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CRISPR/Cas9: New Generation Plant Genome Editing Tool

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Abstract—*Functional genomics aims on identification of gene function that is achieved by reducing or completely disrupting the normal gene expression. Over a decade RNAi serves as a magic bullet in this field. Sequence specific nuclease (SSN) is a potential tool for mediating genome alteration with high precision. ZNF, TALEN and CRISPR are the popular genome editing tools. The CRISPR/ Cas 9 system, basically a type II bacterial immune system, is a very powerful site specific genome editing tool that can be applied for genome editing of nearly all organisms. Single guide RNA (sg RNA) is complementary to a target gene and is anchored by a PAM that guides the cas 9 nuclease to cleave the target sequence which is subsequently repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms. This review illustrates the mechanism and potentiality of CRISPR/Cas 9 system as a tool for plant genome editing to meet current challenges in agriculture.*

Keywords*: SSN; ZNF; TALEN; CRISPR; Cas 9; sg-RNA; PAM; NHEJ; HDR.*

1. INTRODUCTION

Identification of gene function is ambiguous for manipulation of gene expression which is achieved by reducing or completely disrupting its normal expression. Over a decade RNAi serves as a magic bullet in this field. Targeted genome editing with site specific nucleases opens an efficient and precise pathway for reverse genetics, genome engineering and targeted transgene integration experiments. The revolutionary invention of CRISPR/Cas9 technology opens a new era of genome editing. The CRISPR/Cas system is basically a prokaryotic immune system that offers a type of acquired immunity against invading bacteriophage or plasmids by degrading the exogenous gene[1]. CRISPR stands for clustered regularly interspaced short palindromic repeats and Cas9 is a CRISPR associated nuclease. These 29 nucleotide repeat sequences separated by various 32-nt spacer sequences were first reported from *iap* gene in *Escherichia coli* as early as 1987[1]. Later, they were found in 40% of sequenced bacterial genomes and 90% of archaea[2]. In 2005, the CRISPR spacer sequence was found to be highly homologous with exogenous sequences from bacterial plasmids and phages that enable CRISPR to cut foreign DNA[3-5]. The CRISPR/Cas system was developed as a genome editing tool in 2013. CRISPR/Cas requires a short guide RNA sequence to recognize the target loci and the end nuclease activity of Cas cleaves the target DNA by forming DNA double-strand breaks (DSBs) followed by stimulating 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)[6,7] and transcription nactivator–like effector nucleases (TALENs)[8,9],CRISPR/Cas is more efficient and it can edit multiple target genes simultaneously. Based on the advantages, applications of CRISPR/Cas are rapidly developing. This technology will have an impact on the progress of medicine and agriculture fields as it allows the direct and fast genetic modifications of model systems used in these fields.

Among the three types of CRISPR/Cas system[10], most biological research is focused on the application of type II CRISPR/Cas system. The system requires CRISPR-associated 9 protein, crRNA (CRISPR RNA), tracrRNA (transactivating crRNA) and RNase III (Ribonuclease III) to edit target genes. Single guide RNA is formed by fusing crRNA to tracrRNA[11]. In 2013 CRISPR/Cas 9 system was applied successfully for target specific gene editing in mammels[12,13]. Then CRISPR/Cas 9 system was succefully implemented in plants that brings a revolution in plant molecular biology research[15-17]. 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 a tremendous impact on bioengineering and molecular biology. This review combines the mechanism and application of CRISPER/Cas9 system in plant genome editing as well as in crop improvement with its advantages and future prospects.

2. IMPLICATION OF CRISPR/CAS9 SYSTEM FROM BACTERIAL IMMUNITY TO GENOME EDITING:

Before 2013, the dominating genome editing tool was ZNF[18,19] and TALEN[8]. The latest ground-breaking technology for genome editing is based on RNA-guided engineered nucleases, which already hold great promise due to their simplicity, efficiency and versatility. The most widely

used system is the type II CRISPR/Cas9 system from *Streptococcus pyogenes*[11]. The CRISPR/Cas systems can be classified into types I, II, and III[10], with the type II system requiring only the Cas9 nuclease to degrade DNA that has sequence similarity with single guide RNA (sgRNA)[2]. It was found that viruses are unable to infect archaeal cells having sequence similarity with the own genome. CRISPR/Cas systems are part of the adaptive immune system of bacteria and archaea. The immunity is acquired by the integration of short fragments of the invading DNA known as spacers between two adjacent repeats at the proximal end of a CRISPR locus. The spacer sequences of CRISPR were found to be originated from phage genomes[3–5]. The CRISPR arrays, including the spacers, are transcribed during subsequent encounters with invasive DNA and are processed into small interfering crRNAs approximately 40 nt in length, which combine with the tracrRNA to activate and guide the Cas9 nuclease[20]. This cleaves homologous double-stranded DNA sequences known as protospacers in the invading DNA[20]. The presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5′-NGG-3′ is essential for cleavage[11]. Specificity is provided by the so-called 'seed sequence' approximately 12 bases upstream of the PAM, which must match between the crRNA and target DNA(Fig: 1 A).

