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CRISPR/Cas 9 A novel genome editing technology for cotton improvement

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CRISPR-Cas9 is a valuable gene editing tool in plant science research. The presence of clustered repeats was first discovered by Ishino *et al.*, (1987) when the loci containing repeat sequences with an unknown function found in the *Escherichia coli* genome. Prokaryotes have CRISPR family throughout the genome was discovered by Mojka *et al.*, (2000) and the name CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) was coined and designed as Cas genes by Jansen *et al.*, (2002). First experimental evidence was reported for CRISPR adaptive immunity and the CRISPR complexes cleave the RNA and the tracr RNA forms a duplex structure with CrRNA in association with Cas 9 by Barrangou *et al.*, (2007). The PAM (Protospacer Adjacent Motif) is a motif with sequence NGG (any, guanine, guanine) is specific to Streptococcus pyogenes and 52 -NAG (any, adenine, guanine) the origin of spacers and adaptive immune system was reported by Bolotin *et al.*, (2005) and Pourcel *et al.*, (2005). The CRISPR acts upon DNA targets and the spacers are converted in to mature crRNAs that act on small guide RNAs (Marrafini *et al.*, 2008, Brouns *et al.*, 2008). The Cas 9 is guided by spacer sequences and cleaves target DNA *via* Double Stranded Breaks (Garneu *et al.*, 2010).

CRISPR system has Cas proteins of different components and mechanisms of action, were divided into two major classes (Makarova *et al.*, 2015). The class 1 system are classified as type I, III, and IV. In these types RNA guided target cleavage requires a large complex of several effector proteins. The class 2 systems are classified as type II, putative types V (Zetsche *et al.*, 2015) and VI (Shmakov *et al.*, 2015). Type II requires only one RNA-guided endonuclease Cas9 and Cpf1 in type V to mediate cleavage of invading genetic material. A large variety of Cas9 proteins exist in different bacterial type II CRISPR systems. These Cas9 nucleases range from 900 to 1,600 amino acids and classified as three sub classes type II-A, type II-B, and type II-C (Makarova *et al.*, 2015). The most commonly used Cas9 for genome engineering has been adapted from the type II-A CRISPR system from Streptococcus pyogenes (Sp). The Sp Cas9 has a simple PAM for genome editing Type II CRISPR-Cas9. Genome editing requires a DNA binding sequence specific and an effector domain that enables DNA cleavage creating a Double Stranded Break (DSB) and stimulates DNA repair pathway at the desired sequence and regulates transcription near the binding site. Nucleases were engineered to target site specific gene editing and the RNA guided nucleases (RGNs) from the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR associated proteins (Cas) system are the endogenous DNA repair pathways create mutations at the desired DSB sites(Vander Oost, 2013).

The CRISPR/Cas9 system is a two component system composed of Cas9 and a gRNA (Baltes *et al.*, 2014). Once Cas9 finds a PAM site, the gRNA binds to the DNA, a double break occurs three base pairs upstream the PAM. The Cas9 nuclease is the one among the nucleases, which recognizes target DNA and pairs between its guide RNA(s) and DNA. Cas9 is the simplest one and became the most popular and powerful tool for genome engineering. The advanced CRISPR/Cas9 technology not only provides a molecular tool and also enables the development of innovative and practical applications (Pennisi *et al.*, 2013; Doudna and Charpentier, 2014). The advent of CRISPR/Cas9

technology has revolutionized the field of genome editing. The CRISPR/Cas9 system can be used to engineer, so-called 'non transgenic' varieties (Woo *et al.*, 2015). A major advantage of targeting is that CRISPR/Cas9 can be introduced as transgenes to create the genome edits, and then progeny plants can be selected that carry the desired edits but have lost the Cas9 transgene through segregation. (Kanchiswamy, 2016). To select the transformed plants additional selection markers are required. This Cas9 technology was demonstrated in the model plant system Arabidopsis and also broadly used in different plant species. CRISPR/Cas9 vectors are available in the public plasmid repository of Add gene (http://www.addgene.org/crispr/plant/). Bioinformatics platforms help in gRNA design for different Cas9 proteins with variable PAM specification. Bioinformatics platforms help in gRNA design for different Cas9 proteins with variable PAM specification.

