Advances in the Application of CRISPR/Cas9 Technology in Non-model Plants: Postprint

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Abstract

The emergence of genome editing technology has profound significance for plant genetic breeding and crop trait improvement. CRISPR/Cas (clustered regularly interspaced short palindromic repeats) is an immune system composed of clustered regularly interspaced short palindromic repeat sequences and their associated proteins. Its function is to enable prokaryotes (40% of bacteria and 90% of archaea) to resist invasion by foreign genetic material (phages and viruses). This technology enables simultaneous editing of multiple target genes in the genome and is simpler, more cost-effective, and more efficient compared to the previous two generations of gene editing technologies: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Currently, CRISPR/Cas9 gene editing technology has achieved targeted genome editing in model plants and most major crops such as Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa, Triticum aestivum, Zea mays, and tomato, and its application scope continues to expand to various plant species. However, compared with model plants and some major crops, the application of CRISPR/Cas9 gene editing technology in non-model plants, especially in minor crops, still requires further refinement in areas such as vector construction, target design, off-target detection, and homologous recombination. This article summarizes the latest research progress of CRISPR/Cas9 technology in non-model plants and minor crops, discusses the current limitations of its application in these plants, and proposes corresponding improvement strategies. Finally, it provides an outlook on the research prospects of the CRISPR/Cas9 system in non-model plants to serve as a reference for researchers in the field.

Full Text

Preamble

CRISPR/Cas9 Technology and Its Application in Non-model Plants

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Abstract

The emergence of genome editing technology has profound significance for plant genetic breeding and crop trait improvement. CRISPR/Cas (clustered regularly interspaced short palindromic repeat) is an immune system composed of clustered regularly interspaced short palindromic repeats and their associated proteins. Its function is to enable prokaryotes (40% of bacteria and 90% of archaea) to resist invasion by foreign genetic material (phages and viruses). This technology enables simultaneous editing of multiple target genes in the genome. Compared with the previous two generations of gene editing technologies—zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)—it is simpler, cheaper, and more efficient. Currently, CRISPR/Cas9 gene editing technology has achieved targeted genome editing in model plants such as Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa, Triticum aestivum, Zea mays, and Solanum lycopersicum, as well as in most major crops, and its application scope continues to expand to various types of plants. However, compared with model plants and some major crops, CRISPR/Cas9 gene editing technology in non-model plants, especially in some small crops, still has issues such as vector construction, target design, off-target detection, and homologous recombination that need further improvement. This paper summarizes the latest research progress of CRISPR/Cas9 technology in non-model plants and small crops, discusses the current limitations of this technology in non-model plants and small crops, and proposes relevant improvement strategies based on this analysis. Finally, the research prospects of the CRISPR/Cas9 system in non-model plants are discussed to provide references for relevant researchers.

Keywords: CRISPR/Cas9 system, plant genetic breeding, genome editing, non-model plants

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Genetic mutations are crucial for studying plant gene function and crop genetic improvement. In the past, characterization of natural mutants has revealed the importance of genetic diversity, and many studies have employed physical methods (radiation), chemical methods (ethyl methanesulfonate), or biological methods (such as T-DNA/transposons) to generate mutants through point mutations, deletions, rearrangements, and gene recombination. However, random mutagenesis produces many undesirable mutations, and large-scale mutant screening is not only laborious but also expensive. The emergence of sequence-specific nucleases (SSNs) has achieved a breakthrough in targeted mutagenesis. SSNs can induce specific double-strand breaks (DSBs) at chromosomal loci, triggering two distinct DNA repair mechanisms: non-homologous end-joining (NHEJ) and homology-directed repair (HDR). Zinc finger nucleases (ZFNs), as the first generation of SSNs, were used to edit plant genomes. However, ZFN structures are difficult to manipulate and costly, greatly hindering their application across various plant species. Subsequent development and application of TALENs in plants, while easier for vector construction and operation than ZFNs, still required building complex tandem repeat domains in TAL proteins and remained expensive.

The advent of CRISPR technology has accelerated the progress of genetic engineering, overcoming the limitation of previous generations that required different nucleases for each target gene. Compared with the first two generations of gene editing technologies, the CRISPR/Cas9 system only requires synthesis of one target sgRNA (single guide RNA) per gene target site, enabling precise targeted editing of the genome. Additionally, the CRISPR/Cas9 system offers advantages of simple operation, low cost, and high editing efficiency. Currently, this technology has achieved precise targeted mutagenesis in model plants such as Arabidopsis, rice, and maize, and its application value is being extended to an increasing number of plants.

