficient base-editing system and found that their construct BE3 (APOBEC-XTEN-nCas9-UGI, UGI: uracil DNA glycosylase inhibitor) had a high base-editing efficiency both in human and mammalian cells. Similarly, cytidine deaminase PmCDA1 can be used to generate point mutations in yeast and mammalian cells [33]. In addition, two groups have combined AID, dCas9, and multiplex sgRNAs, and generated diverse point mutations in mammalian cells [34,35]. Also, Yang et al. [36] fused cytidine deaminases with the ZF or TALE-DNA-binding modules, and created site-specific point mutations in *Escherichia coli* and human cells.

Recently, precise base editing has been applied in rice, *Arabidopsis*, wheat, maize, and tomato. Lu and Zhu [37] constructed an sgRNA-APOBEC1-XTEN-nCas9 base-editing plasmid for *Agrobacterium*-mediated rice transformation, which had an editing efficiency of 1–10%, generating plants with point mutations of the expected phenotype. Li et al. [38] introduced an sgRNA-APOBEC1-XTEN-nCas9-UGI construct into rice calli. They found that the base-editing efficiency was dependent on the target sequence and could achieve a maximum efficiency of 20%. Chen et al. [39] synthesized a maize codon-optimized BE3 and transformed the vector into *Arabidopsis*; they detected a C-to-T mutation efficiency of ∼1.7% in the T1 generation and obtained herbicide-resistant mutants. Zong et al. optimized the base-editing plasmid for cereal plant codons and tested the editing efficiency of nCas9 and dCas9 in rice, wheat, and maize. Whereas dCas9 yielded low frequencies of base editing, nCas9 yielded high frequencies in all three plants. The base-edited rice and maize plants were produced by *Agrobacterium*-mediated transformation, and those of wheat were generated by particle bombardment [40]. Using a fusion of nCas9 or dCas9 with PmCDA1, Shimatani et al. [41] induced point mutations in rice [41]. They simultaneously transformed three sgRNAs and nCas9–PmCDA1 into rice and obtained multiple herbicide-resistant plants, and they also detected homozygous DNA substitutions in transgenic tomatoes produced by *Agrobacterium*-mediated transformation [41]. In addition to SpCas9, which recognizes the NGG PAM (protospacer adjacent motif), CRISPR systems from other bacteria and archaea, or a modified SpCas9 recognizing other PAM sequences, has been reported [42]. Ren et al. [43] demonstrated that both APOBEC1-XTEN-nCas9-UGI and APOBEC1-XTEN-nCas9(VQR)-UGI recognized the corresponding PAM sequence and produced point mutations at targeted sites in rice. These findings indicate that Cas9 variants and alternative CRISPR systems work well in plants, and will expand the applications of base editing.

Although HR-mediated gene replacement is infrequent, base editing can still be achieved by this method, usually combined with some strategies. Endo et al. [44] found that when DNA ligase 4 was disrupted, gene targeting efficiency in rice increased dramatically and bi-allelic targeted gene replacement at the ALS locus was obtained. Sun et al. [45] found that using two sgRNAs and a repair template, nucleotide substitution through particle bombardment worked efficiently in rice. Similarly, this dual-sgRNA/Cas9-mediated targeted gene replacement strategy was successfully conducted in *Arabidopsis*, with an efficiency of 0.8% [46]. Sauer et al. [47] reported that using single-stranded oligonucleotides (ssODNs) and CRISPR/Cas9, precise EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene replacement was achieved in flax protoplasts, and EPSPS edited flax plants were regenerated. Precise gene replacements can also be achieved in plant cells by harnessing NHEJ. Using a pair of sgRNAs targeting adjacent introns and a donor DNA template flanking the same sgRNA targeting sites, Li et al. [48] achieved gene replacements in the rice EPSPS gene at a frequency of 2.0%,

