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CRISPR/Cas9: A powerful tool for crop genome editing

*Gaoyuan Song, Meiling Jia, Kai Chen, Xingchen Kong, Bushra Khattak, Chuanxiao Xie, Aili Li, Long Mao**

National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

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ABSTRACT

The CRISPR/Cas9 technology is evolved from a type II bacterial immune system and represents a new generation of targeted genome editing technology that can be applied to nearly all organisms. Site-specific modification is achieved by a single guide RNA (usually about 20 nucleotides) that is complementary to a target gene or locus and is anchored by a protospacer-adjacent motif. Cas9 nuclease then cleaves the targeted DNA to generate double-strand breaks (DSBs), which are subsequently repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms. NHEJ may introduce indels that cause frame shift mutations and hence the disruption of gene functions. When combined with double or multiplex guide RNA design, NHEJ may also introduce targeted chromosome deletions, whereas HDR can be engineered for target gene correction, gene replacement, and gene knock-in. In this review, we briefly survey the history of the CRISPR/Cas9 system invention and its genome-editing mechanism. We also describe the most recent innovation of the CRISPR/Cas9 technology, particularly the broad applications of modified Cas9 variants, and discuss the potential of this system for targeted genome editing and modification for crop improvement.

Contents

* *Corresponding author*. Tel.: +86 10 82105861.
E-mail address: maolong@caas.cn (L. Mao).
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1. Introduction

Since the inception of genetic engineering, methodologies for modifying a specific genetic locus of a target organism with a single-base resolution have been eagerly pursued. The invention of CRISPR/Cas9 technology has made this dream come true and opens a new era for genome editing. The technique is extremely simple, economical, and versatile in many applications with minor modifications. CRISPR/Cas9 is commonly used in mammals and plants, for both basic scientific research and genetic engineering. The technique is rapidly evolving and its application is constantly expanding. In this review, we describe how CRISPR/Cas9 works and how it can be applied in plants, especially crop plants. We also discuss the pitfalls of this technique and its future development for crop genetic improvement.

2. The CRISPR/Cas9 system: from bacterial immunity to genome editing

CRISPR is an acronym for clustered regularly interspaced short palindromic repeats and Cas9 is a nuclease associated with CRISPRs. These 29-nucleotide (nt) repeat sequences separated by various 32-nt spacer sequences were first reported in bacteria as early as 1987 [1]. Later, they were found in 40% of sequenced bacterial genomes and 90% of archaea [2]. Meanwhile, several types of Cas genes were found to be well conserved and adjacent to repeat elements [3]. These CRISPR/Cas systems can be classified into types I, II, and III, with the type II system requiring only the Cas9 nuclease to degrade DNA that matches a single guide RNA (sgRNA) [2]. The year 2005 was remarkable in the CRISPR/Cas9 epoch; in that year the spacer sequences were found to be originated from phage genomes [4–6]. Based on this discovery and the findings that viruses are unable to infect archaeal cells carrying sequences matching their own genomes, CRISPR/Cas systems were hypothesized to serve as a critical immune system to protect owners from pathogen invasion [5]. By 2011, the mechanism by which Cas9 works with CRISPR RNA (crRNA) and trans-activator crRNA (tracrRNA) to attack foreign DNA that matches the crRNA was decoded [7]. Soon, the tracrRNA and crRNA were combined into a single guide RNA molecule, an advance that has since rapidly accelerated the application of the CRISPR/Cas9 system in practice (Fig. 1-A) [8].

3. The Cas9 nuclease: the structure and the working mechanism

Unlike random mutagenesis, such as EMS mutagenesis and radiation [9], targeted genome-editing provides precise and