1. Basic Composition of the CRISPR/Cas9 System

A complete CRISPR sequence component should contain repeat regions and spacer regions [Figure 1: see original paper]. The spacer region functions in the recognition and capture of foreign DNA sequences. Short and conserved repeat regions contain palindromic sequences that can form hairpin structures to degrade foreign genes. Upstream of the sequence lies a leader region that initiates CRISPR expression, and degradation of foreign invasive genes requires the cooperation of Cas protein gene families located upstream of the leader region. Currently, multiple types of Cas genes, including Cas1-Cas10, have been discovered. Finally, the CRISPR sequence and Cas protein genes combine to form a highly conserved CRISPR/Cas9 system.

The CRISPR system is divided into three types: Type 1, Type 2, and Type 3. Type 1 is distributed in both bacteria and archaea and is the most complex

composition among the three types, containing not only multiple subtypes but also various Cas proteins, with the core protein component being Cas3 protein. This protein is a multi-domain protein where each domain has different functions, primarily serving as nuclease and helicase. Type 2 has Cas9 protein containing HNH and RuvC nuclease domains as its core component. The HNH nuclease domain plays an important role in processing crRNA and degrading foreign genes in the CRISPR system. Currently, the most widely used and simplest to operate is the Type 2 CRISPR system. The Type 3 system exists in a few bacteria and archaea, containing Cas6 and Cas10 protein components that mainly participate in crRNA processing and degradation of invasive DNA.

2. Mechanism of Action of the CRISPR/Cas9 System

The Type 2 CRISPR-Cas system requires only three components: Cas9, tracrRNA, and crRNA, which recognize and locate pathogen genetic material through a three-step process of acquisition, expression, and interference. The first stage captures foreign DNA [Figure 2: see original paper]: when phages and viruses invade, the bacterial spacer sequences capture a small fragment of the foreign gene DNA sequence. The sequence recognized by the foreign gene and spacer sequence is called the protospacer adjacent motif (PAM site: NGG, where N is any base). The second stage involves crRNA expression [Figure 3: see original paper]: the spacer sequence adjacent to the PAM is modified, processed, and integrated into the CRISPR locus, transcribed into CRISPR precursor RNA (pre-crRNA), which is then further processed into crRNA (short CRISPR-derived RNA). The third stage is targeted interference [Figure 4: see original paper]: in addition to mature crRNA, degradation of foreign genes requires the assistance of some small molecule RNAs with good specificity (tracrRNA, trans-activating RNA). crRNA and tracrRNA form a new composite RNA, which, guided by sgRNA and together with the Cas9 protein, achieves degradation by complementing foreign DNA at the PAM site.

3. Application of CRISPR/Cas9 System in Non-model Plants and Small Crops

Non-model plants typically refer to plants with relatively long growth cycles and whose genomes have not been thoroughly studied. CRISPR/Cas9 gene editing technology has become an ideal approach for plant genetic improvement and molecular design through functions such as frameshift mutations, fragment deletion, and gene modification. The application of CRISPR/Cas9 gene editing technology in more non-model plants not only enhances biodiversity but also holds significant importance for developing the potential of more plants. This technology has gradually achieved targeted genome editing in many non-model plants (Table 1).

3.1 Parasponia

Parasponia is a tropical tree species belonging to the Ulmaceae family and is known as the only non-leguminous plant capable of establishing nitrogen-fixing symbiosis with rhizobia. Van et al. (2018) used the Arabidopsis AtU6 promoter to drive sgRNA expression and the 35S promoter to drive Cas9 protein expression, enabling CRISPR/Cas9-mediated mutagenesis of four genes—PanHK4, PanEIN2, PanNSP1, and PanNSP2—and demonstrated that PanNSP1 and PanNSP2 are essential for nodule formation.

3.2 Poplar

Poplar (Populus) comprises approximately 100 species in the Populus genus. In addition to furniture, papermaking, and match production, poplar wood is widely used in ecological shelter forests, Three-North Shelter Forests, agricultural shelter forests, and industrial timber forests. Poplar is also an excellent species for roadside greening and landscape gardening. Elorriaga (2016) used pK2GW7 as a CRISPR/Cas9 gene editing vector to target mutate the LEAFY and AGAMOUS genes, revealing that these genes are necessary for inducing male and female characteristics in poplar.

