An Era of CRISPR/Cas9-mediated Plant Genome Editing

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Abstract

Recently the engineered nucleases have revolutionized genome editing to perturb gene expression at specific sites in complex eukaryotic genomes. Three important classes of these genome editing tools are Zinc Finger Nucleases (ZFN), Meganucleases and Transcription Activator-Like Effector Nucleases (TALEN) which work as hybrid systems comprising of target-specific DNA binding domains and molecular scissors or restriction endonucleases. Moreover, the more recent type II clustered regularly inter-spaced short palindromic repeats (CRISPR)-associated protein (CRISPR/Cas9) system has become the favourite plant genome editing tool for its precision and RNA based specificity unlike its counterparts which rely on protein based specificity. Plasmid-mediated co-delivery of multiple sgRNAs and Cas9 to the plant cell can simultaneously alter more than one target loci which enables multiplex genome editing. In this review, we discuss recent advancements in the CRISPR/Cas9 technology mechanism, theory and its applications in plants and agriculture. We also suggest that the CRISPR/Cas9 as an effective genome editing tool, has vast potential for crop improvement and studying gene regulation mechanisms and chromatin remodelling.

CRISPR/Cas9: ultimate tool for multiplex genome editing in plants

The small size of Cas9 and the relative ease of delivery of Cas9 and multiple sgRNAs to the cell makes it possible to simultaneously alter more than one target loci in a single genome, which is known as multiplexing or multiplex genome editing. On the contrary, the larger size of ZFN and TALEN which requires a pair of proteins recognizing complementary strands of DNA for introducing DSBs makes these techniques less suitable for multi locus gene editing (Bortesi and Fischer, 2015). Moreover, a number of studies exploited the use of two or more sgRNAs for multiplex genome editing in the model plant *Arabidopsis* (Li *et al.*, 2013; Mao *et al.*, 2013), rice (Zhang *et al.*, 2014) and tomato (Brooks *et al.*, 2014). Through multiplex genome editing chromosomal deletions from a few hundred base pairs up to tens of thousands have been made in *N. benthamiana* (Belhaj *et al.*, 2013), *Arabidopsis* (Mao *et al.*, 2013; Li *et al.*, 2013) and rice (Zhou *et al.*, 2014). This enables researchers to delete entire cluster of genes by deleting chromosomal fragments.

Recently methods have been proposed (Xing *et al.*, 2014) for developing gRNA modules and configuring expression cassettes having multiple