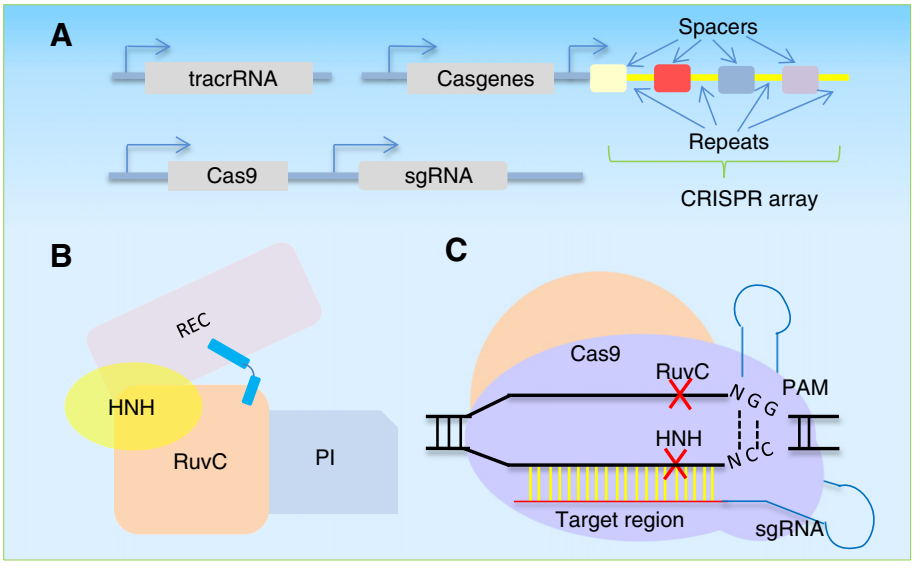

Fig. 1 – Components of the type II CRISPR/Cas system. A. Genomic structures of the native bacterial CRISPR/Cas system (top) and the engineered CRISPR/Cas9 system (bottom). tracrRNA, trans-activator RNA; sgRNA, single guide RNA; B. A schematic representation of the Cas9 protein structure. Domains include REC (large recognition lobe) and RuvC (a nuclease domain) which is linked with an arginine-rich region. HNH is a second nuclease domain. PI, PAM-interacting domain. C. The conformation of Cas9-sgRNA complex in the process of DNA cleavage.

highly efficient modifications in specific genome regions. The CRISPR/Cas9 system achieves its sequence specificity by the special structure and conformation of the Cas9 protein. As shown in Fig. 1-B, the Cas9 protein contains a conserved core and a bi-lobed architecture including adjacent active sites and two nucleic acid binding grooves: a large recognition (REC) lobe and a small nuclease (NUC) lobe that are connected by a helix bridge [10–12]. REC determines the Cas9-specific function, whereas the NUC incorporates two nuclease domains, RuvC and HNH, and a protospacer-adjacent motif (PAM)-interacting domain (PI). Under natural conditions, Cas9 is inactive. It is activated when combined with the sgRNA at its REC lobe. The Cas9-sgRNA complex scans a DNA double strand for rigorous PAMs (the trinucleotide NGG) using Watson–Crick pairing between sgRNA and targeted DNA. Once anchored at the proper PAMs, the HNH nuclease domain cleaves the RNA–DNA hybrid, while RuvC cleaves the other strand to form a double-strand break (DSB) (Fig. 1-C). DSBs can be repaired by non-homologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms that are endogenous to both prokaryotes and eukaryotes [13]. NHEJ employs DNA ligase IV to re-join the broken ends, an operation that can introduce insertion or deletion mutations (indels), whereas HDR repairs the DSBs based on a homologous complementary template and often results in a perfect repair. The error-prone NHEJ has advantages for gene knock-out. HDR is used for gene replacement and gene knock-in in plants [14, 15].

The CRISPR/Cas9 system supersedes previous genome editing techniques such as zinc finger nucleases (ZFNs) and engineered transcription activator-like effector nucleases (TALENs), both of which rely on the nuclease domain of *Fok* I endonucleases to break the double-strand DNA [16–18]. Compared with ZFN and TALANS, CRISPR/Cas9 is much easier to manipulate and hence has broader application. ZFN, for example, consists of an array of Cys2–His2 ZF domains, with each finger binding to specific PAMs, which make it difficult to select proper target sequences. When at work, two ZFNs form a dimer to locate a unique 18–24 bp DNA sequence. Owing to off-target risks, difficulty in engineering modular DNA-binding proteins, and context-dependent binding requirements, the application of ZFN and TALEN technologies remains very limited [19].

