CRISPR/Cas9-mediated genome editing in plants

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Abstract

The increasing burden of the world’s population on agriculture necessitates the development of more robust crops. As the amount of information from sequenced crop genomes increases, technology can be used to investigate the function of genes in detail and to design improved crops at the molecular level. Recently, an RNA-programmed genome-editing system composed of a clustered regularly interspaced short palindromic repeats (CRISPR)-encoded guide RNA and the nuclease Cas9 has provided a powerful platform to achieve these goals. By combining versatile tools to study and modify plants at different molecular levels, the CRISPR/Cas9 system is paving the way towards a new horizon for basic research and crop development. In this review, the accomplishments, problems and improvements of this technology in plants, including target sequence cleavage, knock-in/gene replacement, transcriptional regulation, epigenetic modification, off-target effects, delivery system and potential applications, will be highlighted.

1. Introduction

With the world’s population expected to grow from the current figure of 6.8 billion to 9.1 billion by 2050, there is a great need for basic plant research and crop improvement to increase food production. Information regarding the genomes of many types of plants is rapidly increasing due to the unprecedented throughput, scalability, speed and low cost of genomic sequencing technologies, which have led to the rapid generation of whole-genome sequencing data [49]. Although crops have been improved through conventional plant breeding techniques, these methods are now constrained by declines in genetic variation, which are hampering the growth in production that will be required to feed future generations [13]. Genome editing technology may facilitate a better understanding of the information derived from genomic sequencing and of the mechanisms underlying important traits while allowing the creation of new crop cultivars through genome engineering [61]. Many of the earliest approaches to what has been referred to as “genome editing” relied on site-specific recognition of DNA sequences through the use of meganucleases, zinc finger...

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nucleases (ZFNs) and TALENs; however, each of these systems had both advantages and disadvantages. The CRISPR/Cas9 system represents the most recent genome editing technology, and advances in both gene mutation and gene targeting in plants using this system are described herein [19] (see Fig. 1).

The CRISPR/Cas9 system comes from the adaptive immune system of bacteria and archaea, which detects and degrades invasive DNA from bacteriophages and plasmids [24]. Although there are various types of CRISPR/Cas9 systems, the most commonly used system for genome editing is the Cas9 nuclease from the type II CRISPR/Cas9 system of Streptococcus pyogenes [219], which requires an NGG (N, any nucleotide; G, guanine) PAM sequence for DNA targeting [30,45]. Exploitation of this system for biotechnological advances in plants has led to the design of two components, one encoding the Cas9 endonuclease and the other a synthetic single guide RNA (sgRNA), with the latter usually under the regulatory control of a U6 or U3 small nuclear RNA promoter [17]. Several excellent reviews on the CRISPR/Cas9 system are available, outlining the broad application of CRISPR/Cas9 systems in biology, medicine and crops [8,34,83,94,97,126]. The present review will focus on the technological accomplishments, problems and methods for optimizing CRISPR/Cas9 technology in plants; it will also discuss the prospects of the CRISPR/Cas9 system with respect to gene knockouts, SNP or gene replacement, transcription modulation, delivery systems and regulatory issues.

2. Targeted gene knockout

Within one year of the first breakthrough in CRISPR/Cas9 technology in human and mouse cells [17], several reports were published demonstrating the function of the Cas9/sgRNA system in a variety of plant species [23,44,58,69,75,77,95,108,119]. Follow-up studies have provided evidence of functional CRISPR/Cas9-mediated genome editing in various important crops [67]. Furthermore, improvements in several aspects of the system have been made to increase its knockout efficiency.

Optimization of the codons in Cas9, the promoter for Cas9 expression and the position of the T-DNA insertions can be used to improve Cas9 expression levels and targeting efficiencies [25,64,70,112,123]. In rice, target sequences with a higher G-C content (50–70%) have relatively higher editing efficiencies. Furthermore, the formation of a stem-loop structure between the target sequence and the sgRNA scaffold sequence may affect binding to the genomic target strand and result in lower editing efficiency [64]. Modifications to the sgRNA, such as extending the duplex length or mutating the fourth thymine of the polythymine sequence to cytosine or guanine, can also improve editing efficiency [18].

