REVIEW

Application of CRISPR/Cas9 in plant biology

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Abstract The CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) system was first identified in bacteria and archaea and can degrade exogenous substrates. It was developed as a gene editing technology in 2013. Over the subsequent years, it has received extensive attention owing to its easy manipulation, high efficiency, and wide application in gene mutation and transcriptional regulation in mammals and plants. The process of CRISPR/Cas is optimized constantly and its application has also expanded dramatically. Therefore, CRISPR/Cas is considered a revolutionary technology in plant biology. Here, we introduce the mechanism of the type II CRISPR/Cas called CRISPR/Cas9, update its recent advances in various applications in plants, and discuss its future prospects to provide an argument for its use in the study of medicinal plants.

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1. Introduction

CRISPR/Cas acts as a type of adaptive immunity in prokaryotes that was formed over a long evolutionary history. It can degrade exogenous genes from an invading phage or plasmid and was first observed in 1987. Ishino et al. found an interval approximately 32 nt of non-repetitive sequences and “tandem repeats” downstream from the \textit{iap} gene in \textit{Escherichia coli}. In 2002, the “tandem repeats” were called “clustered regularly interspaced short palindromic repeats” (CRISPR)\textsuperscript{2,3}. In 2005, the CRISPR spacer sequence was found to be highly homologous with exogenous sequences from bacterial plasmids and phages\textsuperscript{4-6}. As a result of this homology between host and exogenous substances, CRISPR is able to cleave foreign DNA. Notably, the vital site-specific gene editing tool called the CRISPR/Cas system was developed in 2013. CRISPR/Cas only requires a short guide RNA sequence to recognize the target loci according to Watson–Crick base pairing, the endonuclease activity of Cas can lead to gene modification by cleaving the target DNA and forming DNA double-strand breaks (DSBs) that stimulate DNA repair mechanisms \textit{in vivo}, resulting in gene mutation (e.g., insertion, deletion and replacement).

Compared with previously developed gene editing tools zinc finger nucleases (ZFNS)\textsuperscript{7,8}, and transcription activator–like effector nucleases (TALENs)\textsuperscript{9,10} (Table 1)\textsuperscript{11,12}, CRISPR/Cas is more efficient and it can edit multiple target genes simultaneously\textsuperscript{13}. Based on these advantages, applications of CRISPR/Cas are rapidly developing. The ZFN and TALEN gene editing tools search valid sequences with proteins, while CRISPR/Cas depends on guide RNA (gRNA). Recently, a new genome editing technology was developed called NgAgo, which is applicable for editing genes in human cells with the DNA-mediated NgAgo endonuclease\textsuperscript{11}. To date, only one study using NgAgo has been published\textsuperscript{14}. This study reports that gDNA/NgAgo led to a gene knockdown that resulted in an abnormal phenotype in zebrafish. Unfortunately, other groups have not successfully repeated the utilization of NgAgo for genome editing, and therefore NgAgo is still a topic of discussion in the field. Gene editing technologies are developing rapidly, including those using the CRISPR/Cas system. Foreseeably, gene editing technologies will have an impact on the progress of medicine, agriculture, and other scientific fields because it will allow for direct and fast genetic modifications of model systems used in these fields.