3.3 Marchantia polymorpha

Marchantia polymorpha is one of the most widely distributed species among bryophytes. Sugano et al. (2014) targeted the auxin regulatory factor ARF1 gene, using the U6-1 promoter to drive sgRNA and a 35S promoter-driven Cas9 protein containing a nuclear localization signal, successfully implementing CRISPR/Cas9 in Marchantia polymorpha.

3.4 Alfalfa

Alfalfa is the general name for Medicago species, commonly known as golden clover, and is a perennial flowering plant. Meng et al. (2017) used pFGC5941 as a CRISPR/Cas9 gene editing vector, with the alfalfa U6 promoter driving specific sgRNA and the 35S promoter driving Cas9 protein to achieve targeted knockout of the PDS gene in Medicago truncatula, obtaining 10.35% homozygous knockout mutants in the T0 generation and providing a new research tool for functional genomics studies in alfalfa and other legume forages.

3.5 Birdsfoot Trefoil

Birdsfoot trefoil (Lotus corniculatus) is a perennial herbaceous plant in the Fabaceae family that can be used not only as forage but also for soil improvement. Wang et al. (2016) utilized CRISPR/Cas9 gene editing technology to edit symbiotic nitrogen fixation (SNF) related genes in birdsfoot trefoil.

3.6 Sweet Orange

Sweet orange (Citrus sinensis) is a tree species with few or no thorns on its branches. Oranges are rich in vitamins C and P, which can enhance body resistance. Jia and Wang (2014) used pBI121 as a CRISPR/Cas9 gene editing vector to target the CsPDS gene, detecting mutation frequencies of 3.2%-3.9%, thereby establishing a foundation for future citrus variety improvement using this technology.

3.7 Banana

Banana (Musa spp.) is an important tropical fruit and food crop. Hu et al. (2017) constructed a pYLCRISPR/Cas9-sgRNA vector targeting the MaPDS gene, where the Cas9 protein was driven by the PUbi promoter and sgRNA was driven by rice-derived U6a, successfully obtaining resistant plants.

3.8 Grape

As one of the world' s most important fruit crops, grape (Vitis vinifera) has tremendous economic value. Ren et al. (2016) developed a pCACRISPR/Cas9 binary vector targeting the IdnDH gene, where the Arabidopsis U6 promoter (AtU6) drove sgRNA and the 35S promoter drove Cas9 protein expression, successfully implementing the CRISPR/Cas9 system in grape and ultimately obtaining transgenic plants.

3.9 Petunia

Petunia, belonging to the Solanaceae family and Petunia genus, is a perennial herbaceous plant widely used in flower bed arrangement, flower trough configuration, scenic spot decoration, windowsill embellishment, and home decoration. Zhang et al. (2015) used PGGE as a vector to target edit the PDS gene, a key enzyme in carotenoid biosynthesis, successfully obtaining mutants.

Table 1. Application of CRISPR/Cas9 System in Different Non-model Plant Species

Plant	Target Gene
Parasponia	PanHK4, PanEIN2, PanNSP1, PanNSP2
Populus	LEAFY, AGAMOUS
Marchantia polymorpha	ARF1
Medicago	PDS
Lotus corniculatus	SNF-related genes
Citrus sinensis	CsPDS
Musa spp.	MaPDS
Grape	IdnDH
Petunia	PDS

4. Current Limitations of CRISPR/Cas9 System in Non-model Plants

The CRISPR/Cas9 system has been fully utilized in model crops and some major crops such as Arabidopsis, maize, rice, and wheat. However, its extension to non-model plants, especially small crops with tremendous development potential and practical value yet to be enhanced—such as castor, buckwheat, luffa, and shepherd' s purse—still faces certain limitations.

4.1 Vector Construction Issues for CRISPR/Cas9 in Non-model Plants

During CRISPR/Cas9 system vector construction, model plants and major crops have been thoroughly studied, allowing the use of their specific promoters to drive knockout vectors. In contrast, most non-model plants, particularly small crops, can only use universal strong promoters (such as Arabidopsis U6) to drive CRISPR/Cas9-sgRNA. Through continuous research, some scientists have constructed a set of universal vectors that can be used for both dicotyledonous and monocotyledonous plants. These universal CRISPR/Cas9 vectors only require distinguishing between monocots and dicots for gene editing. However, whether these universal vectors offer better adaptability and knockout efficiency compared to plant-specific knockout vectors remains to be verified.