One of the main challenges with knockout technology in plants is that mutations can occur independently in plant somatic tissue, resulting in chimerism; only mutations that are present in germ-line cells are transmitted to the next generation. However, the reported frequency of chimerism differs among studies [22,70,112]. Chimerism was suggested to arise due to poor expression of the Cas9 gene in germ cells. To increase germline expression, promoters expressing Cas9 specifically in germline cells were used to increase the frequency of mutation in subsequent generations in Arabidopsis. Using an egg cell-specific promoter, a higher frequency of biallelic or homozygous mutations was detected in T1 plants, which simplified identification of the mutant phenotype [112]. Similarly, Cas9 expression that is driven by alternate promoters, such as the SPOROCYTELESS promoter and the YAO gene promoter can also facilitate greater efficiency in germline genome editing [70].

Another problem with knockout technology is that the blunt cutting activity of Cas9 typically generates indels of only 1 bp in plants, which are difficult to detect by methods other than DNA sequencing. The most immediate solution to this problem is to programme gRNAs to target two adjacent sites to create larger deletions; however, this strategy always displays low efficiency. Alternatively, blocking or inhibiting components of the classical non-homologous end-joining (NHEJ) pathway (e.g., Ku70, Ku80,
Lig4) to promote microhomology-based repair is another strategy that does not influence efficiency. The production of larger deletions (>10 bp) with this strategy has been demonstrated in rice and *Arabidopsis* with ZFNs and TALENs [91,89]. Similarly, DNA ligase may also be inhibited by the small molecule Scr7 to promote microhomology-based alternative NHEJ, as demonstrated in mammalian cells [72].

Several methods have also been developed for multiplex genome engineering. The first method assembled multiplex targeting sequences into a single vector by GoldenGate cloning or Gibson assembly [20]. The final vector contains a maize codon-optimized Cas9 sequence driven by a ubiquitin promoter and two gRNAs driven by U6 promoters. This tool can be used to efficiently edit DNA in maize and *Arabidopsis* [65,66,121]. A second technique was also recently published. In one case, six gRNA expression cassettes were placed into a single vector with Cas9 in three steps [131]. In another study, an assembly system based on an isocaudamer technique with compatible restriction enzymes was also developed [109]. Although laborious and time-consuming, this approach allowed for the simultaneous editing of multiplex target sites. By using the precise processing of tRNA precursors, an elegant system was created, in which multiple (up to 8) sgRNAs with exact start sites could be processed in vivo from a single transcript by the endogenous cellular tRNA-processing machinery [118]. Furthermore, this strategy has been functionally verified in maize [90].

In the past, resources such as the *Arabidopsis* and rice T-DNA gene knockout libraries have made a significant contribution to the rapid advancement of basic plant biology research and/or crop improvement. For further applications, systematic investigation of gene function and hypothesis-free discovery of novel pathways that underlie biological processes require targeted perturbation of specific genes across the genome. Recently, a number of studies have harnessed the programmable nature of CRISPR-Cas9 to conduct powerful genome-wide screens [14,51,110]. Genes that are essential for cell viability, as well as genes that are involved in resistance to specific small-molecule drugs, have been revealed by loss-of-function gene knockout screens in both human and mouse cells. With the improved knockout efficiency of CRISPR/Cas9 system, such genome-wide screens in plants have the potential to expand our knowledge of functional genomics and lead to crop improvements in the future.

### 3. Gene knock-in and replacement

A major challenge in all eukaryotes is to harness gene replacement methods to accurately and precisely introduce new alleles into the genome. This is particularly important for precisely defining SNP functions or for creating improved crops. Although gene knockout using CRISPR/Cas9 has become feasible due to its high efficiency in many plants, so far only a few cases of CRISPR/Cas9-based gene knock-in or replacement in plants have been reported. To achieve gene knock-in using CRISPR/Cas9, double-strand breaks (DSBs) must be repaired through HDR using a DNA template as the donor sequence, which is substituted into the specified region. CRISPR/Cas9-mediated site-directed mutagenesis and knock-ins have been used in rice and *Arabidopsis* protoplasts [58,95]. Recently, this technique was also successfully used to introduce two substitutions into an acetolactate synthase gene in rice, thereby conferring resistance to the herbicide bispyribac sodium [99,132]. Although there have been some successful cases, generating a universal gene knock-in and replacement strategy in plants remains challenging.