CRISPR/Cas can be divided into three major types, I, II and III\textsuperscript{15}. At present, most research is focused on the principles and applications of the type II CRISPR/Cas9 more than the other two types. The CRISPR/Cas9 system requires CAS-associated 9 protein, crRNA (CRISPR RNA), tracrRNA (transactivating crRNA) and RNase III (Ribonuclease III) to edit target genes. Jinek et al.\textsuperscript{16} demonstrated that a single guide RNA (sgRNA) formed by fusing crRNA to tracrRNA plays the same role as a crRNA-tracrRNA hybrid. Zhang et al.\textsuperscript{17} and Church et al.\textsuperscript{18} reported the use of CRISPR/Cas9 in mouse and human cells, respectively, and showed that they could edit target specific genes of mammalian cells successfully in March 2013. Then, three research teams\textsuperscript{19-21} were able to use CRISPR/Cas9 to target genes in plants and the technology has since obtained widespread attention in plant biology (Table 2)\textsuperscript{22-78}. CRISPR/Cas9 has been rapidly developed and successfully applied to alter metabolic pathways and improve crop quality and drug development \textit{via} gene mutation, gene silencing, and transcriptional regulation. The applications of type II CRISPR have had a tremendous impact on bioengineering and molecular biology, however, scientists are still searching for more flexible and applicable CRISPR-derived systems, such as dCas9 nickase\textsuperscript{79}, fCas9\textsuperscript{80}, Cpf1\textsuperscript{81}, and other similar nuclease systems to apply to molecular biology research. This review summarizes some of the sophisticated applications of CRISPR/Cas9 in plants in order to facilitate its application in medicinal plant research.

2. The mechanism of CRISPR/Cas9

CRISPR/Cas9 cleaves foreign DNA \textit{via} two components, Cas9 and sgRNA (Fig. 1A). Cas9 is a DNA endonuclease that can be derived from different bacteria, such as \textit{Brevibacillus laterosporus}\textsuperscript{82}, \textit{Staphylococcus aureus}\textsuperscript{83}, \textit{Streptococcus pyogenes}\textsuperscript{84}, \textit{Streptococcus thermophilus}\textsuperscript{85}, and \textit{Streptococcus pyogenes} is the most widely used for Cas9 isolation. Cas9 contains two domains, \textit{i.e.}, HNH domain and RuvC-like domain. The HNH domain cuts the complementary strand of crRNA, while the RuvC-like domain cleaves the opposite strand of the double-stranded DNA. The sgRNA is a synthetic RNA with a length of about 100 nt. Its 5’-end has a 20-nt sequence that acts as a guide sequence to identify the target sequence accompanied by a protospacer adjacent motif (PAM) sequence, which is often the consensus NGG (N, anynucleotide; G, guanine). The loop structure at the 3’-end of the sgRNA can anchor the target sequence by the guide