Sequence specific Cas9-mediated genome editing requirement are as follows :

- A Cas9 protein (CRISPR associated 9) nuclease, an enzyme with a nuclear localization signal specialized for cutting DNA.
- gRNA consisting of a guide sequence as a single transcript that is approximately 100 nucleotides long and is referred to as a single guide RNA (sgRNA) (a construct/chimera of CRISPR RNA (crRNA) and trans activating CRISPR RNA (tracrRNA) at the 52 end that matches the DNA sequence of the target site and a conserved 32 end scaffold with a special stem loop structure that binds Cas9.
- PAM, sequence 52 -NGG-3, a protospacer adjacent motif in the genomic sequence downstream of the targeted DNA.

CRISPR/Cas systems divided broadly into two classes: Class I uses multiple Cas proteins to cleaveforeign DNA, whereas class II employs a single nuclease. Class II is most suitable for genome engineering and in recent years has been extensively used for both NHEJ- and HDR-mediated genomeengineering in diverse organisms. Class II systems encompass two DNA nucleases, Cas9 and Cas12a (previously Cpf1). Cas9 creates blunt ends, whereas Cas12a leaves staggered cuts in DNA.

The Cas 9 nuclease is a type II CRISPR/Cas9 system of *Streptococcus pyogenes* is the most commonly used in genome editing (Gasiunas *et al.*, 2012). In the CRISPR/Cas9 system prototype, Cas9 was directed to the DNA target by an RNA duplex of crRNA and tracrRNA, but a single guide RNA (gRNA) is used for genome editing. Cas9 targets sequence specific sites of the target DNA by Watson Crick base pairing and its guide RNA and makes a cleavage. RNA guided nuclease directly interacts between Cas9 and a short protospacer adjacent motif (PAM) of DNA. Cas 9 has natural endonuclease activity for sequence-specific genome editing.

Genomic DNA sequence bearing a PAM could be edited by Cas9 with a specific gRNA due to the occurence of PAMs in genomes and Cas9/gRNA can target almost every gene. This Cas9 mediated plant genome editing is feasible using plasmid vectors containing the Cas9 and gRNA expression cassettes of CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). Promoter of DNA dependent RNA polymerase II (Pol II) and a Pol II transcriptional terminator are used to express Cas9 which is fused with a nuclear localization signal peptide (nuclease), a Pol III promoter and a terminator in the gRNA. The Cas9 and gRNA expression cassettes are put in one plasmid, which can be delivered into plant cells using genetic transformation methods. This genome editing approach has sparked new breeding technologies based on CRISPR/Cas9. The use of two sgRNAs targeting two sites across a gene can lead to gene deletion.

Site specific integration with CRISPR Cas 9

In precision crop breeding the site-directed mutagenesis and site specific integration of a gene (a knock-in) is of great value

for improvement programme. This technology uses designer nucleases and the cellular DNA repair system to precisely modify genomic sequence (Voytas, 2013).This can be achieved by inserting a reporter to track the protein expression of a particular gene location. Site directed mutation of specific amino acids to change protein activity and also introduce foreign DNA fragments to confer new traits.

Two type of site directed mutagenesis occur

1, Homology Directed Repair (HDR)

2, Non Homologous End Joining (NHEJ) followed by double stranded break (DSB). In eukaryotes a double strand break (DSB) is the most lethal type of DNA damage has evolved major DNA repair pathways known as non-homologous endjoining and homology directed repair. In practice, a synthetic sequence specific nuclease is designed to recognize the targeted genomic site and is transfected/tranformed into the cell, where it creates a double strand DNA break (DSB) at the specific site. This DSB is usually repaired by the endogenous, error-prone non homologous end joining (NHEJ) pathway, which introduces a small insertion or deletion (InDel) at the site, thus knocking out the gene. In a gene knock-in a donor DNA fragment with homology to the flanking sequence is repaired through homology-directed repair (HDR). As a result, the sequence of the donor fragment is integrated into the genome at the DSB site. Homology Directed Repair (HDR) takes place in the double stranded break (DSB) region made by Cas9/gRNA-mediated site-directed mutagenesis results in the donor sequence being substituted into the specified region and gene knock-in has been reported in plants. Non Homologous End Joining (NHEJ) occurs at a much higher frequency during DSB repair than HDR (Ray and Langer, 2002). The DSB repair system (NHEJ and HDR) is a ubiquitous component of all living cells. Therefore, an artificial nuclease Cas 9 whose recognition site is reprogrammable is the most critical part of genome editing. DNA *geminivirus* replicons of high copy number were used as the donor template to increase gene knock-in efficiency in plants (Baltes *et al.*, 2014).

