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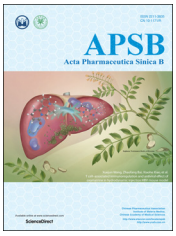

REVIEW

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[1]. Ishino et al.[1] found an interval approximately 32 nt of non-repetitive sequences and "tandem repeats" downstream from the *iap* gene in *Escherichia coli*. In 2002, the "tandem repeats" were called "clustered regularly interspaced short palindromic repeats" (CRISPR)[2,3]. In 2005, the CRISPR spacer sequence was found to be highly homologous with exogenous sequences from bacterial plasmids and phages[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 *in vivo*, resulting in gene mutation (*e.g.*, insertion, deletion and replacement).

Compared with previously developed gene editing tools zinc finger nucleases (ZFNs)[7,8], and transcription activator–like effector nucleases (TALENs)[9,10] (Table 1)[11,12], CRISPR/Cas is more efficient and it can edit multiple target genes simultaneously[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[11]. To date, only one study using NgAgo has been published[14]. This study reports that gDNA/NgAgo led to a gene knockdown that resulted in an abnormal phenotype in zebrafish, but no gene mutation could be detected. 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[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.[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.[17] and Church et al.[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[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)[22–78]. CRISPR/Cas9 has been rapidly developed and successfully applied to alter metabolic pathways and improve crop quality and drug development *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[79], fCas9[80], Cpf1[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 *via* two components, Cas9 and sgRNA (Fig. 1A). Cas9 is a DNA endonuclease that can be derived from different bacteria, such as *Brevibacillus laterosporus*[82], *Staphylococcus aureus*[83], *Streptococcus pyogenes*[84], *Streptococcus thermophilus*[32], and *Streptococcus pyogenes* is the most widely used for Cas9 isolation. Cas9 contains two domains, *i.e.*, HNH domain and RucV-like domain. The HNH domain cuts the complementary strand of crRNA, while the RucV-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[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 *Drosophila*
NgAgo-gDNA	5′ phosphorylated ssDNA	NgAgo	21.3–41.3	24	1:1 Nucleotide pairing	Low	Human cells

Table 2 List of CRISPR/Cas9 gene editing in plants.

Plant	Target gene	Cas9 version	Cas9 promoter	sgRNA promoter	Delivery method	Editing method	Mutation frequency (%)	Ref.
Arabidopsis thaliana	*BRI1*, *JAZ1*, *GAI*	Human codon-optimized Cas9	2 × 35S	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	30–84	22
	A non-functional *GFP*	*Chlamydomonas Reinhardtii* codon-optimized Cas9	CaMV 35S	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	N/A	23
	AtPDS3, AtFLS2, AtRACK1b, etc.	Plant codon-optimized Cas9	35 SPPDK	AtU6	*Agrobacterium* infiltration	NHEJ	1.1–7.7	19
	CHLI1, *CHLI2*, *TT4*	Human codon-optimized Cas9	OsUBQ1	OsU3	*Agrobacterium*-mediated transformation	NHEJ	10–89	24
	ADH1, TT4, RTEL1	*Arabidopsis* codon-optimized Cas9	PcUbi4-2	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	2.5–70.0	25
	ADH1	*Arabidopsis* codon-optimized Cas9	PcUbi4-2	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ, HDR	42.8	26
	TRY, CPC, ETC2	Maize codon-optimized Cas9	2 × 35S	U6-26, U6-29	*Agrobacterium*-mediated transformation	NHEJ	42–90	27
	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	Plant codon-optimized Cas9	Ubi, 35S	AtU3b, AtU3d, AtU6-1, AtU6-29	*Agrobacterium*-infiltration	NHEJ	81.4–90.0	30
	AP1, TT4	Plant codon-optimized Cas9	AtUBQ1, SPL	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	3–56	31
	ADH1	*Streptococcus thermophilus* and *Staphylococcus aureus* codon-optimized Cas9	PcUbi4-2	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	6.1–98.5	32
	ETC2, TRY, CPC, etc.	*Zea mays* codon-optimized Cas9	EC1.2	U6-26p, U6-29p	*Agrobacterium*-mediated transformation	NHEJ	N/A	33
	BRI1	Human codon-optimized Cas9	2 × 35S, *YAO*	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	4.3–90.5	34
	PYR1, *PYL1*, *PYL2, etc.*	Human codon-optimized Cas9	AtUBQ1	AtU6-26, AtU3b, At7SL-2	*Agrobacterium*-mediated transformation	NHEJ	13–93	35
Brassica oleracea	*BolC.GA4.a*	*Streptococcus pyogenes* Cas9	35S	U6-26	*Agrobacterium*-mediated transformation	NHEJ	10	36
Citrus sinensis	*CsPDS*	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*	Plant codon-optimized Cas9	ZmUbi	AtU6, GmU6	*Agrobacterium*-mediated transformation	NHEJ	11.7–48.1	40
	GFP, 01gDDM1, *11gDDM1*, *etc.*	Human codon-optimized Cas9	2 × 35S	MtU6.6	*Agrobacterium*-mediated transformation	NHEJ	> 70	41
	DD20, DD43	Soybean codon-optimized Cas9	GmEF1A2	GmU6	Particle bombardment	NHEJ, HDR	59–76	42

