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CRISPR-Cas9: A Genome Editing Tool in Crop Plants: A Review

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ABSTRACT

Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease 9 (CRISPR-Cas9) system is a rapid technology for gene editing. CRISPR-Cas9 is an RNA guided gene editing tool where Cas9 acts as endonuclease by cutting the target DNA strand. Double Stranded Breaks (DBS) can be repaired by non-homologous end joining (NHEJ) and homology-directed repair (HDR). The NHEJ employs DNA ligase IV to rejoin the broken ends which cause insertion or deletion mutations, whereas HDR repairs the DSBs based on a homologous complementary template and results in perfect repair of broken ends. CRISPR-Cas9 impart diverse advantageous features in contrast with the conventional methods. In this review article, we have discussed CRISPR-Cas9 based genome editing along with its mechanism of action and role in crop improvement.

Key words: Cas9, CRISPR, DSBs, Genome editing, sgRNA.

Abbreviation: CRISPR: Clustered regularly interspaced short palindromic repeats, Cas9: CRISPR-associated protein 9**,** sg RNA-Single guide RNA, NHEJ: Non-homologous end joining, HDR: Homology-directed repair, DSBs: Double stranded break.

CRISPR exposition

CRISPR-Cas9 can be extended as clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. CRISPR is the DNA sequences found in prokaryotes in response of bacteriophages infection (Barrangou, 2015a). Cas genes near to the CRISPR is essential for its functions and also codes for proteins essential to the immune response and provide immunity in up-position to viruses and plasmids in bacteria and archaebacteria (Barrangou *et al*., 2007; Brouns *et al*., 2008; Barrangou and Marraffini, 2014; Sorek *et al*., 2013; Barrangou, 2013). CRISPR sequences along with Cas9 enzymes constitute CRISPR-Cas9 system which is vastly used to edit genes within genome of an organism (Barrangou *et al*., 2007). The CRISPR-Cas system is divided into three phases; Adaptation, Expression and Interference. In the adaptation phase new spacers from target are being inserted into CRISPR locus. During expression CRISPR is transcribed into CRISPR RNA (pre-crRNA) and cas genes becomes functional to be expressed as cas proteins which help in processing of pre-crRNA into mature crRNA. Target nucleic acid is recognized and destroyed in interference phase by crRNA and cas proteins (Koonin and Krupovic, 2014; Rath *et al*., 2015) (Fig 1).

Classification

CRISPR-Cas9 systems are classified based on structural, functional properties and complement of associated *cas* genes. CRISPR-Cas system is divided into two classes Class 1 and Class 2. Class 1 utilizes multiple Cas proteins complex while Class 2 utilizes single large Cas protein for degradation of foreign nucleic acids. Class 1 is further divided into types I, III and IV and class 2 is further divided into types II, V and VI (Wright *et al*., 2016). These six system types are further categorized into 19 subtypes

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(Westra *et al*., 2016). Signature genes are characteristic feature of each type and subtypes. Signature protein Cas3 is present in Type I which are structurally quite complex. Cas3 protein has both helicase and DNase activity for degrading the target DNA (Sinkunas *et al*., 2011). Type I system is further classified into six sub types depending upon number of Cas genes *viz.,* Type I-A to Type I-F. Type III CRISPR-Cas systems contain the signature protein Cas10 with unknown activity which are further divided into Type III-A and Type III-B (Rouillon *et al*., 2013; Spilman *et al*., 2013; Osawa *et al*., 2015). Class 2 types are rarer in nature but have utility in biotechnology especially genetic engineering. Type II systems of Class 2 encode Cas1, Cas2, Cas4 and Cas9 signature protein. Cas9 take part in adaptation and crRNA processing which cleaves the target DNA (Deltcheva *et al*., 2011; Garneau *et al*., 2010; Wei *et al*., 2015; Heler *et al*., 2015). Type II systems are further classified into subtypes II-A and II-B and II-C (Koonin*et al*., 2013; Chylinski *et al*., 2013). All three Type I and II and Type III systems target DNA. Type II systems have been found in bacteria while the

Type I and Type III systems occur both in bacteria and archaea (Makarova *et al*., 2011) (Fig 5).