CRISPR/Cas9 cleavage coupled with HDR has the potential to overcome this challenge. Significant enhancements of gene replacement rates by homologous recombination have been reported in mammalian cells by targeting genes with Cas9/gRNA complexes while simultaneously inhibiting DNA ligase VI with the compound Scr7 and/or suppressing genes required for NHEJ (i.e., genes encoding KU70 and KU80) [60,68,73]. Such treatments greatly suppress the NHEJ DNA repair process and stimulate increased homologous recombination efficiency at the sites of the Cas9-gRNA-induced DNA breaks. Indeed, one report using ZFN-mediated gene targeting showed an increase in gene editing and homologous recombination (HR) in *Arabidopsis* containing mutations in SMC6B and in ku70 or lig4, which are involved in NHEJ repair [91]. Most recently, efficient gene replacement by HR was demonstrated in maize and soya bean. Five different target genes in immature maize embryos were successfully targeted for biallelic editing using biolistic delivery of Cas9 genes and sgRNA genes using either double-stranded or single-stranded DNA molecules as replacement strands [113].

With CRISPR/Cas9 cleavage, HDR rates can be improved by approximately one order of magnitude by increasing the copy number of the donor DNA. In a recent study, nuclease constructs were encoded on a modified bean yellow dwarf virus genome that is capable of undergoing replication within the plant cell. Also encoded on this replicon was the template, flanked on each side by sequences with homology to the DNA flanking the cleavage site of the target gene. This replicon sequence was introduced into tomato cells on an *Agrobacterium* T-DNA. Furthermore, it was predicted to initiate rolling circle replication and to generate hundreds to thousands of copies. A gene targeting frequency of approximately 10% was observed using this replicon, representing an order of magnitude increase over that observed for a non-replicating T-DNA vector sequence. Interestingly, no evidence of an off-target replicon sequence or a T-DNA insertion was observed [10]. This study, coupled with previous results from the same laboratory [6], demonstrates that a high copy number of the repair sequence coupled with site-specific DNA cleavage efficiently promotes gene targeting via homology-directed repair processes.

With the still-low efficiency of gene knock-in/replacement, the selection of correctly edited cells is very important, as in most cases gene knock-in/replacement of endogenous genes does not confer a selectable phenotype. Positive-negative selection, a target gene-independent selection system for cells in which a gene is replaced using selectable markers located on the template DNA, has been developed. Because it does not rely on gene-specific selection, the positive-negative selection system represents a universal strategy that can be used for any target gene [41,74,80,102,117]. Moreover, several strategies to eliminate selectable marker genes, such as site-specific recombination [42,48,86], transposition systems [28,79], HR [87] and a self-cleaning mechanism of CRISPR/Cas9 [4,106,125], have been developed. The combination of a highly efficient CRISPR/Cas9 nuclease with a universal positive-negative selection-dependent gene knock-in/replacement and subsequent elimination of the positive selection marker gene can be used to induce mutations in any gene of interest (unpublished data from the DaBeiNong Group).

In addition to genetic integration via HR, targeted gene insertion or replacement can also be accomplished with NHEJ. In this case, the linear donor molecules, which do not contain flanking homology arms, are captured at DSBs during repair by NHEJ. While this method has not been used extensively in plants [18,114], it has been employed frequently in mammalian cells [27]. With additional improvements in the efficiency with which foreign DNA is captured at DSBs [71,122], this method is a potentially effective approach for targeted DNA integration into plant genomes. Most recently, gene replacements in the endogenous rice gene 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) at a frequency of 2.0% has been achieved by using a pair of sgRNAs targeting adjacent introns and a donor DNA template that includes the same pair of sgRNA sites [57]. These newly developed approaches can be
generally used to insert or replace targeted gene fragments in rice and other plants.

In addition to the template-assisted gene knock-in/replacement methods mentioned above, a simplified approach has recently been developed through a fusion of CRISPR/Cas9 with a cytidine deaminase enzyme that retains the ability to be programmed with a guide RNA. This combination enables the direct, irreversible conversion of cytidine to uridine, thereby effecting a C → T (or G → A) substitution in a programmable manner without requiring dsDNA backbone cleavage or a donor template [50]. The resulting ‘base editors’ convert cytidines within a window of approximately five nucleotides and can efficiently correct a variety of point mutations relevant to human disease. Similarly, CRISPR/Cas9 and the activation-induced cytidine deaminase (AID) orthologue PmCDA1 were engineered to form a synthetic complex (Target-AID) that performs highly efficient target-specific mutagenesis. Specific point mutation was induced primarily at cytidines within the target range of five bases [78]. Another method, termed ‘CORRECT’, was also developed to have increased HDR efficiency for genome-editing; in this method, silent CRISPR/Cas-blocking mutations were incorporated along with pathogenic mutations [82]. Further testing of these methods in plants may provide an alternative and convenient approach for important SNP replacement.