4. Applications of CRISPR/Cas9 in plant genome editing

With its high efficiency and simplicity, CRISPR/Cas9 and its modified versions have been widely explored in various organisms with many applications: gene mutation, gene expression repression or activation, and epigenome editing. In plants, the application of CRISPR/Cas9 is just emerging. In *Arabidopsis*, a model plant, several genes including *AtPDS3*, *AtFLS2*, *AtADH*, *AtFT*, *AtSPL4*, and *AtBRI1* are targeted with varying mutational efficiencies, from 1.1% to as high as 84.8%, in the first generation (Table 1) [15, 20–26]. These mutations are stably heritable across multiple generations with high percentages (up to 79.4%) [25]. A single CRISPR/Cas9 with two sgRNA expression cassettes has been developed to modify two genes (*CHLOROPHYLL A OXYGENASE1* and *LAZY1*) simultaneously [26]. In tobacco (*Nicotiana benthamiana*), CRISPR/Cas9 has been coupled with VIGS (virus-induced gene silencing) technology, a transient expression system [27–29], while in tomato, the knockout of *ARGONAUTE 7* causes clear morphological changes in compound leaves [30]. Several cases have been successful in rice [21, 31–38]. The knockouts of rice *OsPDS* and *OsBADH2* genes were achieved with mutation rates of 9.4% and 7.1%, respectively [31]. Later, much higher mutation rates were obtained with improved CRISPR/Cas9 components that reached an average of 85.4% mutation rate, with mostly biallelic and homozygous mutations [37]. With appropriate sgRNA combinations, the system has been used to delete chromosomal fragments (115–245 kb) in rice, removing a cluster of genes [38]. These achievements provide strong demonstrations of the CRISPR/Cas9 system as a potential and practical technology for crop genome editing.

Successful examples have also been reported for several other crops with more complex genomes, such as sorghum (*Sorghum bicolor*), maize (*Zea mays*), citrus (*Citrus sinensis*), poplar (*Populus tricocarpa*), tomato (*Solanum esculentum*) and wheat (*Triticum aestivum*) (Table 1) [21, 39–41]. In sorghum, a gene (*DsRED2*) was targeted with a 33% mutation rate [21], whereas the mutation rate for maize *ZmIPK* was 13.1% in a protoplast cell assay [39]. Four additional maize genes, *LIG1*, *MS26*, *MS45*, and *ALS1* were edited with the CRISPR/Cas9 system in transformed plants with a mutation rate lower than 5% [40]. In citrus, a test of the *PDS* gene showed a low mutation rate of 3.9% [41], whereas in poplar, a 51.7% mutation rate was obtained [42]. The flexibility and precision make CRISPR/Cas9 suitable for crops with multiple genomes, or polyploids. Soybean (*Glycine max*), for example, is a paleopolyploid with most genes present in two copies. Two genes, *Glyma01g38150* and *Glyma11g07220*, orthologs of the *Arabidopsis deficient in DNA methylation 1* (*DDM1*), a gene that is considered to be a chromatin-remodeling factor, has been successfully edited using one sgRNA that targets both genes [43]. Two additional soybean genes, *DD20* and *DD43*, were targeted simultaneously with 59% and 76% mutation rates, respectively [44]. More dramatically, in bread wheat, a hexaploid crop with high repetitive sequence content [45–48], three homoeoalleles (*TaMLO-A*, *TaMLO-B*, *TaMLO-D*) that confer resistance to powdery mildew can be edited simultaneously with a moderate mutation rate of 5.6% [45]. Thus, the potential of CRISPR/Cas9 in crop genome editing and its applications are certain to be further developed over time.

5. Technical pitfalls in using the CRISPR/Cas9 system

Similarly to ZFNs and TALENs, the CRISPR/Cas9 system has the problem of off-target effects that may introduce unexpected mutations. First, an improper concentration ratio between Cas9 and sgRNA may lead to off-target cleavage, and the higher the Cas9:sgRNA ratio, the more severe the effect [49, 50]. Optimal mutagenesis was reported in *Arabidopsis* with a Cas9:sgRNA ratio of 1:1 when two genes (*AtPDS3* and *AtFLS2*) were tested with different Cas9:sgRNA ratios [20]. Second, promiscuous PAM sites may lead to undesired cleavage of DNA regions [51]. To avoid this event, bioinformatics tools such as E-CRISPR and CasOT [52–58] can be used to assist in sgRNA design with reference to whole genome sequence information. Third, insufficient Cas9 codon