Cas9 activation

The Cas9 protein is derived from type II CRISPR. It is an RNA guided DNA endonuclease which can target current sites by modifying its guide RNA (sg RNA) sequence (Wang *et al*., 2016). Cas9 activation is the crucial process in CRISPR-Cas9 genome editing. Guide RNA loading is critical step in Cas9 activation. Large-scale conformational rearrangement occurs in Cas9 protein after the binding of sgRNA and target DNA. Crystal structures of Cas9 bound to single guide RNA divulge a conformation definite from both the apo and DNA bound condition in which the 10 nucleotide RNA sequence is essential for initial DNA interrogation. This segment of the guide RNA is crucial for Cas9 to form a DNA recognition-competent structure that is poised to attract double-stranded DNA target sequences (Sternberg *et al*., 2014; Jiang *et al*., 2015 and 2016). Catalytic nuclease lobes of Cas9 rotate and generate nucleic acid-cleaving activity (Jinek *et al*., 2014). Activity of Cas9 is increased by the sgRNAs with +85 nucleotide tracrRNA tails which induced higher level of indels *in vivo* (Hsu *et al*., 2013). Novel sequence changes to the tracrRNA significantly improve Cas9 activity when delivered as an RNP. A dual guide RNA (dgRNA) with a modified tracrRNA can refine reporter knockdown and indel creation at many targets within the long terminal repeat (LTR). The sequence modified tracrRNAs boost Cas9-mediated reduction of CCR5 surface receptor expression in cell lines which indicates higher levels of indel development (Scott *et al*., 2019). More sgRNA: Cas9 complexes promote higher editing efficiency. However, excessive sgRNA: Cas9 complexes may give rise to off-target effects as a result of the inevitable complementarity of nonspecific sequences in the genome (Fu *et al*., 2013). The capacity of Cas9 to generate high levels of indels decides effectiveness of Cas9 technology. Toxic activity of Cas9 has been detected due to unrestricted production of Cas9 along with non-specific guiding by sgRNA for non-specific binding and cleavage of non-target DNA (Cho *et al*., 2018). The toxicity of Cas9 has impeded its extensive applications due to its abundant intracellular expression (Wang *et al*., 2019).

CRISPR genome editing

CRISPR-Cas technologies have been revolutionized genome editing by the prokaryotic RNA-guided defense system (Mir *et al*., 2018). CRISPR-Cas9 genome editing requires a single guide (sg) RNA that directs the Cas9 endonuclease to a specific region of the genomic DNA, resulting in double stranded nicks in the target DNA (Fig 5) (Jinek, *et al.,* 2012). The CRISPR-Cas9 system cleaves specific nucleotides based on complementary sequence with Cas9 protein and sgRNA (Jinek, *et al*., 2012 and Peng *et al*., 2016). Cas9 protein accommodates two nucleic acid binding grooves *viz*., a large recognition (REC) lobe and a small nuclease (NUC) lobe that are linked by a helix bridge (Nishimasu *et al*., 2014; Anders *et al*., 2014) (Fig 2A).REC regulates the Cas9 specific function and the NUC integrate two nucleases, RuvC and HNH and protospacer adjacent motif (PAM) interacting domain (PI). The presence of PAM flanking the target sites are required for target recognition and succeeding R-loop formation.These sgRNAs are non-coding short RNA sequences which bind to the complementary target DNA sequences which confers target sequence specificity to the CRISPR-Cas9 system (Jiang and Doudna, 2017).