	GS1, *CHI20*	Soybean codon-optimized Cas9	2 × 35S	U6	*Agrobacterium*-mediated transformation	NHEJ	N/A	43
	Glyma06g14180, Glyma08g02290, Glyma12g37050	Codon-optimized cas9	CaMV 35S	AtU6-26, GmU6-10	*Agrobacterium*-mediated transformation	NHEJ	3.2–20.2	44
Hordeum vulgare	*HvPM19*	*Streptococcus pyogenes* Cas9	35S	U6-26	*Agrobacterium*-mediated transformation	NHEJ	10–23	36
Marchantia polymorpha	*MpARF1*	Human codon-optimized Cas9	CaMV 35S and MpEF1α	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
	NbPDS	Plant codon-optimized Cas9	35S PPDK	AtU6	*Agrobacterium* infiltration	NHEJ, HDR	9–39	19
	NbFLS2, NbBAK1	Plant codon-optimized Cas9	35S	AtU3, AtU6	*Agrobacterium*-mediated transformation	NHEJ	N/A	46
	NbPDS	Human codon-optimized Cas9	35S	AtU6	*Agrobacterium* infiltration	NHEJ	1–3	21
	Nbpds	Human codon-optimized Cas9	CaMVE 3S	CaMVE35S	*Agrobacterium*-mediated transformation	NHEJ	12.7–13.8	47
	NbPDS, NbIspH	Plant codon-optimized Cas9	35S	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	75–85	48
	XT	Plant and Human codon-optimized Cas9	35S	U6-26	*Agrobacterium* infiltration	NHEJ	11	49
Nicotiana tabacum	*NtPDS*, *NtPDR6*	Plant codon-optimized Cas9	2 × 35S	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	81.8–87.5	50
	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
	OsSWEET11,OsSWEET14	*Streptococcus pyogenes* Cas9 and rice codon-optimized Cas9	CaMV 35S	OsU6	PEG-mediated transformation	NHEJ	N/A	23
	OsMYB1	Human codon-optimized Cas9	OsUBQ1	OsU3	*Agrobacterium*-mediated transformation	NHEJ	50–89	24
	CAO1, *LAZY1*	Rice codon-optimized Cas9	OsUbi	OsU3	*Agrobacterium*-mediated transformation	NHEJ	83–92	52
	OsPDS, OsMPK2, OsBADH2, etc.	Rice codon-optimized Cas9	2 × 35S	OsU6	Particle bombardment	NHEJ, HDR	7.1–50	20
	OsMPK5	Human codon-optimized Cas9	CaMV 35S	OsU6	*Agrobacterium*-mediated transformation	NHEJ	3–8	53
	OsPDS, OsDEP1	Rice codon-optimized Cas9	2 × 35S	OsU3	Particle bombardment	NHEJ, HDR	33–38	54
	OsBEL	Plant codon-optimized Sp Cas9	2 × 35S	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	2–16	55
	OsPDS, *OsPMS3*, *OsEPSPS*, etc.	Human codon-optimized Cas9	35S, OsUBQ1	OsU6, OsU3	*Agrobacterium*-mediated transformation	NHEJ	21.1–66.7	56
	SWEET1a, *SWEET1b*, *SWEET11*, etc.	Rice codon-optimized SpCas9	OsUbi1	OsU6	*Agrobacterium*-mediated transformation	NHEJ	12.5–100	57
	ALS	Rice codon-optimized SpCas9	2 × P35S	OsU6	*Agrobacterium*-mediated transformation	HDR	0.147–1	58

Table 2 (*continued*)