Table 1 – Applications of the CRISPR/Cas9 system in plants. [a, b, c, d]

Plant species	Target genes	Cas9 promoter	Version of Cas9	sgRNA Promoter	References
A. thaliana	*AtPDS3*, *AtFL2*, *AtRACK1b*, *AtRACK1c*	35S PPDK[a]	Plant codon-optimized	AtU6	Li et al. [20]
A. thaliana	*AtADH1*, *AtTT4*	Ubi4	*Arabidopsis thaliana* codon-optimized	AtU6	Fauser et al. [22]
A. thaliana	*AtBRI1*, *AtJAZ1*, *AtGAI*	CaMV 35S	Human codon-optimized	AtU6	Feng et al. [23]
A. thaliana	*AtFT*, *AtSPL4*	ICU2[b]	*Arabidopsis thaliana* codon-optimized	AtU6	Hyun et al. [24]
A. thaliana	*AtCHL1*, *AtTT4*, *AtAP1*, *AtGUUS*	CaMV 35S	Human codon-optimized	AtU6	Feng et al. [25]
C. sinensis	*CsPDS*	CaMV 35S	Human codon-optimized	CaMV 35 S	Jia et al. [41]
S. esculentum	*SlAGO7*, *Soliyc08g041770*, *Soliyc07g021170*, *Soliyc12g044760*	2 × 35S	Human codon-optimized	AtU6	Brooks et al. [30]
N. benthamiana	*NbPDS*	35S PPDK	Plant codon-optimized	AtU6	Li et al. [20]
N. benthamiana	*NbPDS*, *NbPCNA*	CaMV 35S	Human codon-optimized	PEBV[d]	Ali et al. [27]
N. benthamiana	*NbPDS*	CaMV 35S	Human codon-optimized	AtU6	Nekrasov et al. [29]
N. benthamiana	*NbPDS*, *NbPDR6*	2 × 35S	Nicotinana codon-optimized	AtU6	Gao et al. [28]
G. max	*Glyma07g1450*, *GmDDM1s*, *GmMIRs*	2 × 35S	Human codon-optimized	MtU6	Jacobs et al. [43]
G. max	*GmDD20*, *GmDD43*	EF1A2[c]	Soybean codon-optimized	GmU6	Li et al. [44]
O. sativa	*OsSWEET11*, *OsSWEET14*	CaMV 35S	Wild-type SpCas9	OsU6	Jiang et al. [21]
O. sativa	*OsROC5*, *OsSPP*, *OsYSA*	CaMV 35S	Human codon-optimized	AtU6	Feng et a. [23]
O. sativa	*OsPDS*, *OsBADH2*, *Oso2g23823*, *OsMPK2*	2 × 35S	Rice codon-optimized	OsU3	Shan et al. [31]
O. sativa	*OsCAO1*, *OsLAZY*	Ubi	Rice codon-optimized	OsU3	Miao et al. [32]
O. sativa	*OsDERF1*, *OsEPSPS*, *OsPDS*, *OsPMS3*, *OsMSH1*, *OsMYB1*, *OsROC5*, *OsSPP*, *OsYSA*	CaMV 35S	Rice codon-optimized	OsU3	Zhang et a. [33]
O. sativa	*OsMPK5*	CaMV 35S	Human codon-optimized	OsU3/U6	Xie et al. [34]
O. sativa	*OsBEL*	2 × 35S	Plant codon-optimized	AtU6	Xu et al. [35]
O. sativa	*OsMPKs*	Ubi	Rice codon-optimized	OsU3	Xie et al. [36]
O. sativa	46 genomic targets	Ubi/35S	Plant codon-optimized	OsU3/U6	Ma et al. [37]
O. sativa	*OsSWEET11*, *OsSWEET14*	Ubi	Rice codon-optimized	OsU6	Zhou et al. [38]
T. aestivum	*TaMLO-A1*	Ubi	Plant codon-optimized	TaU6	Wang et al. [45]
T. aestivum	*TaLOX2*	2 × 35S	Rice codon-optimized	TaU6	Shan et al. [46]
Z. mays	*ZmIPK*	CaMV 35S	Rice codon-optimized	ZmU3	Liang et al. [39]
Z. mays	*ZmLIG1*, *ZmM26*, *Zm45*, *ZmALS1*	Ubi	Maize codon-optimized	ZmU6	Svitashev et al. [40]