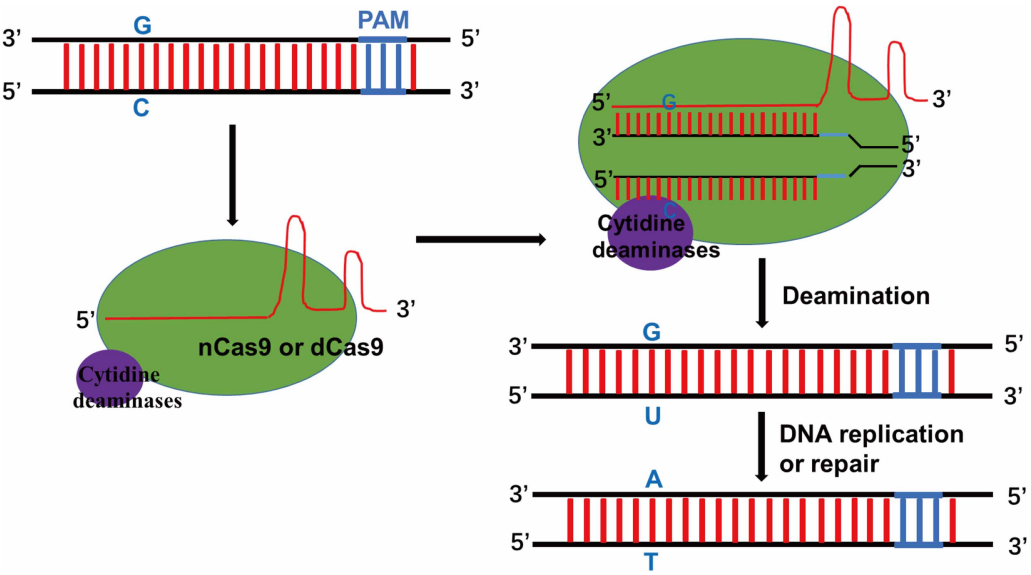

Figure 2. Precise base editing in plants.

In this approach, nCas9 or dCas9 is fused with a cytidine deaminase that can catalyze the deamination of cytosine (C) to uracil (U). When a C occurs at the appropriate position in the target site, it may be changed to U, and U will be replaced by T via base-pairing after DNA replication or repair.

this rather high efficiency makes this intron-mediated site-specific gene replacement method a valuable alternative to HR.

Conclusions and future perspectives

Recent years have witnessed the development of DNA-free delivery methods and precise base-editing systems in plants, but there are still many problems to be solved before these methods can be widely used. To date, both protoplast- and bombardment-mediated DNA-free genome editing is restricted to certain species; this means that DNA-free genome-editing methods need to be optimized and plant transformation technologies improved. For example, it is helpful to optimize DNA-free genome editing by detecting the relationship between initial CRISPR/Cas9 RNA/RNP yield and RNA/RNP yield that transformed into plant cells, by understanding the time-course degradation of CRISPR/Cas9 RNA or RNP in plant cells, and by titration of CRISPR/Cas9 RNA or RNP input dosage for editing efficiency. Furthermore, simplifying the plant tissue culture methods, minimizing plant tissue culture time and utilizing plant genes involved in developmental reprogramming or wound response could boost plant transformation technologies [49].

For cytidine deaminase-mediated base editing, one disadvantage is that base editors usually convert all the Cs within the ~5 bp editing window to Ts, and this may introduce undesired changes at the target locus. Fortunately, a recent study has shown that the editing window can be narrowed from ~5 nt to as little as 1–2 nt by using base editors containing mutated cytidine deaminase domains [50]. We believe that engineered base editors could also be used in plants to increase the precision of editing. Another limitation for cytidine deaminase-mediated base editing is that it most frequently converts C to T (or G to A), which is not applied for other nucleotide substitution. The best solution for this limitation is increasing HR efficiency in plants, which is still currently very difficult; and it is also promising to conjugate Cas9 with another enzymatic or chemical catalyst that has base conversion activity. A combination of DNA-free and precise base editing will prove to be of great value, especially in crop improvement. Now that Kim et al. have succeeded in delivering BE3 mRNA and RNP into mouse zygotes to create base-edited mice [51], similar applications in plants will be not too far distant. With continuous improvements in transformation technologies and more precise control of

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gene mutagenesis, we expect that CRISPR-based genome editing will revolutionize plant biology research and crop development.

Summary

- DNA-free editing and precise base editing based on CRISPR system have been used in plants. DNA-free genome editing methods can reduce off-target effects and base editing systems are highly effective in generating point mutations.
- Problems such as species-dependent and low accuracy are needed to be solved before DNA-free and base editing methods can be widely used. And a combination of DNA-free and precise base editing will be of great value, especially in crop improvement.
- With continuous improvements in CRISPR delivery technologies and more precise control of gene mutagenesis, CRISPR-based genome editing will revolutionize plant biology research and crop development.

Abbreviations

AID, activation-induced cytidine deaminase; CRISPR, clustered regularly interspaced short palindromic repeat; dCas9, dead Cas9; DSBs, double-strand breaks; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase gene; HR, homologous recombination; nCas9, Cas9 nickase; NHEJ, non-homologous end joining; PAM, protospacer adjacent motif; PEG, polyethylene glycol; RNP, ribonucleoprotein; UGI, uracil DNA glycosylase inhibitor.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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