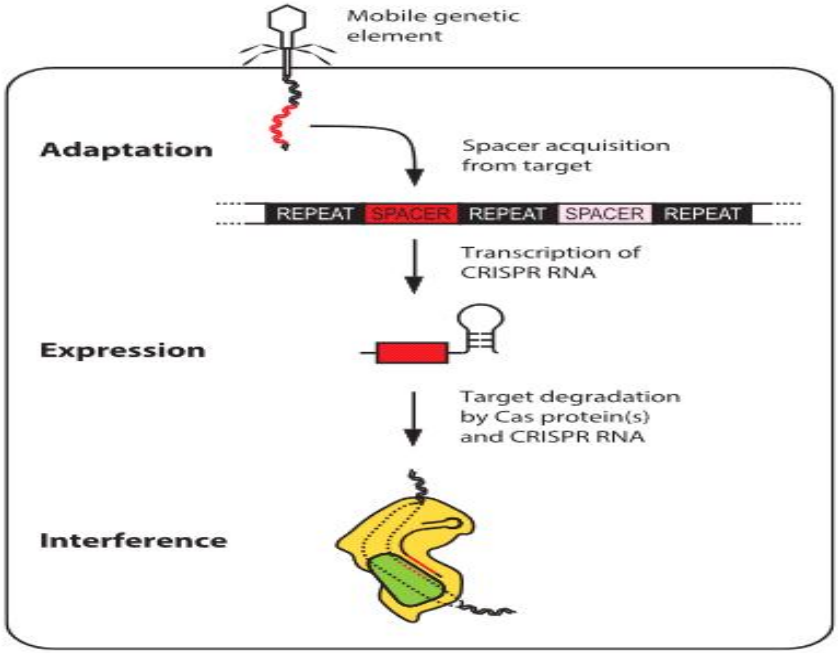

Fig 1: Steps of CRISPR-Cas exposition (Courtesy: Rath *et al*., 2015).

Cas9 is inactive under natural state and activated after binding with the sgRNA at its REC lobe. The Cas9-sgRNA complex scans a DNA double strand resulted due to pairing between sgRNA and targeted DNA. After finding complementary target DNA, the Cas9-sgRNA complex attaches, the sgRNA starts interrogating the double stranded DNA by base pairing with the target DNA strand from the PAM proximal position with the help of the PI (PAM interacting) domain. After tying up with PAMs the HNH nuclease cleaves the RNA–DNA hybrid whereas Ruv C cleaves the other strand in order to form a double-strand break (DSB) (Fig 2B and 3).

Cas9 uses its endonuclease activity to generate double-strand breaks in target DNA during bacterial immune response (Mali *et al*., 2013; Cong *et al*., 2013; Jiang *et al*., 2013; Bao *et al.,* 2019). DSBs can be repaired by non-homologous end joining (NHEJ) and homology directed repair (HDR) mechanisms (Puchta, 2005). NHEJ utilize DNA ligase IV to re-join the broken ends an action that introduce insertion or deletion mutations (indels). In plants, the cleaved ends of DNA are usually rejoined by non-homologous end-joining (NHEJ) (Xiea *et al.,* 2014). During the NHEJ processing insertion/deletion can occur resulting in a frameshift or introduction of a premature stop codon (Fu *et al.,* 2013). HDR repairs the DSBs based on a homologous complementary template and results in a perfect repair. HDR is generally used for gene knock-out plants (Schiml *et al*., 2014). A transgenic DNA can be generated by providing a donor DNA in trans and the double strand break will be repaired by the host cell. This pathway is useful in generating loss-of-function (knockout) of the gene of interest (Costa *et al*., 2017) (Fig 4).

Implementation of the CRISPR-Cas9 technology for crop improvement

Genome editing is used to decipher gene function and opens a novel door to create genetic diversity for breeding. Transcriptional alleles can be generated by targeting regulatory elements, which improves desirable characters. Genetic diversity present in wild species and uncultured varieties of crops as an origin of allele-mining and widely enlarging the crop germplasm pool could not be exploited properly so use of CRISPR-Cas 9 allow to create genetic diversity by gene editing. (Barrangou, 2015b; Wolter *et al* 2019). (Antonia *et al*., 2016 revealed applications of CRISPR–Cas9 for multiplexed, inducible gene regulation and genome-wide screens. Application of CRISPR/Cas9 technology has been initiated in economically important crop *Oryza sativa*. Cas9/sgRNA-mediated minute deletions/ insertions at single cleavage at transient and stable transformations were described in rice and *Arabidopsis*. Four sugar efflux transporter genes were modified in rice at high efficiency using Cas9/sgRNA-induced large chromosomal segment deletions (Wu *et al*., 2011; Zhou *et al*., 2014).

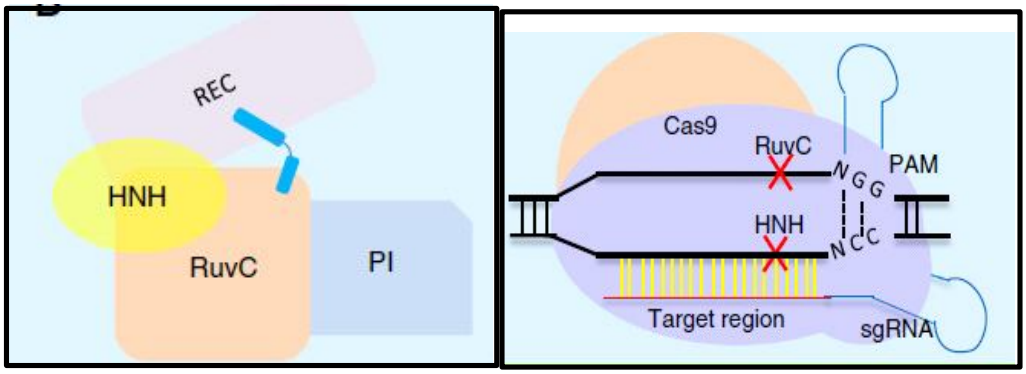

Fig 2: A. schematic representation of the Cas9 protein structure B. Cas9-sgRNA complex in the task of DNA cleavage. (Courtesy: Anders *et al*., 2014).