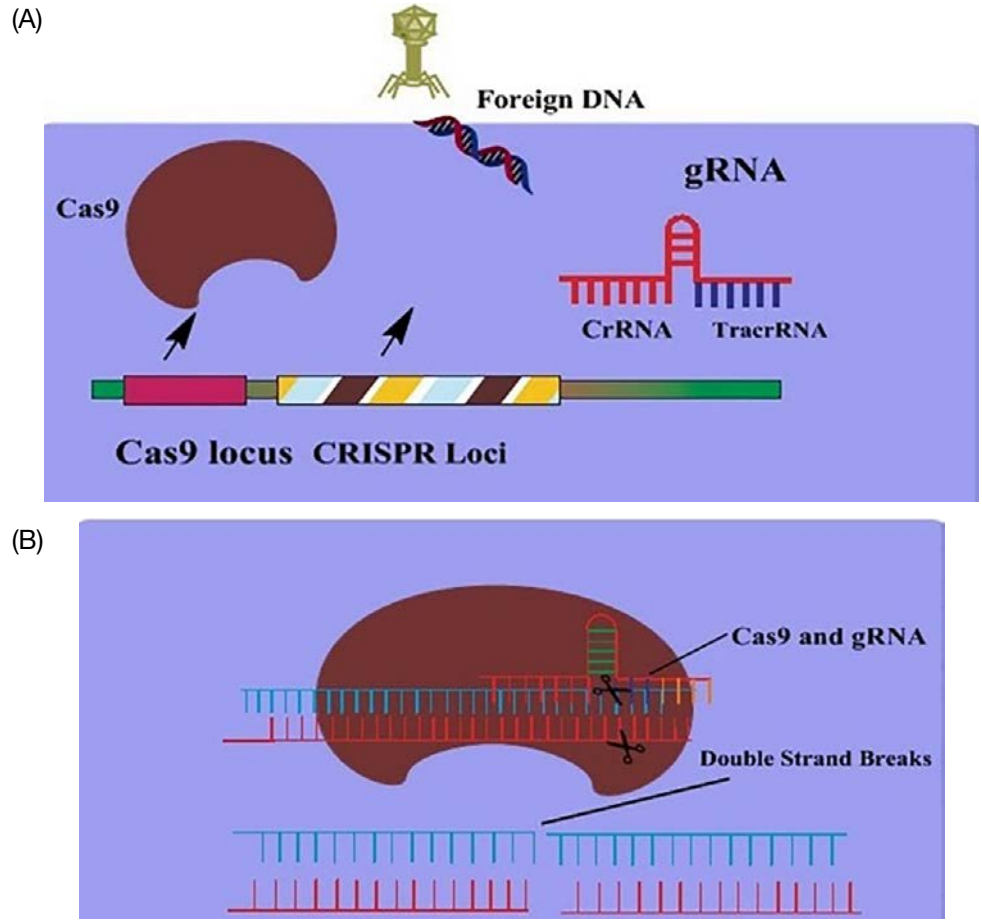

Figure 4.1 (A) Bacteria and archaea have genetic defence mechanisms against the invading DNA of bacteriophages. Their genome has Cas protein as well as CRISPR loci, which upon invasion by foreign DNA transcribe respective Cas proteins and guide RNA. Guide RNA or gRNA is a chimera formed by CRISPR RNA and trans-activating CRISPR RNA or tracrRNA. (B) Cas9/gRNA complex intercept target DNA which is complementary to gRNA and restriction sites are recognized through PAM sequence, i.e. 5′-NGG-3 located adjacent to the target sequence. Once localized, the RuvC and HNH sub-domains of NUC domain of Cas9 start endonuclease activity by inducing double stranded breaks (DSBs) in the target DNA.

gRNAs in plants. The application of these broad range resources comprised of binary vectors and gRNA are highly compatible with the requirements of a number of plants systems under complex conditions. This offers researchers with capability not only to customize their gRNA module according to specific plant systems but also to employ multiple gRNAs in a single cassette for multiplex genome editing.

Applications in plants

Since its discovery as modern genome editing tool, numerous research studies have evaluated CRISPR/Cas9 Type II system's possible role in gene expression regulation, gene knock-in/-out, site-specific mutation and epigenetic mechanisms in various organisms (Figs. 4.1 and 4.2). Moreover, its application in plants and agriculture are gradually becoming the focus of researchers as the tool offers new and efficient ways of manipulating a plant's genome to meet a broad set of enforced conditions.

As a model plant, vast genomic data of *Arabidopsis* have made this species an ideal target for CRISPR/Cas9 based genome editing and number of genes including *ADH1* (Schiml *et al.*, 2014), *AtPDS3*, *AtFLS2* (Li *et al.*, 2013), *ADH1* and *TT4*