4. Transcriptional regulation of target genes

Manipulating the expression levels and timing of specific genes during plant growth and development with artificial transcription factors is very useful for academic research and crop improvement. Cas9 can provide a versatile platform for delivering different regulatory components to several specific sites. After deactivating its nuclease function (by point mutations in its two catalytic domains, HNH and RuvC), general transcription activators or repressors can be fused to the C-terminus of Cas9 without affecting its gRNA binding ability. When gRNAs targeting the promoter sequence of a gene are coupled with this dCas9-activator/repressor, the complex becomes a powerful tool for the transcriptional control of target genes.

This modified system has been used in CRISPR interference (CRISPRi) for highly efficient and precise gene silencing. Compared with the RNAi concept of transcript degradation and/or translational blocking, the CRISPRi system blocks transcription initiation and elongation [33,89]. A critical question for repression is the impact of gRNA location on silencing efficiency. A region between –50 and +300 base pairs (bp) relative to the transcription start site (TSS) were defined as having the highest silencing efficiency by CRISPRi [32]. The dCas9 C-terminus was fused with the SRDX repression domain to produce transcriptional repressors, and such constructs were used to selectively repress both artificial reporter transgenes and endogenous genes. In plants, robust silencing has been most clearly shown with dCas9 fused to the SRDX repressor motif [85]. Therefore, the dCas9/sgRNA system offers a general platform for RNA-guided DNA targeting for stable and efficient modulation of transcription. In mammalian cells, CRISPRi can reduce gene expression levels and is comparable to RNAi, but it also has several key advantages, such as low cytotoxicity, high variability of function between organisms and experimental conditions due to its reliance on cytoplasmic machinery for silencing and fewer off-target effects. Investigation of such features in plants will expand the further applications of the dCas9/sgRNA repression system.

Target gene transcriptional activation in plants is also possible with dCas9 proteins fused to the EDLL domain (a plant-specific transcriptional activator) or the TAL activation domain (dCas9: TAD) [85,105]. This system has been shown to induce robust activation of synthetic reporters and endogenous genomic loci [32,62,81]. dCas9-VP64 could also overcome CpG methylation and activate the imprinted FIS2 gene in Arabidopsis [62]. Taking advantage of this observation, many groups fused a large number of VP64 protein repeats directly to dCas9. Multiple enhanced activator tools emerged, including dSpCas9-VP160 (10 repeats of VP16) [15], dSpCas9-VP192 (12 repeats of VP16) [5] and VP64-dSpCas9-BFP-VP64 (with four repeats of VP16 on the N-terminus of dSpCas9 and blue fluorescent protein plus four more repeats of VP16 on the C-terminus) [11].

Several methods have been developed to further improve target transcriptional regulation efficiency. The SuPerNova tagging (SunTag) system employs 10 repeats of a short epitope tag fused directly to dCas9 (SunCas9), allowing for the trans-expression and recruitment of 10 single-chain variable fragments (scFvs) fused to VP64 [101]. As a result, a single guide is able to induce robust gene expression, activating gene expression up to 45-fold with the SunCas9 system. An sgRNA-based recruitment strategy has also been developed. RNA hairpins from the male-specific bacteriophage-2 (MS2) were repetitively fused to the 3’ end of an sgRNA. In tandem, an MS2 coat protein (MCP), which binds each MS2 hairpin as a homodimer, was fused to the VP64 activator domain. When the modified guide, MCP-VP64 fusion protein, and dSpCas9 are co-expressed, expression of the target gene is activated by 50–300-fold in cultured mammalian cells [51,127].

Building on the sgRNA-based recruitment strategy, a novel chimeric activator p65-HSF1 (p65 is a subunit of the ubiquitous NF-kB transcription factor complex, and HSF1 is heat shock factor 1, which is responsible for transcriptional changes in response to temperature stress) may be used to replace VP64. The synergistic activation mediator (SAM) system incorporates two MS2 hairpins into exposed loops within the sgRNA and fuses MCP to p65-HSF1, resulting in four copies of MCP-p65-HSF1 scaffolding onto the SAM sgRNA backbone to dSpCas9-VP64. With this advanced recruitment strategy, single guides were shown to successfully induce high levels of expression, with a median improvement of 105-fold over the dSpCas9-VP64 activator [51]. Both SunCas9 and the dSpCas9 SAM activator were found to be between –200 and +1 bp relative to the TSS. In a simplified yet robust system, three separate activation domains, namely VP64, p65, and Rta, were fused in tandem directly to dSpCas9. This dSpCas9-VP64-p65-Rta (dSpCas9-VP-R) system showed 20- to 300-fold higher expression than dSpCas9-VP64 [12]. Each technology induced high expression levels of endogenous genes, clearly showing that Cas9-based transcription factors can be further tested in plants to activate endogenous gene expression.