4.2 Target Design Issues for CRISPR/Cas9 System in Non-model Plants

Since the inception and application of the CRISPR/Cas9 system, more than 20 sgRNA design software tools have been developed for humans, animals, and plants, including online and standalone versions such as CRISPR Design and CRISPR-P. These tools determine target sites for genes through more than 30 evaluation indicators based on operating platform, species selection, sequence input, parameter setting, and result output. However, these design software tools primarily cover most model plants and major economic crops, while a large portion of non-model plants and small crops currently lack corresponding sgRNA design software. Researchers must manually design multiple sgRNAs according to conventional target design principles: (1) target sequence length of 18–22 bp, generally selecting 20 bp; (2) target sequences should be as close as possible to the start codon, with the first or second exon being optimal; (3) select target sequences with good specificity, avoiding repetitive sequences, poly-A sequences, and poly-T sequences, with GC content of 40%–50% being optimal; (4) the 5' end of the target allows 1–2 bp mismatches, but the number of matched bases should not be less than 18 bp. To ensure accurate knockout sites, researchers must manually design multiple sgRNAs by finding PAM sites through gene exon nucleotide sequences, which increases workload for later detection and identification of specific sgRNAs.

4.3 Assessment of Editing Efficiency and Off-target Effects of CRISPR/Cas9 System in Non-model Plants

For model plants, in addition to experimental methods such as enzyme digestion, SMART sequencing, SSA reporter vector activity detection, and Sanger sequencing, quantitative analysis software for gene knockout efficiency has been developed based on comprehensive off-target detection methods, such as CRISPR-GA (CRISPR Genome Analyzer, http://54.80.152.219/). Currently, non-model plants can only evaluate target sgRNA specificity through in vitro transcription of sgRNA and in vitro enzyme digestion of target DNA fragments, comparing them with standard sgRNA targets of known activity. While this activity assay is not difficult, the process is relatively cumbersome and requires repeated experiments to ultimately determine highly active sgRNA, making it time-consuming.

4.4 Homologous Recombination Efficiency Issues with CRISPR/Cas9 System in Non-model Plants

Low recombination efficiency is a common problem of CRISPR/Cas9 systems in both model and non-model plants. Conventional CRISPR uses guide RNA (gRNA) coupled with nuclease (most commonly Cas9), which together target specific DNA base regions; the nuclease then cleaves the double helix. Cellular repair mechanisms attempt to rejoin the cut DNA ends, but occasionally insert or delete some bases, which scrambles the DNA code and may inadvertently knock out target genes. To repair point mutations, the CRISPR/Cas9 system must also introduce a "donor" DNA strand with correct bases, relying on a second cellular mechanism called homology-directed repair (HDR). However, this repair mode depends on the cell division state, and HDR functions poorly in cells with poor division capacity.

4.5 Genetic Transformation Issues with CRISPR/Cas9 System in Non-model Plants

Obtaining transgenic plants through genetic transformation depends heavily on transformation efficiency. Current research indicates that transformation efficiency is inversely proportional to vector size. In the CRISPR/Cas9 gene editing system, the Cas9 protein component is typically larger than 5 kb, thus affecting genetic transformation efficiency to some extent. Although some studies have found that sequential transformation (first obtaining hCas9 transgenic plants, then transforming a small sgRNA plasmid without Cas9 into hCas9 plants) can produce mutant plants similar to direct CRISPR/Cas9 transformation, this method is mostly applicable to genetic transformation systems using leaves as receptors, and whether the hCas9 gene integration into the plant genome has any toxic effects remains unknown.

4.6 Application Issues of Single-base Gene Editing Systems in Non-model Plants

The application of CRISPR/Cas9 single-base editing systems enables base editing through cytidine deaminase using sgRNA for targeted localization without DNA double-strand breaks. The successful correction efficiency of this technology is 23-35%. Currently, this system has been applied in model plants. However, its limitations include the ability to only achieve single-base editing of C→T or G→A, and the activity window of the single-base editing system remains relatively large. Additionally, the single-base editing system can still cause minimal DNA sequence insertions or deletions at target sites.

5. Strategies for Improving CRISPR/Cas9 System Application Efficiency in Non-model Plants

5.1 Complete Whole-genome Sequencing of Non-model Plants

The purpose of whole-genome sequencing is to obtain species genome sequences, which can study species growth, development, evolution, and origin at the genome level, deepen understanding of species, and play important roles in discovering new genes and improving breeding. The application of CRISPR/Cas9 systems for genome editing research can comprehensively mine gene functions to obtain important genetic information. Many non-model plants have broad ecological adaptability and growth capacity in various extreme habitats, and CRISPR/Cas9 technology application will promote exploration of special trait genes that grow in extreme habitats, such as drought-resistant, salt-tolerant, and cold-resistant genes. Furthermore, CRISPR/Cas9 system application accelerates exploration of gene metabolic pathways and transcriptional regulatory mechanisms, enabling gene modification through gene knockout and insertion mutations.