| Table 1 | Comparison of ZFN, TALEN, CRISPR/Cas9 and NgAgo\textsuperscript{11,12}. |
| --- | --- |
| Technology | DNA binding determinant | Endonuclease | Mutation rate (%) | Target site length (bp) | Binding specificity | Off-targeting | Application |
| ZFN | Zinc finger protein | FokI | 10 | 18–36 | 3 Nucleotides | High | Human cells, pig, mice, tobacco, nematode and zebrafish |
| TALEN | Transcription-activator-like effector | FokI | 20 | 30–40 | 1 Nucleotide | Low | Human cells, water flea, cow and mice |
| CRISPR/Cas9 | crRNA/sgRNA | Cas9 | 20 | 22 | 1:1 Nucleotide pairing | Variable | Human cells, wheat, rice, maize and \textit{Drosophila} |
| NgAgo-gDNA | 5’ phosphorylated ssDNA | NgAgo | 21.3–41.3 | 24 | 1:1 Nucleotide pairing | Low | Human cells |
| Plant | Target gene | Cas9 version | Cas9 promoter | sgRNA promoter | Delivery method | Editing method | Mutation frequency (%) | Ref. |
|-------|-------------|--------------|---------------|----------------|-----------------|----------------|------------------------|------|
| Arabidopsis thaliana | BRII1, JAZ1, GAI | Human codon-optimized Cas9 | 2 × 35S | AtU6-26 | Agrobacterium-mediated transformation | NHEJ | 30–84 | 22 |
| A non-functional GFP | Chlamydomonas | Plant codon-optimized Cas9 | 35 SPPDK | AtU6 | Agrobacterium infiltration | NHEJ | 1.1–7.7 | 19 |
| AtPDS3, AtFLS2, AtRACK1b, etc. | | | | | | | |
| CHL1, CHL2, TT4 | Human codon-optimized Cas9 | CaMV 35S | AtU6-26 | Agrobacterium-mediated transformation | NHEJ | N/A | 23 |
| ADH1, TT4, RTEL1 | Arabidopsis codon-optimized Cas9 | PcUbi4-2 | AtU6-26 | Agrobacterium-mediated transformation | NHEJ | N/A | 25 |
| ADH1 | | | | | | | |
| TRY, CPC, ETC2 | | | | | | | |
| FT, SPL4 | Human codon-optimized Cas9 | AtICU2 | AtU6 | Agrobacterium-mediated transformation | NHEJ | 10.00–84.78 | 28 |
| AtCRU3 | Arabidopsis codon-optimized Cas9 | 35S | U6-26 | Agrobacterium-mediated transformation | NHEJ | N/A | 29 |
| At1g16210, At1g56650, At5g55580 | | | | | | | |
| AP1, TT4 | Plant codon-optimized Cas9 | | | | | | |
| ADH1 | Streptococcus thermophilus and Staphylococcus aureus | | | | | | |
| ETC2, TRY, CPC, etc. | Zea mays codon-optimized Cas9 | EC1.2 | U6-26p, U6-29p | Agrobacterium-mediated transformation | NHEJ | N/A | 33 |
| BRII | Human codon-optimized Cas9 | 2 × 35S, YAO | AtU6-26 | Agrobacterium-mediated transformation | NHEJ | 4.3–90.5 | 34 |
| PYR1, PYL1, PYL2, etc. | | | | | | | |
| Brassica oleracea | BolC.GA4.a | Streptococcus pyogenes Cas9 | 35S | U6-26 | Agrobacterium-mediated transformation | NHEJ | 10 | 36 |
| Citrus sinensis | CaPDS | Human codon-optimized Cas9 | CaMV 35S | CaMV 35 S | Agrobacterium infiltration | NHEJ | 3.2–3.9 | 37 |
| Cucumis sativus | eIF4E | Plant codon-optimized Cas9 | 35S | AtU6 | Agrobacterium-mediated transformation | NHEJ | N/A | 38 |
| Glycine max | Bar, GmFEI1, GmFEI2, etc. | Plant codon-optimized Cas9 | 2 × 35S | AtU6 | Electroporation and transformation | NHEJ | 10.0–93.3 | 39 |
| GmPDS11, GmPDS18 | | | | | | | |
| GFP, 01gDDM1, 11gDDM1, etc. | Human codon-optimized Cas9 | | | | | | |
| DD20, DD43 | Soybean codon-optimized Cas9 | GmEF1A2 | GmU6 | Particle bombardment | NHEJ, HDR | 59–76 | 42 |
| Organism          | Gene(s)          | CRISPR/Cas9 | Delivery Method | Tag(s)          | Transformation Type | NHEJ | HDR |
|-------------------|------------------|-------------|----------------|-----------------|--------------------|------|-----|
| Glycine maxima     | Glyma06g14180, Glyma08g02290, Glyma12g37050 | Soybean codon-optimized Cas9 | 2 × 35S U6 | Agrobacterium-mediated transformation | NHEJ | N/A | 43 |
| Hordeum vulgare    | HvPM19           | Streptococcus pyogenes Cas9 | 35S U6-26 | Agrobacterium-mediated transformation | NHEJ | 3.2–20.2 | 44 |