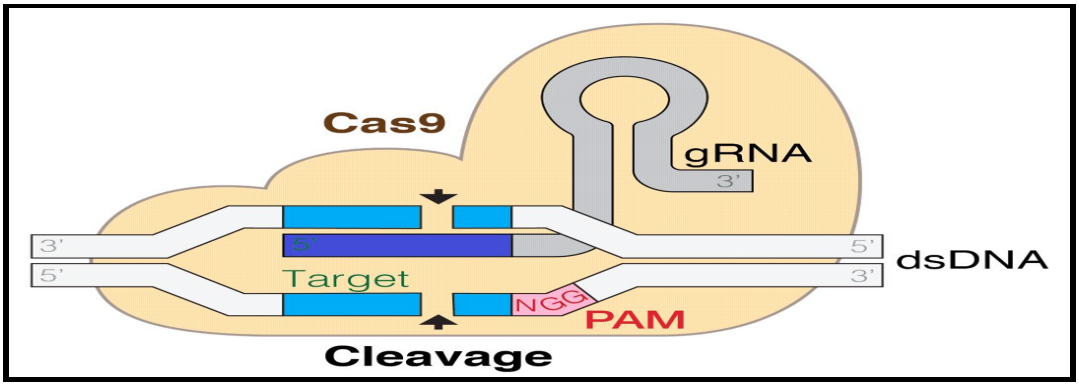

Fig 3: sgRNA-Cas9 complex involved in CRISPR gene editing (Courtesy: Peng *et al.,* 2016.

Powdery mildew resistance in hexaploid bread wheat by simultaneous editing of three homoeoalleles was achieved by Wang *et al*., 2014. Similar research was implemented for powdery mildew resistance in wheat by modification of three homeologs of TaEDR1 by genome editing enhancers (Zhang *et al*., 2017). The capacity to selectively change genomic DNA sequences *in vivo* is a strong drive for elementary and applied research.

CRISPR/Cas9-based binary vector set was used as a genome editing toolkit in plants that was validated using maize protoplasts, maize transgenic lines and *Arabidopsis* transgenic lines (Xing *et al*., 2014). TALENs and the CRISPR/Cas system was used for targeted mutagenesis for modification of genes, ZmPDS, ZmIPK1A, ZmIPK, ZmMRP4 in maize (Liang *et al*., 2014) and in potato (Wang *et al*., 2015). Genome editing was utilized tobacco (*Nicotiana tabacum*) for two genes, *NtPDS* and *NtPDR6* mediated by the CRISPR-Cas9 system (Gao *et al*., 2015). In soybean, CRISPR/Cas9 system is efficacious by knocking out a green fluorescent protein (GFP) transgene which modify nine