[a] 35S PPDK: 35S enhancer fused with pyruvate orthophosphate dikinase basal promoter.
[b] ICU2: the incurvata 2 promoter.
[c] EF1A2: elongation factor-1 alpha 2 promoter.
[d] PEBV: a pea early browning virus promoter.

optimization may lead to inefficient translation of Cas9 proteins in target species. Several codon-optimized versions of Cas9 genes are available, such as for *Arabidopsis* [15, 21, 25], rice [21, 23, 32, 34], and tobacco (*N. benthamiana*) [28]. Codon efficiency should be considered when these vectors are used for other crop plants. Fourth, given that most CRISPR/Cas9 systems use exogenous promoters for Cas9 and sgRNA expression, vectors with optimal promoters should be selected. In eudicots, the 35S promoter of cauliflower mosaic virus (CaMV) is preferred for expressing Cas9 and the U6 promoter for sgRNA. In monocots, both 35S and Ubi work well to express Cas9, but different promoters are used for sgRNAs in different species, such as OsU3 for rice and TaU6 for wheat. It is noteworthy that the sgRNA 5′ $A(N)_{17–19}NGG3'$ favors the U3 promoter, whereas 5′ $G(N)_{17–19}NGG3'$ prefers the U6 [46]. Recently, OsU6 has been identified as superior to the OsU3 promoter for driving sgRNAs when used for improving the mutation rate in rice [59]. Fifth, homologs or gene family members may complicate target sequences to be edited (Fig. 2). To ensure the function of the CRISPR/Cas9 system for knockout mutation, the position of sgRNAs is best located at the 5′ region of the targeted gene. Finally, epigenetic factors such as DNA methylation or histone modification, which are known to limit protein binding or RNA pairing, should also be considered in regions with complex DNA compositions, such as those with repetitive sequences.

6. New developments in the CRISPR/Cas9 technology

Despite its short history, a plethora of modified versions of CRISPR/Cas9 have been invented, permitting several exciting applications besides conventional genome editing operations (Fig. 3).

6.1. *Cas9 nuclease activity modifications*

First, with a modified Cas9 cleavage domain at Cas9-D10A or Cas9-H840A and combined with paired guide RNAs, Cas9 can cleave a targeted region on the opposite DNA strand, improving its specificity 100–1500-fold [60, 61]. Second, Cas9 can be mutated (called dead Cas9, or dCas9) or CRISPRi such as those created by point mutation in RuvC and HNH nuclease domains and become catalytically inactive and thus cannot cleave targeted regions. Co-expression of dCas9 and a specific sgRNA in the coding region