| Marchantia polymorpha | MpARF1         | Human codon-optimized Cas9 | CaMV 35S and MpU6-1 | Agrobacterium-mediated transformation | NHEJ | N/A | 45 |
| Medicago truncatula | GUS             | Soybean codon-optimized Cas9 | 2 × 35S U6 | Agrobacterium-mediated transformation | NHEJ | N/A | 43 |
| Nicotiana benthamiana | GFP            | Chlamydomonas Reinhardtii codon-optimized Cas9 | CaMV 35S AtU6-26 | Agrobacterium infiltration | NHEJ | N/A | 23 |
| Plant and Human codon-optimized Cas9 35S | 2 × 35S AtU6-26 | Agrobacterium-mediated transformation | NHEJ | 11 | 49 |
| Nicotiana tabacum  | NiPDS, NiPDR6   | Plant and Human codon-optimized Cas9 35S | 2 × 35S AtU6-26 | Agrobacterium-mediated transformation | NHEJ | 81.8–87.5 | 50 |
| Nicotiana tabacum  | mCherry         | Plant codon-optimized Cas9 | 35S-PPDK U6 | Agrobacterium-mediated transformation | NHEJ | N/A | 51 |
| Oryza sativa       | ROC5, SPP, YSA  | Human codon-optimized Cas9 | CaMV 35S OsU6-2 | Agrobacterium-mediated transformation | NHEJ | 4.8–75 | 22 |
| Oryza sativa       | OsSWEET11,OsSWEET14 | Streptococcus pyogenes Cas9 and rice-optimized Cas9 | CaMV 35S OsU6 | PEG-mediated transformation | NHEJ | N/A | 23 |
| Oryza sativa       | OsMYB1          | Human codon-optimized Cas9 | OsUBQ1 OsU3 | Agrobacterium-mediated transformation | NHEJ | 50–89 | 24 |
| Oryza sativa       | CAO1, LAZY1     | Rice codon-optimized Cas9 | OsUbI OsU3 | Agrobacterium-mediated transformation | NHEJ | 83–92 | 52 |
| Oryza sativa       | OsPDS, OsMPK2, OsBADH2, etc. | Rice codon-optimized Cas9 | 2 × 35S OsU6 | Particle bombardment | NHEJ, HDR 7.1–50 | 20 |
| Oryza sativa       | OsMPK5          | Human codon-optimized Cas9 | CaMV 35S OsU6 | Agrobacterium-mediated transformation | NHEJ | 3–8 | 53 |
| Oryza sativa       | OsPDS, OsDEP1   | Rice codon-optimized Cas9 | 2 × 35S OsU3 | Particle bombardment | NHEJ, HDR 33–38 | 54 |
| Oryza sativa       | OsBEL           | Plant codon-optimized SpCas9 | 2 × 35S AtU6-26 | Agrobacterium-mediated transformation | NHEJ | 2–16 | 55 |
| Oryza sativa       | OsPDS, OsPMS3, OsEPSPS, etc. | Rice codon-optimized SpCas9 | OsUbI OsU6, OsU3 | Agrobacterium-mediated transformation | NHEJ | 21.1–66.7 | 56 |
| Oryza sativa       | SWEET1a, SWEET1b, SWEET11, etc. | Rice codon-optimized SpCas9 | OsUbI OsU6, OsU3 | Agrobacterium-mediated transformation | NHEJ | 12.5–100 | 57 |
| Oryza sativa       | ALS             | Rice codon-optimized SpCas9 | 2 × 35P3S OsU6 | Agrobacterium-mediated transformation | HDR | 0.147–1 | 58 |
| Plant                          | Target gene | Cas9 version                                      | Cas9 promoter | sgRNA promoter | Delivery method                      | Editing method | Mutation frequency (%) | Ref. |
|-------------------------------|-------------|--------------------------------------------------|---------------|----------------|--------------------------------------|----------------|-----------------------|------|
| CDEA1, CDEA2, CDKB1, etc.     | CDKA1, CDKA2, OsYSA, OsROC5                      | Rice codon-optimized SpCas9                      | 2 × P35S      | OsU3           | Agrobacterium-mediated transformation| NHEJ           | 0–76.9                | 59   |
| OsFTL1, Os07g0261200, Os02g0700600, YSA, CDKB2 | Rice codon-optimized SpCas9                      | 2 × CaMV 35S OsU3                                |               |                | Agrobacterium-mediated transformation| NHEJ           | 7.6–68.7              | 60   |
| OsAOX1a, OsAOX1b, OsAOX1c, etc. | OsYSA, CDKB2 | Plant codon optimized Cas9                       | 35S           | OsU3, OsU6     | Agrobacterium-mediated transformation| NHEJ           | 33.3–53.3             | 46   |
| OsPDS, OsMPK2, Os02g23823      | OsFTL1, Os07g0261200, Os02g0700600, YSA, CDKB2 | Plant codon optimized Cas9                       | 2 × CaMV 35S  | OsU3           | Agrobacterium-mediated transformation| NHEJ           | 81.4–90.0             | 30   |