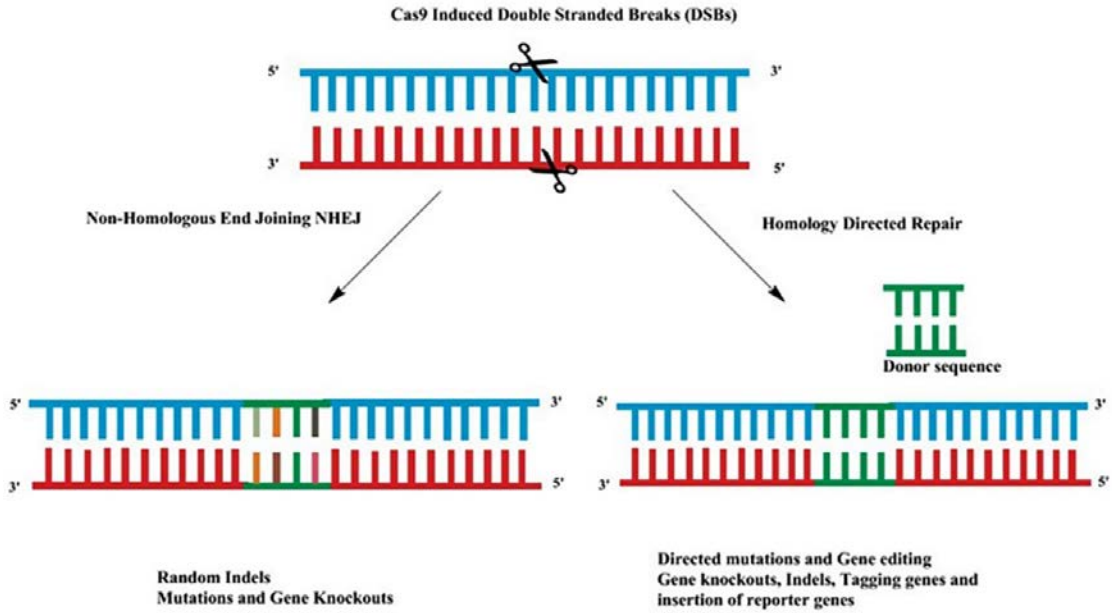

Figure 4.2 In a cell, after double-stranded breaks (DSBs) are introduced, the damaged DNA can be repaired by one of the two automated DNA repair pathways, i.e. the non-homologous end joining (NHEJ), which is more error prone often resulting in insertion deletions (indels), and can be exploited for frameshift mutation to cause gene knockouts. The second is homology directed repair (HDR) pathway which is comparatively precise and repairs DNA in nearly perfect way. In the presence of a user-supplied donor template HDR can be utilized for gene knock-in, gene correction or sequence replacement.

genes (Fauser *et al.*, 2014), *Flowering Locus T (FT)* and *Squamosa Promoter Binding Protein-Like 4* genes (Hyun *et al.*, 2015), and *BRI1, GAI, CHL1* (Mao *et al.*, 2013; Feng *et al.*, 2013) have been targeted. The resultant targeted mutations had varying degrees of efficiency from 1.1% to 85% in T_1 but were found to be stably heritable with as high a percentage as 79.4% (Feng *et al.*, 2013). CRISPR/Cas9-mediated targeted mutations have been induced using two sgRNA expression cassettes to perturb *Chlorophyll A Oxygenase1* and *LAZY1* (Mao *et al.*, 2013). This system has been used in combination with VIGS (virus-induced gene silencing) to study transient expression system in tobacco (*Nicotiana benthamiana*) (Ali *et al.*, 2015; Nekrasov *et al.*, 2013; Gao *et al.*, 2015). In tomato successful silencing of *Argonaute 7* has been achieved through this system resulting in obvious morphological changes in its leaves (Brooks *et al.*, 2014). A number of loci have been successfully targeted for gene perturbation and chromosomal deletion in rice (Jiang *et al.*, 2013; Shan *et al.*, 2014; Miao *et al.*, 2013; Zhou *et al.*, 2014). In rice, *OsPDS* and *OsBADH2* gene knockouts were obtained with mutation rates as high as 9.4% and 7.1%, respectively (Shan *et al.*, 2014). More recently, through improved Cas9/sgRNA platforms, biallelic and homozygous mutations were introduced with a maximum of 85.4% mutation rate (Ma *et al.*, 2015). Site-specific mutations have been effectively introduced through sgRNA and Cas9 delivery into rice protoplast to target promoter regions of *OsSWEET11* and *OsSWEET14*, bacterial blight susceptibility genes (Jiang *et al.*, 2013). Similarly, CRISPR/Cas9 mediated mutations were induced in maize protoplast gene *ZmIPK* (Liang *et al.*, 2014), wheat *LOX2* gene (Shan *et al.*, 2014) and sorghum (*DsRED2*) (Jiang *et al.*, 2013). Furthermore, sgRNA/Cas9 delivery protocol has been devised in important food crops with complex genome like hexaploid wheat, where gene knock-out in a single set of chromosomes may not be sufficient to produce the intended phenotype (Shan *et al.*, 2014). However, through CRISPR/Cas9, researchers have simultaneously edited three homoeoalleles (*TaMLO-A, TaMLO-B, TaMLO-D*) responsible for powdery mildew with a modest mutation rate of 5.6% (Wang *et al.*, 2014). The capability to induce multiple