The transition of the CRISPR/Cas system from biological phenomenon to genome engineering tool came about when it was shown that the target DNA sequence could be reprogrammed simply by changing 20 nucleotides in the crRNA and that the targeting specificity of the crRNA could be combined with the structural properties of the tracrRNA in a chimeric single guide RNA (gRNA), thus reducing the system from three to two components[11]. CRISPR/Cas9 cleaves foreign DNA via two components i.e, Cas9 and sgRNA. Cas9 is a DNA endonuclease that can be isolated from *Brevibacillus laterosporus*[21], *Staphylococcus aureus*[22], *Streptococcus pyogenes*[23], *Streptococcus thermophilus*[24], and among them the most widely used bacterium is *Streptococcus pyogenes*. Cas9 contains two domains namely 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 about100nt. 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-any nucleotide; G-guanine).The loop structure at the 3′end of the sgRNA can anchor the target sequence by the guide sequence and form a complex with Cas9, which cleaves the double- stranded DNA by forming double-strand break (DSB) at that site (Fig: 1B).

Once a DSB is generated, the host DNA breakage machinery activates and repairs the DNA double strand break with non-homologous end-joining (NHEJ) or homology-directed repair (HDR) mechanism (Fig.2). In NHEJ, the host cellular DNA repair systems will tether the DNA double strand break by random insertion or deletion of short stretches of oligonucleotide bases. This mechanism results in the disruption of the codon-reading frame followed by disrupting the gene expression. In HDR, introduction of a segment of DNA with regions having homology to the sequences flanking both sides of the DNA double strand break will lead to the repair by host machinery through the incorporation of the extra segment of the DNA fragment[25].

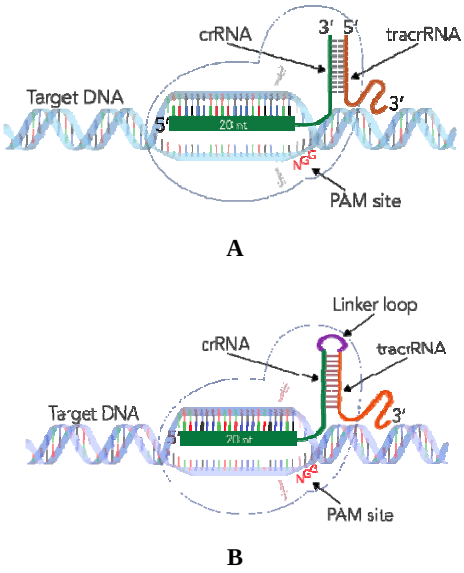

Fig. 1: Schematic diagram of CRISPR/Cas 9 system. A. In the native system the Cas9 protein is guided by the crRNA that has sequence similarity with target DNA and tracrRNA that stabilizes the structure. The presence of PAM motif downstream of the target DNA is the prerequisite for cleavage by Cas9 B. Cas9 can be reprogrammed to cleave DNA by a single guide RNA molecule, a chimera generated by fusing the 3′ end of the crRNA to the 5′ end of the tracrRNA with a loop.

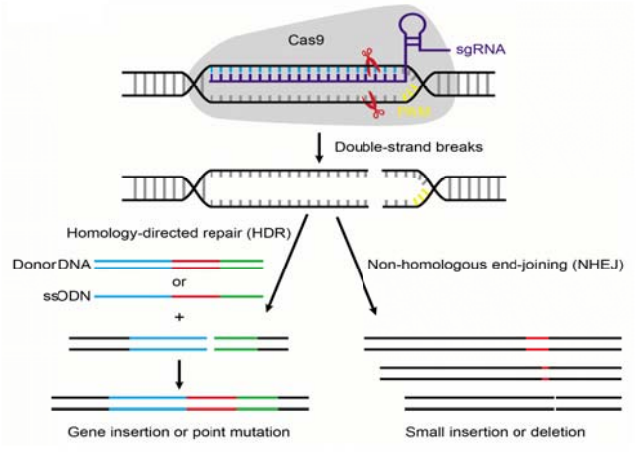

Fig. 2: Schematic diagram of genome editing with site specific nuclease.Cas9 causes DSB that is repaired by either NHEJ or HDR that disrupts or modifies the gene expression.