| OsAOX1a, OsAOX1b, OsAOX1c, etc. | OsYSA, CDKB2 | Plant codon optimized Cas9                       | 35S           | OsU3, OsU6     | Agrobacterium-mediated transformation| NHEJ           | 6.6–81.0              | 62   |
| OsFTL1, Os07g0261200, Os02g0700600, YSA, CDKB2 | Rice codon-optimized SpCas9                      | 2 × P35S OsU3                                    |               |                | Agrobacterium-mediated transformation| NHEJ           | 55.6–87.5             | 65   |
| Petunia hybrid PDS            | PDS         | Plant codon optimized Cas9                       | 35S           | AtU6           | Agrobacterium-mediated transformation| NHEJ           | 55.6–87.5             | 65   |
| Populus tomentosa PtoPDS      | PtoPDS      | Wild-type SpCas9                                 | 35S           | AtU3b, AtU3d, AtU6-1, AtU6-29 | Agrobacterium-mediated transformation| NHEJ           | 51.7                  | 66   |
| Solanum lycopersicum SIAGO7   | SIAGO7      | Codon-optimized Cas9                             | 35S           | AtU6           | Agrobacterium-mediated transformation| NHEJ           | 6.6–81.0              | 62   |
| SHR, SCR                      | Nicotiana   | Codon-optimized Cas9                             | 35S           | AtU6           | Agrobacterium-mediated transformation| NHEJ           | 6.6–81.0              | 62   |
| RIN                           | Codon-optimized Cas9                             | 35S           | AtU6           |                | Agrobacterium-mediated transformation| NHEJ           | 6.6–81.0              | 62   |
| SIPDS, SIPIF4                 | Human codon-optimized Cas 9                      | 35S           | AtU6-26        |                | Agrobacterium-mediated transformation| NHEJ           | 72.7–100              | 70   |
| Solanum tuberosum SIALS1      | Arabidopsis | Codon-optimized Cas9                             | 35S           | AtU6           | Agrobacterium-mediated transformation| NHEJ           | 3–60                  | 71   |
| SIAIA2                        | Rice-codon optimized Cas9                       | 2 × 35S      | StU6           |                | Agrobacterium-mediated transformation| NHEJ           | 6.6–81.0              | 62   |
| Sorghum bicolor DsRED2        | Monocot codon-optimized synthetic Cas9           | Rice Actin 1 | OsU6           |                | Agrobacterium-mediated transformation| NHEJ           | 6.6–81.0              | 62   |
| Triticum aestivum Tainox, Tapds | Human codon-optimized Cas9                      | 35S           | AtU6           |                | Agrobacterium-mediated transformation| NHEJ           | 18–22                 | 47   |
| TaMLO                         | Rice codon-optimized Cas9                       | 2 × 35S      | TaU6           |                | Agrobacterium-mediated transformation| NHEJ           | 26.5–38               | 20   |
| TaLOX2                        | Rice codon-optimized Cas9                       | 2 × 35S      | TaU6           |                | Agrobacterium-mediated transformation| NHEJ           | 26.5–38               | 20   |
| TaMLOA1, TaMLOB1, TaMLOD1     | Plant codon-optimized Cas9                      | 2 × 35S      | TaU6           |                | Agrobacterium-mediated transformation| NHEJ           | 26.5–38               | 20   |
| Vitis vinifera                | IdnDH      | Human codon-optimized Cas9                       | 35S           | AtU6           | Agrobacterium-mediated transformation| NHEJ           | 100% (suspension cell)  | 74   |
sequence and form a complex with Cas9, which cleaves the double-stranded DNA and forms a double-strand break (DSB) at this site.