site-specific mutations in polyploid genomes makes this technology suitable for applications in crops like soybean *(Glycine max)*, a paleo-polyploid with mostly two set of genes. In soybean, two genes, i.e. *Glyma01g38150* and *Glyma11g07220*, which are considered orthologues of the *A. thaliana* deficient in DNA methylation 1 gene (DDM1), a gene with a known function as chromatin remodelling factor, have been effectively targeted through sgRNA/Cas9 co-delivery and more than 70% indels frequencies were reported (Jacobs *et al.*, 2015). CRISPR/Cas9-induced mutagenesis has also been reported in two genomic sites of chromosome 4 of soybean, i.e. *DD20* and *DD43*, producing small insertion deletions with 59% and 76% mutation frequencies, respectively (Li *et al.*, 2015). Efficient gene editing has produced mutant loci in maize by precisely targeting upstream of the *liguleless*1 (*LIG1*) gene, *acetolactate synthase* genes *(ALS1, ALS2)* and male fertility genes (*Ms26, Ms45*) (Svitashev *et al.*, 2015).

CRISPR/Cas9 strategies have been successfully used for many agronomically important crop plants such as model *Nicotiana benthamiana* (Li *et al.*, 2013), *Arabidopsis* plants (Li *et al.*, 2013; Jiang *et al.*, 2013), wheat (Wang *et al.*, 2014), maize (Liang *et al.*, 2014), rice (Miao *et al.*, 2013), sorghum (Jiang *et al.*, 2013) and many more (Fig. 4.3 and Table 4.1). Various promoter systems, i.e. *AtU6* or *OsU6* and *AtUBQ*, *OsUBQ*, or *CaMV 35S*, were exploited to construct sgRNA and Cas9 coding expression vectors for genome editing in these plants (Mao *et al.*, 2013). Several new, quick and novel site-directed mutagenesis protocols are already being established (Shan *et al.*, 2014). About 90% mutation frequency has been calculated in *Arabidopsis* and rice (Miao *et al.*, 2013). The mutation can occur in early embryonic, growth and developmental stages or occur in different part of tissues. The resulting chimera plants contain cells with different genotypes that will be wild type, heterozygous, homozygous or bi-allelic. Different multiple sgRNAs have been employed to create tens to thousands of basepair chromosomal deletions from the genome of *N. benthamiana*, *Arabidopsis* (Li *et al.*, 2013) and tomato (Brooks *et al.*, 2014). This type of deletion sometimes leads to the loss of an entire gene function (Zhou *et al.*, 2014). Other novel methods include homology-directed repair (HDR) of CRISPR/Cas identified genes, domains, or alleles that were replaced. This method has been successfully used by many researchers along with many important reporter genes such as neomycin transferase (*nptII*) (Li *et al.*, 2013; Feng *et al.*, 2013). Successful insertion of the same gene has been reported into the endogenous *ALS* gene in *Arabidopsis* (Schiml *et al.*, 2014).