Site specific transcription with Nuclease deactivated Cas9 (dCas9)

CRISPR-Cas9 may be more suitable for developing transcriptional regulation tools and multiplexing. Nuclease deactivated Cas9 (dCas9) can be engineered to enable sequence specific genomic regulation by fusing to transcriptional activators, repressors, and epigenetic modifiers.

The nuclease deactivated Cas9 (dCas9) and gRNA can be used as a shuttle for transcriptional control to the DNA targets. Nuclease-deactivated Cas9 (dCas9) delivers different regulatory components to a specific site. The catalytic activity of Cas9 is deleted and the dCas9/gRNA fused to its DNA target for transcriptional activation. In this system, multiple molecules could be added to the gRNA scaffold for robust transcriptional activation and a single modified gRNA is sufficient for activating one gene (Konermann *et al.*, 2015; Zalatan *et al.*, 2015). The transcriptional suppressor or other DNA-modifying enzyme replaces AD domain to manipulate gene transcription and epigenetic status (Hilton *et al.*, 2015). Multiple gRNAs are required to target one promoter for robust activation or suppression. The three-component system of dCas9/gRNA-AD should enable more robust transcriptional control in plants, though additional optimization. The dCas9/gRNA targets quantitative traits to produce valuable nutrients or bio agents in plants. CRISPR-dCas9 based activators and repressors were demonstrated in transiently expressed leaves of transformed tobacco by Agro infiltration (Piatek *et al.*, 2015). Targeting with a methylated DNA for 400-fold increase in mRNA expression by transcriptional modulation at multiple genetic loci was reported in Arabidopsis (Lowder *et al.*, 2015). These studies represent the first generation of activators and repressors for transcriptional regulation.

Site specific multiplexing

Targeting of multiple genes can improve more than one trait in crops. In basic research to find the role of multiple genes in a complex network can be made possible by multiplexing CRISPR/Cas9. Cas9 can independently pair with multiple gRNAs, and mutate multiple target sites in a single cell. This multiplexing targets multiple genes and delete large chromosomal segment reported in rice and in Arabidopsis (Zhou *et al.*, 2014; Zhao *et al.*, 2016). Several toolkits have been developed and reported of multiplexing. The first toolkit of multiplexed gene knockout was reported in three Arabidopsis genes (Xing *et al.*, 2014). The second toolkit developed with vectors were validated in monocots and dicots and also a third toolkit with vectors that could be used for genome editing (Wang *et al.*, 2015). Multiplex systems are time consuming and can target more than six target sites.

Cas9/gRNA off target editing

Cas9 of off target activity and gRNA can be designed with the help of bioinformatic tools to reduce the risk (Cradick *et al.*, 2013). The off target effect has been analyzed *in vitro* and *in vivo* approaches can edit a DNA target bearing as many as five mismatches (Mali *et al.*, 2013a; Pattanayak *et al.*, 2013). The off target editing of Cas9 was reported in rice protoplasts (Xie and Yang, 2013). The off target activity occurs when the concentration of Cas9/gRNA is high.The Cas9/gRNA cannot edit a DNA site with any number of mismatches near a PAM (within10–12bp). The higher 52 -NAG-32 PAM sites can be targeted by Cas9/gRNA in bacteria. Cas9 has much less affinity for NAG-PAM than for NGG-PAM. Off target editing can be reduced by fusion of Cas9 with additional DNA binding domains (Bolukbasi *et al.*, 2015).Fidelity of the gRNA can be increased by shortening spacer sequence to 17–18 nt (Fu *et al.*, 2014).