Once a DSB is generated, nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) DNA repair mechanisms are initiated (Fig. 1B). A DSB is usually repaired by NHEJ in most situations and is a simple way to create mismatches and gene insertion/deletions (indel), leading to gene knockout. When an oligo template is present, HDR induces specific gene replacement or foreign DNA knock-ins41,85,86. These processes are all ways that CRISPR/Cas9 can efficiently edit the genome of diverse organisms, including humans, animals and plants.

3. The application of CRISPR/Cas9 in plants

3.1. NHEJ gene knockouts

The major applications of CRISPR/Cas9 include gene knockouts in organisms for elucidating the function of single or multiple gene targets (e.g., enzyme genes or microRNAs) via gene mutation.

3.1.1. Enzyme genes

Jiang et al.23 constructed different binary vectors carrying diverse Cas9 and sgRNA combinations, investigated transient expression of Cas9/sgRNA in Arabidopsis, tobacco, rice, and sorghum by Agrobacterium or PEG-mediated transfection, and confirmed that CRISPR/Cas9 has the capability to edit target genes in these four plants. Jia and Wang37 developed a new tool for transient expression in sweet orange targeting CsPDS (phytoene desaturase gene) via Xanthomonas citri subsp. citri (Xcc)-facilitated agroinfiltration, and found the target gene was successfully mutated with no off-target effects detected. Yin et al. 48 reported a unique sgRNA delivery system named VIGE (virus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing) could be used for transient expression that targets NbPDS3 and NbIspH, which cause a photo-bleaching phenotype when they are expressed in tobacco. The authors demonstrated that newly-grown leaves exhibited the phenotype, thus confirming that VIGE could edit target genes successfully and was an effective mode for genome modification. Wang et al. 73 constructed Cas9/sgRNA vectors that were delivered by particle bombardment to protoplasts of haxaploid bread wheat that targeted the TaMLO (mildew resistance locus) gene. This report confirmed CRISPR/Cas9 as a versatile tool could also be harnessed in haxaploid plants.