In addition to controlling gene expression with repressors and activators associated with dCas9/sgRNA constructs, more recent experimentation with human cells has demonstrated that epigenetic control of gene expression can be achieved by attaching different proteins. When the Kruppel-associated box (KRAB) domain of Kox1 was fused to dCas9, the fusion showed a 15-fold repression of reporter genes [103]. In another approach, four copies of the mSin3 domain (termed SID4X) were fused in tandem to the dCas9 scaffold, and the fusion protein repressed the endogenous gene SOX2 in HEK293T cells more than 29-fold; dCas9 alone showed no effect [15]. Similarly, when histone demethylase LSD1 was fused to a small dCas9 orthologue from Neisseria meningitidis (dNmCas9), the fusion protein removed activating H3K4 methylation marks, leading to enhanced decommissioning and gene repression [47]. Epigenetic transcriptional activation is also possible by fusing the catalytic histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300 to dCas9 and directing it to specific genomic regions using sgRNA targeting. dSpCas9-p300 induced 5- to 270-fold increased gene expression of downstream genes in the locus control region (LCR) of the human β-globin locus by directly acetylating distal enhancer regions [37]. This research...
addresses off-target editing issues in plants. These high-fidelity Cas9 variants may be immediately adopted to producing alanine substitutions at four residues in SpCas9 [3,38]. Another group has described a Cas9 variant named eSpCas9 1.1 with alanine substitutions at three positions [96]. An additional approach to improve CRISPR/Cas9 activity and precision while also providing the ability to target off-target sites is through the use of dCas9-KRAB repressor and the dCas9-SunTag activator. The screens included a loss-of-function (CRISPRi) study for genes that confer sensitivity to a cholera–diphtheria fusion toxin and a gain-of-function screen (CRISPR activation, CRISPRa) for genes that confer resistance to the same toxin. These screens demonstrate a key benefit of Cas9-based screening. While genome-scale screens are laborious and time-consuming, the amount of data and the potential to answer large biological questions are enticing to researchers. Cas9 tools offer undeniable new levels of flexibility, multiplexing capability and precision while also providing the ability to target endogenous loci and to regulate noncoding portions of the plant genome.

5. Accuracy improvement of CRISPR/Cas9

The specificity of CRISPR/Cas9 is an important consideration in mammalian cells, as off-target cleavage of genomic DNA can result in undesirable mutations and chromosomal abnormalities. However, this phenomenon may be alleviated in plants and crop breeding, as negative agricultural traits can be eliminated during the breeding selection process. Negative phenotypes may be removed by crossing with the parental plants followed by phenotype/marker-aided selection in the progeny, if necessary. Still, the off-target phenomenon adds many challenges to functional genome research and molecular crop breeding, especially in high-throughput research programmes. To overcome this pitfall, several methods have been developed to increase Cas9 targeting fidelity. First, the risk of off-target effects can be minimized or avoided by selecting highly specific target sequences by genome searching (nucleotide BLAST) or by using web-based tools for CRISPR/Cas9 target selection [55,76,92]. Among these tools, CRISPR-PLANT and CRISPR-P are specifically designed for CRISPR/Cas9-mediated plant genome editing. CRISPR-PLANT facilitates gRNA design in eight plant species and supports restriction enzyme analysis of target sites [120]. CRISPR-P includes a genome-wide survey of highly specific gRNAs for almost all plant species whose genome sequence is available, and it also provides off-target site analysis [56]. In addition, efforts have been made to optimize the targeting specificity of the CRISPR/Cas9 system, for example by modifying Cas9 into a dCas9-Fold or nickase (i.e., Cas9 with either a D10A or H804A substitution) [26,35,107] or by changing nucleotides in the sgRNA sequence by shortening the gRNA spacer sequence to 17–18 nt [18]. Recently, the off-target problem was addressed using a more elegant approach. New Cas9 variants that are intolerant to any number of mismatches have been engineered by introducing alanine substitutions at four residues in SpCas9 [3,38]. Another group has described a Cas9 variant named eSpCas9 1.1 (enhanced SpCas9 version 1.1), which shows robust on-target activities and has alanine substitutions at three positions [96]. These high-fidelity Cas9 variants may be immediately adopted to address off-target editing issues in plants.