Plant	Target gene	Cas9 version	Cas9 promoter	sgRNA promoter	Delivery method	Editing method	Mutation frequency (%)	Ref.
	*CDKA*1, *CDKA2*, *CDKB*1, etc.	Rice codon-optimized SpCas9	2 × P35S	OsU3	*Agrobacterium*-mediated transformation	NHEJ	0–76.9	59
	OsYSA, OsROC5	Plant codon optimized Cas9	35S	OsU3, OsU6	*Agrobacterium*-mediated transformation	NHEJ	33.3–53.3	46
	*OsFTL1*1, *Os07g0261200, Os02g0700600*	Plant codon optimized Cas9	Ubi, 35S	OsU3, OsU6a, OsU6b, OsU6c	*Agrobacterium*-mediated transformation	NHEJ	81.4–90.0	30
	YSA, CDKB2	Rice codon-optimized SpCas9	2 × CaMV 35S	OsU3	*Agrobacterium*-mediated transformation	NHEJ	7.6–68.7	60
	OsAOX1a, OsAOX1b, OsAOX1c, etc.	Rice codon-optimized SpCas9	OsU3	OsU3	*Agrobacterium*-mediated transformation	NHEJ	N/A	61
	OsPDS, OsMPK2, Os02g23823	Codon-optimized SpCas9	2 × CaMV 35S	OsU3	*Agrobacterium*-mediated transformation	NHEJ	66.4–81.0	62
	Gn1a, DEP1, GS3, etc.	Codon-optimized Cas9	OsUbi	OsU6a	*Agrobacterium*-mediated transformation	NHEJ	27.5–67.5	63
	OsROC5, *OsDEP1*	*Arabidopsis* codon-optimized Cas9	OsUbi	OsU6	*Agrobacterium*-mediated transformation	NHEJ	N/A	64
Petunia hybrid	*PDS*	Plant codon optimized Cas9	35S	AtU6	*Agrobacterium*-mediated transformation	NHEJ	55.6–87.5	65
Populus tomentosa	*PtoPDS*	Wild-type SpCas9	35S	AtU3b, AtU3d, AtU6-1, AtU6-29	*Agrobacterium*-mediated transformation	NHEJ	51.7	66
Solanum lycopersicum	*SlAGO7*	Codon-optimized Cas9	35S	AtU6	*Agrobacterium*-mediated transformation	NHEJ	N/A	67
	SHR, SCR	*Nicotiana* codon optimized Cas9	35S	AtU6	*Agrobacterium*-mediated transformation	NHEJ	N/A	68
	RIN	Codon-optimized Cas9	Ubi4	AtU6	*Agrobacterium*-mediated transformation	NHEJ	N/A	69
	SlPDS, SlPIF4	Human codon-optimized Cas 9	CaMV 35S, AtUBQ	AtU6-26	*Agrobacterium*-mediated transformation	NHEJ	72.7–100	70
Solanum tuberosum	*StALS1*	*Arabidopsis* codon-optimized Cas9	35S	AtU6	*Agrobacterium*-mediated transformation	NHEJ	3–60	71
	StIAA2	Rice-codon optimized Cas9	2 × 35S	StU6	*Agrobacterium*-mediated transformation	NHEJ	N/A	72
Sorghum bicolor	*DsRED2*	Monocot codon-optimized synthetic Cas9	Rice Actin 1	OsU6	*Agrobacterium*-mediated transformation	NHEJ	N/A	23
Triticum aestivum	*Tainox*, *Tapds*	Human codon-optimized Cas9	CaMVE35S	CaMVE35S	*Agrobacterium*-mediated transformation	NHEJ	18–22	47
	TaMLO	Rice codon-optimized Cas9	2 × 35S	TaU6	Protoplast transformation	NHEJ	26.5–38	20
	TaLOX2	Rice codon-optimized Cas9	2 × 35S	TaU6	Particle bombardment	NHEJ	45	54
	*TaMLOA*1, *TaMLOB*1, *TaMLOD1*	Plant codon-optimized Cas9	Ub1	TaU6	Particle bombardment	NHEJ	23–38	73
Vitis vinifera	*IdnDH*	SpCas9	35S	AtU6	*Agrobacterium*-mediated transformation	HR	100% (suspension cell)	74

Zea mays	*ZmIPK*	Plant codon-optimized Cas9	2 × 35S	ZmU3	*Agrobacterium*-mediated transformation	NHEJ	16.4–19.1	75
	ZmHKT1	Human and Maize codon-optimized Cas9	2 × 35S, Ubi1	AtU6-26, OsU3, TaU3	*Agrobacterium*-mediated transformation	NHEJ	N/A	27
	LIG, MS26, MS45, etc.	Maize codon-optimized Cas9	Ubi	ZmU6	*Agrobacterium*-mediated transformation	NHEJ, HDR	0.13–3.9	76
	Zmzb7	Human codon-optimized Cas9	2 × 35S	ZmU3	*Agrobacterium*-mediated transformation	NHEJ	19–31	77
	PSY1	Maize codon-optimized Cas9	ZmUbi2	ZmU6	*Agrobacterium*-mediated transformation	NHEJ	0.18–78.83	78

N/A, not available.