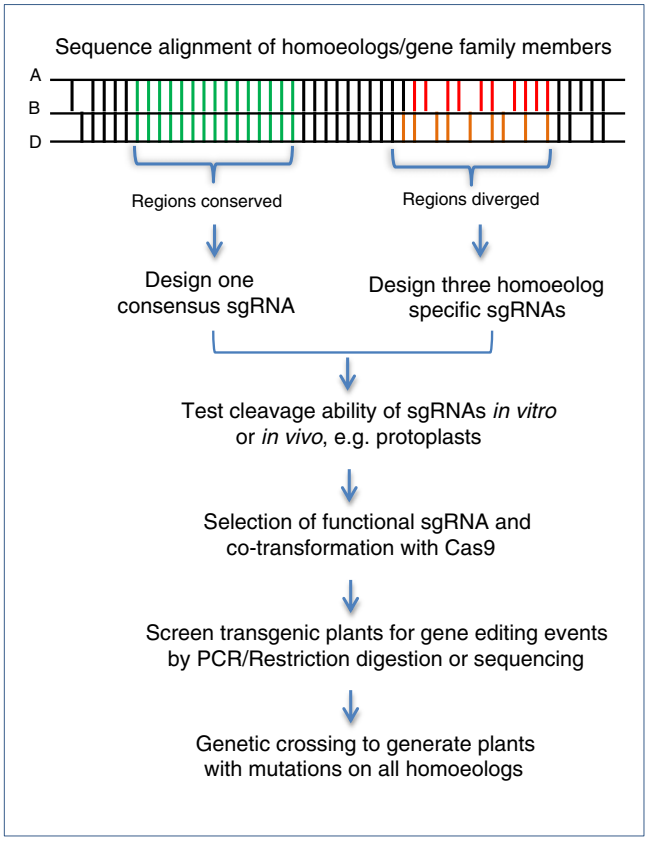

Fig. 2 – A protocol to design sgRNAs for multiple-copy genes. The three genomes (A, B, and D) of hexaploid wheat are used here as representatives for multiple copies of genes (homoeologs) or gene family members.

of a gene can prevent the transcription elongation process, leading to loss of function of incompletely translated proteins. This approach has been used to block transcription initiation by binding to the operator or the promoter of a gene, such as a transcription factor binding site or RNA polymerase binding site. Such binding can markedly decrease gene expression [62].

6.2. Cas9-associated fusion proteins

The dCas9 protein can be fused with transcription activation or repression effectors. dCas9 can be fused with different effector domains (repressor or activator) for recruiting functional proteins to specific genome loci, and then represses or activates gene expression. For instance, dCas9-VP64 (a transcription activator) and dCas9-p65AD (a single copy of the p65 activation domain) can efficiently activate reporter gene expression, showing that the CRISPR/dCas9 system can serve as a generic and modular platform for different types of transcription control [63]. dCas9 can also be combined with epigenetic factors, such as histone-modifying/DNA methylation enzymes, to modulate epigenetic modification of genes [64]. Cas9 can also be fused with fluorescent protein for DNA labeling of a specific region and can be used to provide live-cell images of dynamic chromosome conformation changes and study complex chromosomal architecture and nuclear organization [65].

6.3. The Cas9 gene driven by various promoters

A light-activated CRISPR/Cas9 effector system has been developed by fusion of the light-inducible heterodimer proteins CRY2 and CIB1 to a transactivation domain and a catalytically inactive dCas9, respectively. This system can be easily directed to new DNA sequences for dynamic light regulation of endogenous genes [66]. In addition, using tissue-specific promoters to drive the Cas9/dCas9 gene can accomplish gene mutation and gene activation or repression in various developmental stages and environmental conditions. Examples are the egg cell-specific promoter EC1.2 and germ line-specific promoter SPL to drive the Cas9 expression that may cause heritable mutations in various generations in *Arabidopsis* [67, 68].

6.4. PAM variants

Novel types of Cas9 proteins that can recognize various PAMs have been discovered. The PAM of St1Cas9, for example, has been characterized as NNAGAA, and SaCas9 had three PAMs: NNGGGT, NNGAAT, and NNGAGT, which were more efficient than others in mammalian cells [69, 70]. More excitingly, new versions of Cas9 proteins with point mutations at D1135V/R1335Q/T1337R (VQR) and D1135E/R1335Q/T1337R (EQR) have been generated. VQR-Cas9 can robustly cleave sites bearing NGAN PAMs, whereas EQR-Cas9 prefers NGAG to NGAN and NGNG PAMs in human cells and zebra fish [71]. These altered PAM-specificity variants permit highly efficient editing of endogenous gene sites not currently targetable by wild-type SpCas9. Most recently, a novel nuclease, Cpf1 (CRISPR from *Prevotella* and *Francisella* 1), has been found to employ a T-rich PAM located 5′ to the targeted DNA sequence (5′TTN) and to cleave the DNA *via* a staggered DNA double-strand break that is distant from the PAM, independent of the tracrRNA. This system robustly mediates DNA interference in mammalian cells [72].

6.5. sgRNA length manipulation

Altering the length of sgRNAs may affect the activity of the Cas9 nuclease, an effect that may be used for simultaneous genome editing and transcriptional regulation [73]. Furthermore, a CRISPR/Cas9 multiplex with boosted editing capability has been developed by use of the endogenous tRNA-processing system. These synthetic genes with tandem arrayed tRNA–sgRNA architecture can be efficiently and precisely processed into sgRNAs with desired 5′ target sequences *in vivo* [36]. These new developments make CRISPR/Cas9 a molecular tool with broader applications in plant and animal genome editing.