In addition to herbaceous plants, the CRISPR/Cas 9 system has been recently utilized in woody plants. Fan *et al.* (2015) reported an improved method of Cas9 gene and multiple sgRNAs delivery to poplar plant (*Populus tricocarpa*) cells to precisely

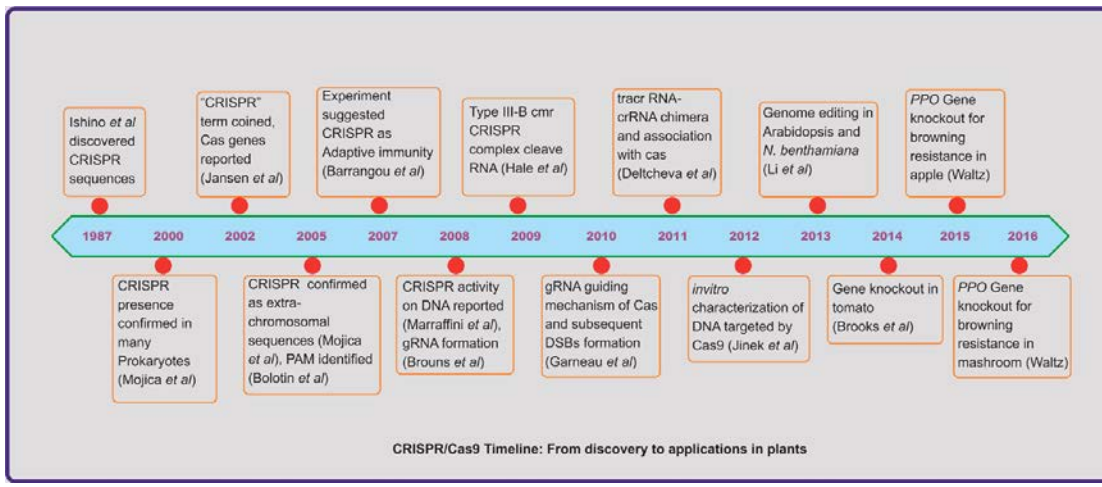

Figure 4.3 CRISPR/Cas9 timeline: from discovery to application in plants.

Table 4.1 CRISPR/Cas9 applications in plants

Species	Trait	Gene Targeted	Mode of action	References
A. thaliana	Multiple traits	*AtPDS3*, *AtFL2*, *AtRACK1b*, *AtRACK1c*, *AtADH1*, *AtTT4*	Knock out	Li *et al*., 2013; Fauser *et al*., 2014
Oryza sativa	Resistance to bacterial blight	*OsSWEET14; OsSWEET13*	Gene knockout	Zhou *et al*., 2015a
Zea mays L.	Leaf development; Male fertility; Herbicide resistance	*LIG1; Ms26*, *Ms45; ALS1*, *ALS2*	Gene knockout	Liang *et al*., 2014; Svitashev *et al*., 2015
S. esculentum	Lignin content; Condensed tannin content	*SlAGO7*, *Soliyc08g041770*, *Soliyc07g021170*,	Gene knockout	Brooks *et al*., 2014
Glycine max L.	Leaf development	*GmDD20*, *GmDD43*, *Glyma07g1450*, *GmDDM1s*, *GmMIRs*	Gene editing	Li *et al*., 2015; Jacobs *et al*., 2015
Triticum aestivum	Powdery mildew resistance, lipoxygenase	*TaMLO-A1*, *TaLOX2*	Gene editing, knock-out	Wang *et al*., 2014; Shan *et al*., 2014
N. benthamiana	Carotenoid biosynthesis	*NbPDS*, *NbPCNA*, *NbPDR6*	Gene knockout	Li *et al*., 2013; Ali *et al*., 2015; Gao *et al*., 2015
Citrus sinensis	Transient protein expression in leaves	*CsPDS*	Gene editing	Jia and Wang, 2014
Populus tricocarpa	Leaf development; male fertility; herbicide resistance		Gene knockout	Zhou *et al*., 2015b
Malus domestica and mushrooms	Browning resistance	*PPO*	Gene knockout	Waltz, 2015, 2016