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-ins[41,85,86]. 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 Wang[37] 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. Lawrenson[36] used CRISPR/Cas9 to edit the *HvPM19* gene in *Hordeum vulgare* and *BolC*.GA4.*a* in *Brassica oleracea via* a transgenic system. The indel frequency of *HvPM19* was 23% in the first generation, while that of *BolC*.GA4.*a* was 10%. In addition, the authors also screened for the expected phenotype in T_0 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 T_0 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.[41] 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 *IPA1* (ideal plant architecture 1) in rice, and found the phenotype of the mutated miR156 was similar to *IPA1* plants. Analysis of the mutants showed that they contained 12 bp or 21 bp deletions, which interrupt the miR156 recognition site. While the deletions did not have an impact on the activity of IPA1 protein, the *IPA1* could be more highly expressed. Thus, utilizing CRISPR/Cas9 to target miRNA is essential in elucidating miRNA regulatory networks.

NHEJ-mediated CRISPR/Cas9 is a formidable system for investigating the function of enzyme genes and facilitating the expression of miRNAs. Additionally, a database should be made which integrates research species, target genes, methods, and results of CRISPR/Cas9. Such a database can be used to contrast the difference between the same or similar species in using CRISPR/Cas9 to edit target genes.

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 *Avr*II 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.[58] 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 tomato[87], maize[76] and soybean[42].

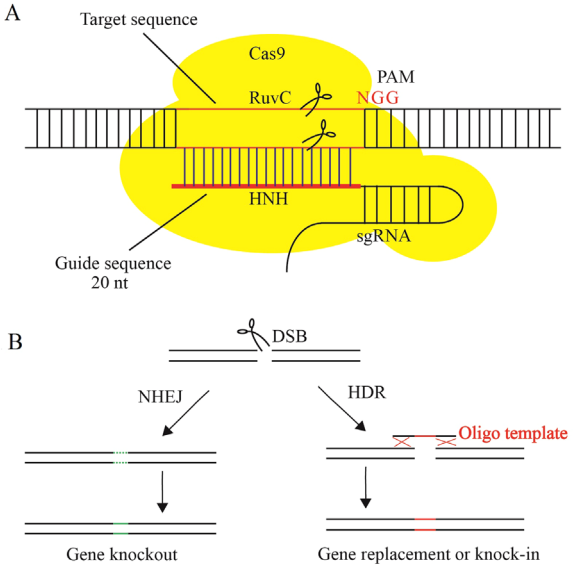

Figure 1 Schematic diagram of CRISPR/Cas9 editing of target genes. (A) A sketch of CRISPR/Cas9 system. The sgRNA (black and red) can identify the target gene, and then the two domains of Cas9 (yellow) cleave the target sequence. (B) Two ways DSB can be repaired. NHEJ is imprecise and always results in 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.

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 cells[88–90] and plants; Piatek et al.[91] 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.[46] 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

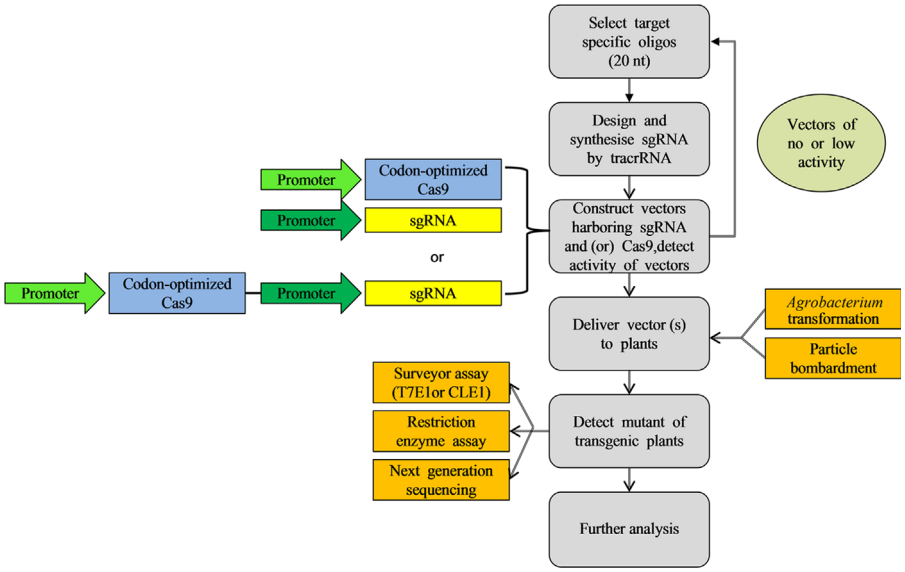

Figure 2 The basic flow of CRISPR/Cas9 editing of target genes.

online tools and stand-alone software have been developed to devise efficient and specific sgRNA. Zhang and coworkers[92] 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[93], CRISPR-P[94], Cas-OFFinder[95], Cas-Designer[96], Cas OT[97], SSFinder[98], which make the design of sgRNA become easier.

The construction of expression vectors is diverse in its methodology. Some researchers[34,55,65,99] have constructed different binary vectors by combining Cas9 with gRNA and induced target gene modification. However, others[28,60] 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*[100], and *Dendrobium officinale*[101]. 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[102,103] 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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