Table 1 – Applications of the CRISPR/Cas9 system in plants

Species	Target gene	Version of Cas9	Promoters (Cas9, gRNA)	Transformation method	References
Arabidopsis thaliana	*BRI1, JAZ1, GAI*	Human codon-optimized Cas9	2x35S, AtU6-26	Agro-transformation by floral dip	Feng et. al (2013)
	TT4, CHLI1, CHL12	Human codon-optimized Cas9	AtUBQ1, AtU6	Agro-transformation by floral dip	Mao et al. (2013)
	FT, SPL4	Human codon-optimized Cas9	AtICU2, AtU6	*Agrobacterium*-mediated transformation	Hyun et. al (2015)
	AtCRU3	Arabidopsis codon-optimized Cas9	35S, U6-26	*Agrobacterium*-mediated transformation	Johnson et. al (2015)
	At1g16210, At1g56650, At5g55580	Plant codon-optimized Cas9	Ubi, 35S, AtU3b, AtU3d, AtU6-1, AtU6-29	Agroibacterium-infiltration	Ma et. al (2015)
	AP1, TT4	Plant codon-optimized Cas9	AtUBQ1, SPL, AtU6-26	*Agrobacterium*-mediated transformation	Mao et. al (2016)
Brassica oleracea	*BolC.GA4.a*	*Streptococcus pyogenes* Cas9	35S, U6-26	*Agrobacterium*-mediated transformation	Lawrenson et. al (2015)
Citrus. sinensis	*CsPDS*	Human codon-optimized Cas9	CaMV 35S, CaMV 35S	*Agrobacterium*-infiltration	Jia et al(2014)
Solanum esculentum	*SlAGO7, Soliyc08g041770, Soliyc07g021170, Soliyc12g044760*	Human codon-optimized Cas9	2x35S, AtU6	*Agrobacterium*-mediated transformation	Brooks et. al (2014)
Nicotiana	*NbPDS*	Plant codon-	35S PPDK	*Agrobacterium*-	Li et
benthamiana		optimized	, AtU6	infiltration	al.
	NbPDS, NbPCNA	Human codon-optimized Cas9	CaMV 35S, PEBV	*Agrobacterium*-mediated transformation	Ali et al.
	NbPDS, NbIspH	Plant codon-optimized Cas9	35S, AtU6-26	*Agrobacterium*-mediated transformation	Yin et. al (2015)
Glycine max	*Glyma07g1450, GmDDM1s, GmMIRs*	Human codon-optimized Cas9	2x35S, MtU6	*Agrobacterium*-mediated transformation	Jacobs et al
Oryza sativa	*ROC5, SPP, YSA*	Human codon-optimized Cas9	CaMV 35S, OsU6-2	*Agrobacterium*-mediated transformation	Feng et.al (2013)
	OsSWEET11,OsSWEET14	*Streptococcus pyogenes* Cas9 andricecodon-optimizedCas9	CaMV 35S, OsU6	PEG-mediated transformation	Jiang et.al (2013)
	OsMYB1	Human codon-optimized Cas9	OsUBQ1, OsU3	*Agrobacterium*-mediated transformation	Mao et.al (2013)
	OsPDS, OsMPK2, OsBADH2	Rice codon-optimized Cas9	2x35S, OsU6	Particle bombardment	Shan et. al (2013)
Triticum aestivum	*TaMLO*	Rice codon-optimized Cas9	2x35S, TaU6	Protoplast transformation	Shan et.al (2013)
	TaMLOA1, TaMLOB1, TaMLOD1	Plant codon-optimized	Ub1, TaU6	Particle bombardment	Wang et.al (2014)
Zea mays	*ZmIPK*	Plant codon-optimized Cas9	2x35S ZmU3	*Agrobacterium*-mediated transformation	Liang et.al (2014)

3. APPLICATION OF GENOME EDITING WONDER IN PLANTS:

CRISPR/Cas9 has been widely used in various organisms for gene mutation, gene expression repression or activation and epigenome editing. In plants, the application of CRISPR/Cas9

is developing day by day. CRISPR/Cas9 has been rapidly developed and successfully applied to plant biological research for crop improvement (Table 1).