Lawrenson36 used CRISPR/Cas9 to edit the HvPM19 gene in Hordeum vulgare and BoI.CGA4.a in Brassica oleracea via a transgenic system. The indel frequency of HvPM19 was 23% in the first generation, while that of BoI.CGA4.a was 10%. In addition, the authors also screened for the expected phenotype in T0 plants and observed that the mutations could be stably inherited in the next generation. This study demonstrated that CRISPR/Cas9 is a powerful tool for investigating the function of target genes in both barley and Brassica oleracea. Ito et al. 69 constructed sgRNA and Cas9 carriers to target the ripening inhibitor gene (RIN) that encodes a transcription factor that regulates fruit ripening in tomato. They found that red pigmentation in the RIN-protein-defective mutants was significantly lower than that of the wild type in T0 transgenic lines, while heterozygous mutants developed ripe red fruits as wild type.
3.1.2. MicroRNAs
MicroRNAs (miRNA) serve as regulators to stimulate or inhibit gene expression in plants. Jacobs et al.63 applied CRISPR/Cas9 to two miRNAs (miR1514 and miR1509) in soybean. Vectors harboring sgRNA and Cas9 were delivered by particle bombardment for transient expression and the authors confirmed that CRISPR/Cas9 could be utilized to target miRNA in soybean, which further extended the application of CRISPR/Cas9 in plants. Li et al.63 used CRISPR/Cas9 to target miR156 recognition site in which further extended the application of CRISPR/Cas9 in plants. CRISPR/Cas9 could be utilized to target miRNA in soybean, ment for transient expression and the authors harboring sgRNA and Cas9 were delivered by particle bombardment for two miRNAs (miR1514 and miR1509) in soybean. Vectors studies have successfully utilized CRISPR/Cas9 editing target precise gene knock-in or gene replacement. But only a few HDR is a highly desirable repair pathway for DSB that lead to 3.2. HDR gene knock-in and gene replacement
HDR is a highly desirable repair pathway for DSB that lead to precise gene knock-in or gene replacement. But only a few studies have successfully utilized CRISPR/Cas9 editing target genes with HDR. Li et al.19 transiently co-expressed Cas9 and gRNA in tobacco protoplasts to target the AvrII site of NbPDS gene using a DNA template. Sanger sequencing found that HDR-mediated gene replacement took a proportion of 9.0%. However, this report did not achieve successful HDR-mediated DSB repair system in Arabidopsis. Subsequently, Schiml et al.26 constructed Cas9/sgRNA vectors targeting the ADH1 (alcohol dehydrogenase 1) gene in Arabidopsis delivered by an Agrobacterium-mediated system for transgenic expression, and obtained mutants made by the HDR-mediated repair system. The authors elucidated that the HDR-mediated repair system was able to target genes in Arabidopsis. Endo et al.26 transformed a Cas9 expression construct, gRNA, and a gene targeting (GT) vector containing an HDR template into the calli of Oryza sativa to target the acetolactate synthase (ALS) gene and successfully obtained bi-allelic rice mutants. Moreover, the HDR-mediated CRISPR/Cas9 system was successfully utilized to create precise and heritable modifications in tomato31, maize37 and soybean32.

There are still great challenges remaining in HDR-mediated CRISPR/Cas9 genome modification, one of the major challenges being how to simultaneously deliver the donor DNA template and the synthetic endonuclease to plant tissues. Thus, if the delivery of donor DNA and endonuclease is elucidated, the efficacy of precise gene knock-in or gene replacement in organisms will increase. Dissecting the functionality of some genes will be quite simple, and it will be possible to produce more new cultivars of medicinal plants with desired traits, such as pest resistance, high yield and high quality. Undeniably, it is essential to do more research on HDR-mediated editing pathways.

3.3. Transcriptional regulation
Transcriptional regulation refers to changes in transcription that induce the changes in gene expression levels. Some research has used CRISPR/Cas9 to regulate transcription in mammalian cells66–69 and plants; Piatet et al.33 was able to target transcription regulation with a catalytically inactive Cas9 (dCas9) combined with a deactivated nuclease function that was still able to bind DNA with gRNA. The results of the experiments with dCas9 demonstrated that the dCas9 C-terminus with a plant-specific transcriptional activator, EDLL, and transcription activator-like (TAL) effectors guided by gRNAs could activate transcription of a PDS target gene, and that the dCas9 C-terminus with SRDX guided by gRNAs could repress transcription of a PDS target gene. Moreover, Lowder et al.34 found that dCas9-VP64 with gRNAs could activate the transcription of AtPAP1 (production of anthocyanin pigment 1) and miR319 2-, 3- and 7-fold in Arabidopsis. Additionally, dCas9-VP64 could reverse methylation-induced gene silencing of AtFIS2 (fertilization-independent seed 2) in Arabidopsis. All three transgenic lines had 200-, 300- and 400-fold changes in AtFIS2 gene expression. Therefore, CRISPR/Cas9 is a powerful tool for transcriptional activation/repression of protein-coding and non-protein-coding genes, and it can also reverse gene silencing caused by methylation, thus proving a significant tool in plant biology.