6. Delivery system for gRNA:Cas9

To mediate genome editing in vivo, constructs carrying the Cas9 and sgRNA expression cassettes must be delivered into plant cells. Agrobacterium-mediated transformation is the most effective method for many plants [31], and the most common delivery method for CRISPR/Cas9 in plants employs T-DNA carrying both the Cas9 and sgRNA expression cassettes that is directly delivered into the plant genome by a type IV secretion mechanism [44,58,69]. Plant material is transformed with a vector containing the sgRNA, a selectable marker, and the Cas9 gene using established transformation procedures. The resulting T0 plants are then screened for mutation at the target site. Biologic transformation of immature embryos and calli can also be used to integrate Cas9 and/or sgRNA expression constructs into wild-type or Cas9-transformed plant cells and produce heritable mutations [95,100]. Quite recently, efficient gene editing and gene replacement by HR has been demonstrated in maize and soybean using biologic delivery of Cas9 genes and sgRNA genes [113]. Additionally, several viral approaches have been recently deployed in plants to combat challenges in transgene delivery [2,6]. Tobacco rattle virus DNA carrying an sgRNA expression cassette was introduced into Cas9-transformed tobacco by an agroinfiltration approach [124]. However, these viral systems are chiefly limited by the size of their nucleic acid cargo; most geminivirus genomes are approximately 3 kb, while the Cas9 coding sequence (from S. pyogenes) alone is longer than 4 kb. Thus, viruses are more practical for delivering gRNA(s) to plants that already stably express Cas9. Although limited with respect to the size of the cargo, viral approaches are still valuable for rapid reverse genetic screening in plants.

Some crop systems, such as citrus plants, have lengthy juvenile phases that result in flowering five to ten years after germination. A vector outbreeding approach in such systems would be lengthy and impractical. However, use of a transient expression system or delivery of a preassembled Cas9:sgRNA may offer viable alternatives [1]. A transient expression system has been used to deliver plasmids carrying Cas9 and sgRNA expression cassettes directly into protoplasts or into tobacco or Arabidopsis leaves via vacuum infiltration [44]. Recently, a DNA-free method of genome editing with CRISPR/Cas9 was reported that directly introduces preassembled gRNA:Cas9 complexes into protoplasts of Arabidopsis, tobacco, and rice. This method was also extended to lettuce (Lactuca sativa) to mutate the BIN2 gene in protoplasts, which can be regenerated into calli and then into full plants at a 46% mutation efficiency rate. The mutations can be transmitted to the next generation, but the CRISPR/Cas9 components are eventually turned over by endogenous cellular degradation mechanisms [115]. Similar results have also been recently achieved in wheat [129]. These methods can produce desired genome modifications in the absence of a vector sequence, eliminating the need for outbreeding and reducing the time and resources required to generate a vector-free plant. These features are important for cultivars that are unable to self-fertilize, for dioecious species, for plants with long juvenility periods and for improving the germplasm of clonally propagated crops. However, the system is only suitable for the creation of indel mutations and not for gene targeting, which would require an exogenous DNA template. Additionally, most transient delivery methods require robust single-cell regeneration systems, which are not presently available for many plants.

7. Alleviation of safety and regulatory issues by technology

In principle, CRISPR/Cas9-induced mutations are substantially equivalent to those produced by random mutagenesis, but they have much higher specificity and efficiency. In addition, the
transgenic Cas9 gene and the antibiotic-resistant marker genes can easily be removed by segregation of the progeny, producing transgene-free gene-edited plant lines. In such cases, CRISPR-edited plants might be exempt from current GMO regulations. As a result, CRISPR-edited plant products may be brought to market more quickly and with significantly less expense than conventional GMO crops. Edited crops with appropriate regulatory structures might prove to be more acceptable than plants that carry foreign DNA in their genomes [52,63]. In addition, the DNA-free delivery method is particularly advantageous because it may not fall under the regulations faced by transgenic crops, as the resulting modified plants are devoid of transgenes encoding CRISPR/Cas9 machinery. Furthermore, direct delivery methods for Cas9 and gRNA using Agrobacterium, viral replicons or nanoparticles may also be very useful for simplifying genome-editing technology and reducing GMO-related issues [36,54,129].