The major applications of CRISPR/Cas9 include gene knockouts in organisms for identifying the function of single or multiple gene targets (e.g., enzyme genes or micro RNAs) via gene mutation. Research was done for investigating the capability of CRISPR/Cas system as a genome editing tool in Arabidopsis, tobacco, rice and sorghum[26]. In sweet orange *CsPDS* (phytoene desaturase gene) gene was successfully mutated by using Cas9/sgRNA via *Xanthomonas citri* subsp. citri(Xcc)-facilitated agro-infiltration[28]. A geminivirus based sgRNA delivery system was developed named as VIGE (virus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing) for be used for transient expression that targets *NbPDS3* and *NbIspH*, which cause a photo-bleaching phenotype when they are expressed in tobacco[29]. Through CRISPR/Cas9 system *TaMLO* (mildew resistance locus)gene was successfully targeted in hexaploid bread wheat[30]. CRISPR/Cas9 is used to edit the *HvPM19* gene in *Hordeum vulgare* and *BolC.GA4.a* in *Brassica oleracea* via a transgenic system[31]. CRISPR/Cas9 was utilized to target miRNA (miR1514 and miR1509) in soybean[32]. NHEJ-mediated CRISPR/Cas9 is a widely used system for investigating the function of enzyme genes and facilitating the expression of miRNAs.

Though HDR leads to precise gene knock-in or gene replacement but success stories of CRISPR/Cas system for gene editing with HDR is very less. The HDR-mediated CRISPR/Cas9 system was successfully utilized to create precise and heritable modifications in tobacco[14], rice[33], Arabidopsis[14], tomato[34], maize[35] and soybean[36]. CRISPR/Cas9 is a powerful tool for transcriptional activation or repression. An catalytically inactive Cas9 that is dCas9-VP64 with gRNAs could activate the transcription of *AtPAP1* (production of anthocyanin pigment1) and miR319 2-,3-and7-fold in Arabidopsis[37].

4. ADVANTAGES OF THE CRISPR/CAS9 SYSTEM:

CRISPR/Cas system is more advantageous than ZNF and TALEN. Like other genome editing tools, CRISPR/Cas9 system does not require any protein engineering or cloning step. Any number of gRNAs can be produced by *in vitro* transcription using two complementary annealed oligonucleotides[38]. CRISPR/Cas9 system brings the genome editing within the budget of any molecular biology laboratory. Unlike ZFNs and TALENs, the CRISPR/Cas9 system can cleave methylated DNA. Approximately 70% of CpG/CpNpG sites are methylated in plants, particularly the CpG islands found in promoters and proximal exons[39]. Thus CRISPR/Cas9 can be used as a versatile tool for plant genome editing purpose. Multiplex editing with the CRISPR/Cas9 system requires the monomeric Cas9 protein and any number of different sequence-specific gRNAs. In contrast, multiplex editing with ZFNs or TALENs requires separate dimeric proteins specific for each target site. These advantages of CRISPR/Cas9 system make it most popular tool for genome editing.

5. CRISPR/CAS9: THE FUTURE OF CROP GENETIC IMPROVEMENT:

CRISPR/Cas9 is a promising tool for genome modification in plants due to its simplicity, efficacy, high specificity and fewer off-target effects. Genome editing can accelerate plant breeding by introducing precise and predictable modifications directly in elite cultivars or accessions, saving the time-consuming backcrossing procedure in conventional breeding schemes. CRISPR/Cas9 system can simultaneously edit multiple traits. For the elimination of genes that negatively regulates the grain quality and disease resistance, NHEJ-mediated gene knockouts is the best implication of CRISPR/Cas9 system leads to increase crop yield as well as confer resistant to pathogens. The plants muted with CRISPR/Cas9 system is not classified under genetically modified crop as for target gene delivery it often uses agroinfiltration, viral infection, or preassembled Cas9 protein-sgRNA ribonucleoproteins transformation technologies[40].

6. CONCLUSION

CRISPR/Cas9 system has many advantages over other genome editing tools by making reverse genetic screening feasible and affordable to a genomic scale [41]. CRISPR/Cas9 can promote research on biosynthetic pathways and regulatory mechanisms of effective components for identifying the excellent germplasm in medicinal plants to develop pharmaceutical botany. The main application of CRISPR/Cas9 system is for genome editing and transcriptional regulation. Though there are reports on application of CRISPR/Cas system for DNA-labelling and epigenome editing but it is not applied in plants till date. It will be interesting to see CRISPR/Cas9 application in plant DNA labelling using fluorescent-labelled Cas9 protein and optimized gRNA and epigenome editing by DNA methylation or histone modifications in the future. Although the CRISPR/Cas9 can be applied to plant genome editing, there are still certain challenges like minimizing off-target effects, knowing the influence of chromatin structure as well as side effects on nearby genes, identifying the mechanisms involved in the different effects of different sgRNAs on mutation efficiency and recognizing the methods for efficient delivery in polyploid plants. Further studies are needed for the improvement of application of CRISPR/Cas9 system in plants to meet current challenges in agriculture.

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