4. The tools of CRISPR/Cas9
The design of sgRNA is one of the key factors in editing target genes successfully using CRISPR/Cas9. Up until now, dozens of

Figure 1 Schematic diagram of CRISPR/Cas9 editing of target genes. (A) A sketch of CRISPR/Cas9 system. The sgRNA (black and red) can identify the target gene, and then the two domains of Cas9 (yellow) cleave the target sequence. (B) Two ways DSB can be repaired. NHEJ is imprecise and always results in a gene knockout mutation. When a template is present, HDR can be activated and results in gene replacement or knock-in. PAM, protospacer adjacent motif; sgRNA, single guide RNA; DSB, double-strand break; NHEJ, nonhomologous end-joining; HDR, homology-directed repair.
online tools and stand-alone software have been developed to devise efficient and specific sgRNA. Zhang and coworkers at the Broad Institute developed an online tool called CRISPR Design (http://www.genome-engineering.org/) to assist in the design of sgRNA and evaluate off-target effects. This tool has two modes; one is the Single Sequence mode that only designs sgRNA 23–500 nt, and the Batch mode can predict several sgRNAs simultaneously. Mismatch and off-target effects can be assessed when sgRNA is designed using this program. In the CRISPR Design program, the available sgRNAs are marked in green, yellow or red, which indicate the different specificity of the sgRNAs. In addition, there are some other tools, including E-CRISPR, CRISPR-P, Cas-OFFinder, Cas-Designer, Cas OT, SSFinder, which make the design of sgRNA become easier.

The construction of expression vectors is diverse in its methodology. Some researchers have constructed different binary vectors by combining Cas9 with gRNA and induced target gene modification. However, others constructed gRNA and Cas9 vectors, respectively, and edited target genes with sequential transformation. Delivering vector(s) effectively is also crucial for high editing efficiency and faces enormous challenges in plants, though the most applied methods for delivering vector(s) to plants include Agrobacterium-mediated transformation, PEG-mediated transfection of protoplasts, and particle bombardment (Fig. 2). All methods have their virtues and faults, and there is still much to be learned and optimized for the use of CRISPR/Cas9 in plants.

5. Conclusions and prospects

CRISPR/Cas9 as an essential technology with specific features, such as simple manipulation, high efficiency and wide application; as a result, it has been rapidly and widely applied to diverse facets of molecular biology. Currently, some medicinal plants have completely sequenced genomes; for instance, *Salvia miltiorrhiza* and *Dendrobium officinale*. Thus, it is feasible to harness CRISPR/Cas9 to edit target genes in these plants and study the synthesis of effective constituents or toxic components to increase the effective constituents or reduce toxicity. Furthermore, using CRISPR/Cas9 to research genetic resources of medicinal plants can select excellent traits and increase yield. Utilizing new technologies like CRISPR/Cas9 can promote research on biosynthetic pathways and regulatory mechanisms of effective components, and screen of excellent germplasm in medicinal plants for rapid development, which is an important part of current pharmaceutical botany.

Currently, the application of CRISPR/Cas9 is mainly about genome editing and transcriptional regulation. Furthermore, DNA labeling and epigenome editing with CRISPR/Cas9 have been reported, but they are not applied in plants. Thus, it will be interesting to see CRISPR/Cas9 application in plant DNA labeling using fluorescent-labeled Cas9 protein and optimized gRNA, and epigenome editing by DNA methylation or histone modifications in the future. The evidence of CRISPR/Cas9 essential functions in genome editing opens many new experimental avenues for gene function analysis and has a tremendous potential in medicinal plant research.

Although the CRISPR/Cas9 can be applied to plant genome editing, there are still certain challenges, such as minimizing off-target rates, elucidating the precise mechanism for this minimization, and how to optimize Cas9 function. Further study is needed to improve the experimental application of CRISPR/Cas9 to promote the development of its basic and applied abilities in the future.

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