perturb *phytoene desaturase* gene (*PtoPDS*). Four gRNAs were used to target phytoene desaturase gene 8 (*PtoPDS*). Many albino genotypes were noted after successful genetic transformation via *Agrobacterium*. Maximum mutation frequency of 51.7% was recorded, in which 30 out 59 were homozygous mutants and other two were heterozygous. They concluded that this system can be used for other important woody plants. In sweet orange *Xanthomonas citri,* facilitated agro-infiltration has demonstrated the successful application of cas9/sgRNA system for genome editing (Jia and Wang, 2014).

Harnessing the potential of CRISPR/Cas9 for disease resistance in crops

CRISPR/Cas9 system was successfully used by two research groups to confer long-term resistance against microbes (Ji *et al.,* 2015). These researchers have shown that Cas9/sgRNA inside plant genomes provides durable resistance against Geminivirus in *Nicotiana benthamiana* and *Arabidopsis thaliana* by inhibiting their replication. The direct CRISPR/Cas9 knock-out system was also used in other important crops (Wang *et al.,* 2014). The barley and wheat susceptible *MLO* (Mildew resistance Locus O) gene was targeted to confer long term resistance against powdery mildew disease in these crops (Varallyay *et al.,* 2012). In last 2 years this system has been used to provide broad-spectrum resistance by altering three homeoalleles encoding Mildew Resistance Locus (*MLO*) proteins against fungi in common hexaploid wheat (Wang *et al.,* 2014).

Recently, in soybean a hairy root based CRISPR/Cas9 system was developed where both the sgRNA and Cas9 cassettes were introduced in a single plasmid to enhance transformation frequency (Cai *et al.,* 2015). In a multiplex system targeting 3 genes simultaneously, one of the sgRNAs targeted the *bar* gene and remaining six sgRNA interrupted *GmFEI2* and *GmSHR* genes. Their work showed that the engineered CRISPR/Cas9 system shared maximum efficiency for both endogenous and exogenous genes. A protocol was optimized for delivering truncated gRNA and Cas9

system into *Arabidopsis* to generate new alleles for the *OST2* gene which acts as proton pump in *A. thaliana* (Osakabe *et al.*, 2016). The newly generated mutants with novel alleles exhibited an altered response against abiotic stress in plants. The rapid advancement in CRISPR/Cas9 technology over time is opening new vistas of easy and efficient genome editing in plants with which this technology is posed to revolutionize plants and agricultural research.

Conclusions

The enormous prospect of using CRISPR/Cas9 in crop genome editing and its applications to increase the commercial value of crops are growing by time as the technology is being further developed. We suggest that compared to other genome editing technologies, the relative ease, precision, robustness and efficiency of CRISPR/Cas 9 gives it a unique place to specifically target desirable or undesirable sites for intended genomic response. A vast number of reported loci associated with qualitative and quantitative plant phenotypes like disease resistance, production of phytochemicals and metabolites, important physiological processes and increased yield etc. can be edited via CRISPR/Cas9. The system not only offers means and ways to trigger accurate gene knock-in or knock-outs, but also helps understand complex mechanism of gene expression regulation, epigenetic mechanisms and chromatin remodelling.

However, challenges like off-target mutations, influence of chromatin structure, influence on flanking genes of the target and the effect of delivery methods remain to be investigated in detail. Advanced bioinformatics tools and a large number of Cas9 variants will enable researchers to design gRNA/Cas9 tools requiring longer PAM, which will increase its specificity and reduce off-target effects. Also, with continuous evolution of new pathotypes, fast and timely CRISPR/Cas9 systems for biotic stress resistance in crops should be developed to cope with the pace of pathogens. Despite these issues, as the most efficient tool among gene editing technologies, the research community is enthusiastically improving CRISPR/Cas9 system for its wide applications in plant sciences.

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