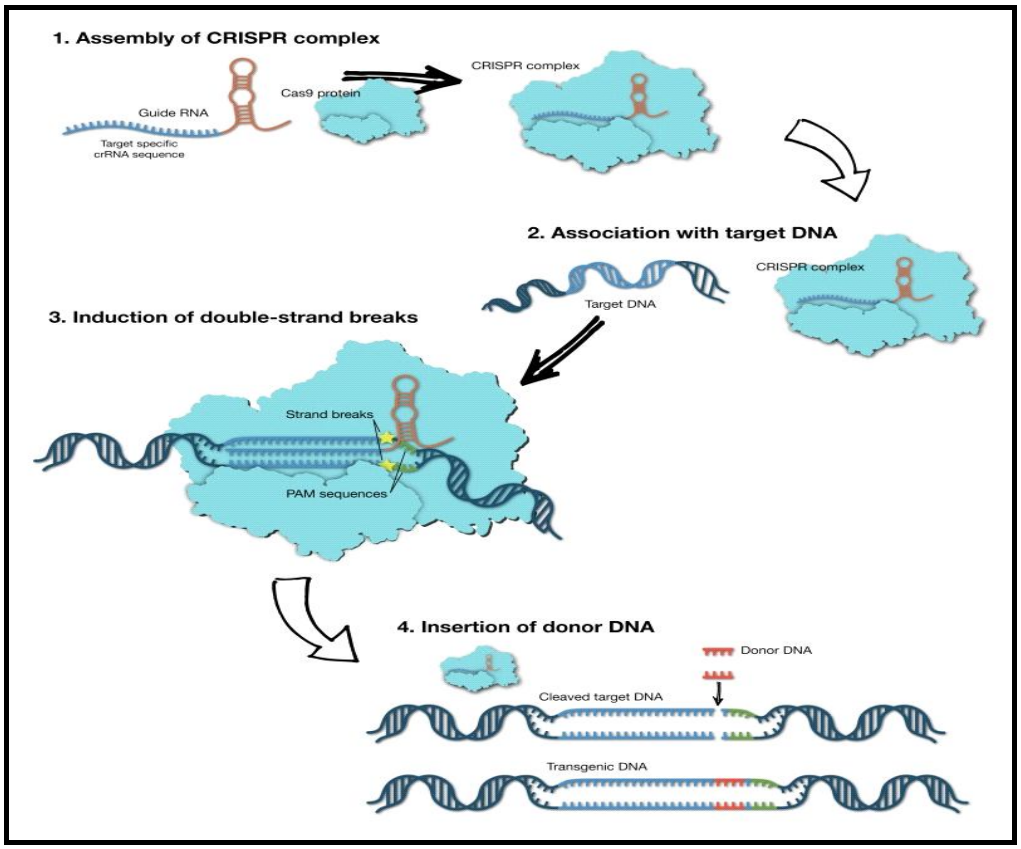

Fig 4: Mechanism of CRISPR-Cas9 genome editing (Courtesy: Jinek, *et al.,* 2012).

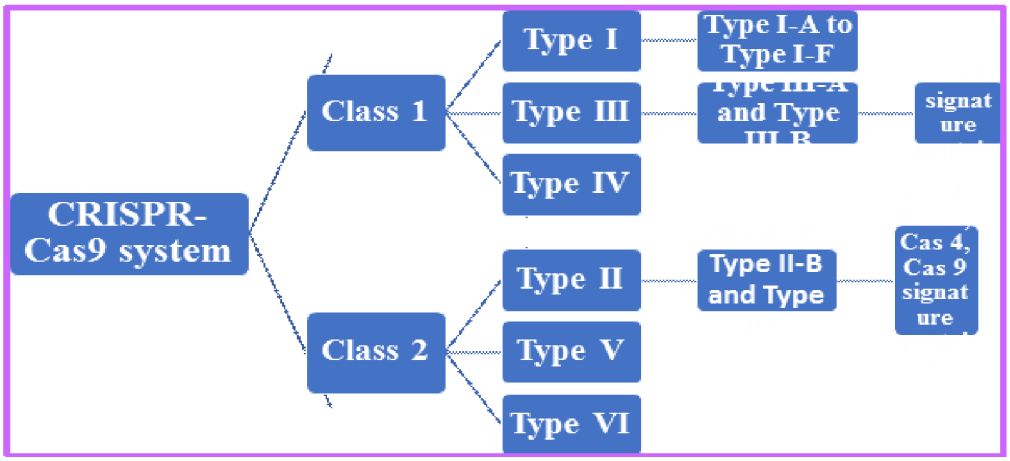

Fig 5: Flowchart depicting classification of CRISPR-Cas9 systems.

endogenous loci (Jacobs *et al*., 2015). The CRISPR-Cas9 system is thoroughly structured in generating targeted mutations in stable transgenic tomato plants where homozygous deletions of a desired size can be generated in the first generation itself (Brooks *et al*., 2014). CRISPR-Cas9-mediated editing functionality has also been demonstrated through transient studies in *Citrus sinensis* (Jia and Wang, 2014). Jiang *et al*., 2013 described expression of the Cas9/sgRNA system in twomonocot and two dicot crop species rice, sorghum, arabidopsis and tobacco. Although CRISPR-Cas9 technology has been utilized for crop plants engineering but wide implementation of this technology will require the development of protocols for plant transformation, species-specific vectors and various genomic resources (Schaeffer and Nakata, 2015).

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