5.2 Design of sgRNA and ssODNs

In CRISPR/Cas9 genome editing technology, Cas9 protein cleavage at specific target sites is primarily determined by the PAM sequence and the 20 nt sequence in single-guide RNA (sgRNA). Therefore, sgRNA sequence selection is targeted and directly determines the localization position of the Cas9 protein nuclease. First, there must be a PAM sequence, which is the NGG sequence (where N is any base). Second, to reduce the deletion rate of Cas9 protein during cleavage, the sgRNA sequence should highly match the target sequence in the genome. Additionally, sgRNA should have minimal homology with other sites in the genome to avoid mismatching. Finally, when designing sgRNA, shortening its length from 20 nt to 17 nt helps reduce mismatch probability.

In CRISPR/Cas9 genome editing technology, sgRNA design and selection are crucial. However, in targeted modification, homology repair template (ssODNs) design is also a key issue. Two different DNA repair modes (HR and NHEJ) are

induced after double-strand breaks. The difference between the two DNA repair methods is that HR requires a homology repair template, namely short-chain DNA or single-strand oligonucleotides (ssODNs). Using this template, HR can perform precise point mutations, insertions, and DNA deletions. When introducing a large DNA fragment sequence at the target site, the repair template can also be a double-strand plasmid containing homology arms. In recent years, ssODNs have replaced double-strand plasmids in genome site modification applications. To improve repair efficiency, ssODNs should include sequences complementary to the target sequence extending at least 40 nt in both the 3' and 5' directions. Based on this, it is best to introduce restriction enzyme sites to conveniently detect whether the gene editing system is effective through restriction fragment length polymorphism (RFLP) experiments.

5.3 Modification of Cas9 Protein

With the development of CRISPR/Cas9 technology, researchers have developed new members in addition to the wild-type SpCas9 system. In 2015, Zhang Feng' s team discovered a simpler nuclease (Cpf1) that can independently complete crRNA processing and maturation without relying on ribonuclease (RNase) and tracrRNA. Cpf1 can not only cleave DNA sequences but also act on RNA, and shows no off-target effects. In the same year, Zhang Feng' s research team also discovered a new protein, C2C2, which specifically cleaves particular RNA sequences in bacteria. These new discoveries can provide convenience for researchers in solving future problems.

Since its introduction in 2013, CRISPR/Cas9 technology has rapidly spread to genetic breeding of multiple plants. Compared with the previous two generations of TALENs/ZFNs gene editing technologies, it offers higher knockout efficiency, simpler operation, fewer consumables, and is suitable for many laboratories. In the plant field, the CRISPR/Cas9 system has been mainly applied in genome editing of model plants and some major crops, but to achieve widespread application to more non-model plants and small crops, further improvement and perfection are needed in the future: (1) Discovery of specific genes. Although whole-genome sequencing of many model plants, especially major crops, has been completed, genome sequences of many non-model plants remain unclear, and many genes related to important traits await identification, hindering the application of CRSPR/Cas9 technology in plant genetic engineering breeding; (2) Improvement of vector construction efficiency. In non-model plants, sgRNA design currently relies on manual design by researchers through plant gene exons, and sgRNA specificity must be further verified through experiments, seriously affecting later knockout efficiency and being time-consuming and labor-intensive without relevant auxiliary software; (3) Establishment of non-model genetic transformation systems. Currently, many non-model plants cannot apply CRISPR/Cas9 gene editing technology due to the lack of effective genetic transformation systems, and the relatively large size of the system vector also affects transformation efficiency; (4) Improvement of HDR occurrence

frequency. Current CRISPR/Cas9 technology research primarily obtains transgenic plants by knocking out target sites, so how to modify the CRISPR/Cas9 system to introduce relevant functional genes at genomic target sites through HDR will become a key breakthrough direction for CRISPR/Cas9 technology in plant genetic engineering improvement. It is believed that with further development of CRISPR/Cas9 technology, these problems will eventually be overcome, and its emergence will certainly bring better development to plant genetic engineering.

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Note: Figure translations are in progress. See original paper for figures.