8. Application of the CRISPR/Cas9 system for functional genomics research

With its high efficiency for generating biallelic mutants in one or more genes at the T0 stage, CRISPR/Cas9 has and will have a large impact on functional genomic studies in plant systems, for both forward and reverse genetic studies. CRISPR-mediated gene knock-ins and knockouts will also likely play a significant role in crop functional genomics. This technique can be exploited to understand spatial and temporal gene expression patterns or to determine the cellular localization of proteins by fusing a GUS or GFP tag in-frame with endogenous genes. Gene replacement may also be utilized to determine the functions of specific genetic regions by allowing the addition or removal of specific regions of the genome, including introns, exons and regions encoding signal peptides and functional domains. A CRISPR/Cas9-mediated gene-editing approach can also be used to identify genes that are associated with specific traits. Using marker-assisted selection, breeders identify genomic regions that are associated with traits; these are known as quantitative trait loci (QTLs). For example, multiple targeted genomic deletions can be generated within the domains of a QTL. This approach is feasible, as the generation of deletions >100 kb is already possible in plants [133].

Genome-scale Cas9 transcriptional technologies, such as transcriptional activation or repression and RNA cleavage, may be utilized as alternatives in generating populations for phenotypic screening, which would allow researchers to easily probe gene function and to more deeply understand genomic regulatory networks. In plants, the use of such tools to precisely control gene expression may help us better understand gene regulatory networks [84,88] and thus to understand more complex traits. This system overcomes many issues associated with ectopic overexpression or RNAi knockdown by allowing gains or losses of gene function to be studied in their genomic contexts. Moreover, CRISPR/Cas9 can be used for multiplex gene activation or repression. With the great advancements that have been achieved for synergistic activation in mammalian systems, testing activation domains derived from plant proteins will be an additional prospect for transcriptional regulation in plants [51].

9. Application of the CRISPR/Cas9 system for crop improvement

The CRISPR/Cas9 system will have a revolutionary effect on plant research and crop breeding [93,113,116]. The ability to achieve DNA modifications allows molecular breeders not only to remove sources of unwanted or undesired DNA but also to improve or adjust genomic and transcriptional regulatory sequences. In the context of plant pathogen defence, simultaneous knockout of the three TaMLO homologues in wheat produced resistance to powdery mildew [111]. Similarly, knockout of the disease-susceptibility genes OsSWEET13 and OsERF922 led to increased disease resistance [134]. Cas9/gRNA technology also has been successfully applied to many important crops and horticulture plants, including maize [59,100], wheat [111], barley [53], soybean [40], tomato [9], sweet orange [43], petunia [98,128] and poplar [21]. Compared with medical and clinical research, genome editing in plants does not involve ethical issues and thus is more suitable for further application research [133,53].

Given that geminiviruses use rolling-circle replication to copy DNA, DSBs generated within the viral DNA sequence could fundamentally compromise the gene-editing process. Using viral technology, tobacco (Nicotiana benthamiana) has been modified to enhance resistance to three different geminiviruses, namely bean yellow dwarf virus, beet severe curly top virus and tomato yellow leaf curl virus [2,6]. The recently reported DNase H activity of Cas9 on ssDNA derived from Neisseria meningitidis may be harnessed for even greater targeting efficiency towards the viral genome [39,130]. Further testing of CRISPR/Cas9 technologies to target plant pathogens and to explore potential applications in pest control and plant-pathogen interactions will supply important data applicable to plant breeding for crop protection in the future [7].

10. Conclusion and remarks

With the emergence of functional genomics achievements and the discovery of important FNPs (functional nucleotide polymorphisms), the CRISPR/Cas9 system can also be further used for the production of cisgenic modifications, for the targeted introduction or deletion of large genomic regions, for transcriptional regulation, and even for alterations in ploidy level. The method has a low off-target effect, thus enabling breeders to exert specific control over various traits and phenotypes. With further development of the different technologies and their combinations, researchers may achieve activation, repression and mutation in multiple target genes by deploying sgRNAs of different lengths for each target site in a single cell [29]. Given its simplicity, flexibility, versatility and efficiency, the future of gene discovery, functional genomics and crop trait improvements is likely to rely heavily upon CRISPR/Cas9 Technologies.

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