With more versions of modified CRISPR/Cas9 components, including new types of components, more precise and efficient genome editing tools can be expected.

7. CRISPR/Cas9 applications in crop genetic improvement

The CRISPR/Cas9 system is simple, efficient, and highly specific and produces fewer off-target events. It is thus a promising tool for genome modification in plants. CRISPR/Cas9 is expected to

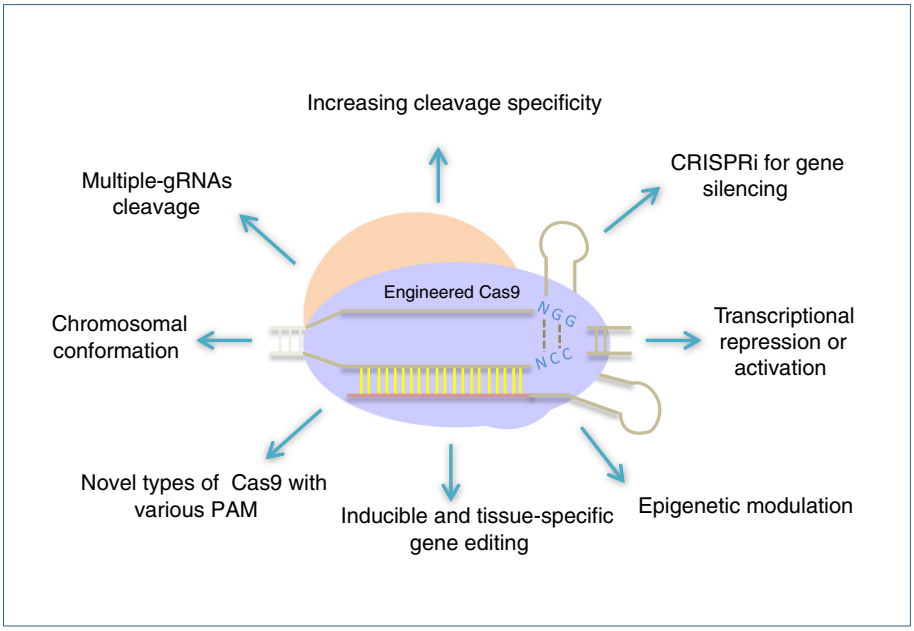

Fig. 3 – The development of engineered CRISPR/Ca9 systems. New functions derived from modified CRISPR/Cas9 are shown (see text for more details).

have a large impact on basic and applied research in plant biology. It should also have a large impact on crop breeding. Genome editing allows precise and predictable modifications directly in elite cultivars or accessions, saving the time-consuming backcrossing procedure in conventional breeding schemes. With multiple traits being modified simultaneously, the CRISPR/Cas9 system should provide a more efficient approach to pyramid breeding [74]. NHEJ-mediated gene knockouts are the most direct application of CRISPR/Cas9. Negative regulators of both grain development and disease resistance can be modified to increase crop yield and equip the host with resistance to targeted pathogens. Other gene modification methods, such as gene expression regulation and epigenetic modulation, can also be used for agricultural purposes. In addition, CRISPR/Cas9 provides alternative approaches for delivering target genes into crops with no transgenic footprint, such as by agroinfiltration, viral infection, or preassembled Cas9 protein-sgRNA ribonucleoproteins transformation so as to circumvent the traditional regulations on genetically modified organisms [75].

8. Perspectives

Although much progress has been made in CRISPR/Cas9-based genome editing technology in the last few years, some problems remain to be solved: off-target effects, influence of chromatin structure, side effects on nearby genes, mechanisms underlying the different effects of different sgRNAs on mutation efficiency, and methods for efficient delivery in polyploid plants. Despite these challenges, with the tremendous enthusiasm of the research community, gene editing technologies as represented by the CRISPR/Cas9 system will improve rapidly. This simple, affordable, and elegant genetic scalpel is expected to be widely applied to enhance the agricultural performance of most crops in the near future.

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