Genome wide survey of gRNA design reveal a sufficient number of highlyspecific gRNAs could be designed to cover 90 per cent of genes for seven out of eight plant species (Xie *et al.*, 2014). Next generation sequencing, such as GUIDE seq (Tsai *et al.*, 2015), Digenome seq (Kim *et al.*, 2015), and ChIP seq (Kuscu *et al.*, 2014), identify off target sites though highly specific gRNA. The off target activity of Cas9 reduces the number of its targetable sites. Fusion of Cas9 with additional DNA binding domains also reduces off target editing (Bolukbasi *et al.*, 2015). Shortening the gRNA spacer sequence to 17–18 nt increases targeting fidelity. Many bioinformatics tools have been developed to facilitate Cas9-mediated genome editing. Bioinformatics platforms help in gRNA design for different Cas9 proteins with variable PAM specificities and predict the on target editing efficiency and off target risk of gRNAs, and the best target sites for Cas9. In polyploidy or outcrossing species, the sequence variation between different alleles should be considered for gRNA design (Fu *et al.*, 2014).

CRISPR/Cas9 for improving traits in cotton

CRISPR/Cas9 genome editing tool undertake functional studyof the genes as well as for improving cotton traits. CRISPR/Cas9 genome editing in cotton was first employed in the function of MYB-25-like transcription factor in cotton fiber development (Li *et al.* 2017). MYB-25 like gene was dominantly expressed during cotton fiber initiation and early development. Two gRNAs were designed for targeting two different locations in MYB25-like gene which were conserved in both the A and D sub genomes. Both gRNAs individually and/or together generated lots of deletion/insertion mutations with a high efficiency without any off target effects (Li *et al.*, 2017). ALARP is a protein rich in alanine, which is preferentially expressed in cotton fibers. By using CRISPR/Cas9 technology, gene knock out of ALARP was reported deletion together with large insertion without detecting any potential off target effect (Zhu *et al.*, 2018). Based on sequence analysis of the target site containing CRISPR/Cas9, the

mutation frequencies of GhALARP-A and GhALARP-D target sites were 71.4 {100% and 92.9 {100%, respectively (Zhu *et al.*, 2018).

In cotton, gene knock out of two copies of 14-3-3d gene by CRISPR/Cas9 genome editing technology showed higher resistance to Verticillium dahliae infestation compared to the wild type plants (Zhang *et al.*, 2018). After inoculating with *V. dahliae* for 18 days, the CRISPR/Cas9 edited plants showed significantly reduced disease symptoms, and the disease index was less than that in the wild-type CCR35 plants. The infected plants severity was reported to decrease from ~90 pre cent in wild type to ~30 pre cent in the CRISPR/Cas-edited plants. The disease index decreased by more than 50% in the genome-edited plants as compared to the wild type (Zhang *et al.*, 2018). Virus-resistant plants using CRISPR/Cas9 can be developed in two ways, by directly targeting conserved regions of the virus genome and by mutating relevant S-genes. Cas9 expressing transgenic plants were developed using tobacco rattle virus as a vector for the delivery of sgRNAsto target the replication-associated protein, coat protein (CP), and intergenic region (IR) of the begomo virus Cotton leaf curl Khokhran virus (CLCuKoV) (Ali *et al.*, 2015a, b). The sgRNAs reduced the accumulation of virus in comparison to control plants but targeting the origin of viral virion strand DNA replication in the IR region proved to be successful in interfering with virus replication. The use of CRISPR/Cas generated virus resistance can select for editing-resistant variants of the viruses (Ali *et al.*, 2016; Mehta *et al.*, 2019).

Cotton crop is exposed to many environmental stresses like drought, salinity, extreme low (chilling) and high (heat) temperatures. These abiotic stresses significantly affect cotton growth and development. CRISPR/Cas9 genome editing technology achieves resilience to these stresses. The down regulation of GhHB12 gene, a HD-ZiP transcription factor subfamily I gene, enhance the tolerance to abiotic stresses in cotton, suggesting that knockout of this gene can help developing drought tolerant cotton lines with strong environmental adaptability (He *et al.*, 2020). The miRNAs control almost all biological process in plant development (Li and Zhang 2019). The miR156 controls seedling development and timing of various developmental stages of vegetative growth to reproductive growth. CRISPR/Cas9 knock out miR156 in cotton; resulted in the miR156 knockout lines of cotton showed restricted growth and are also found sensitive to abiotic stresses, including drought, salinity, and water logging.

Improving the cotton plant morphology with deep root system, the cotton plants will uptake more nutrients and water from the deeper layers of soil. Thus, a cotton plant can sustain drought stress with stronger and healthy roots help plant variety of abiotic and biotic stresses. Nitric oxide (NO) is an important regulator for root development in plants. NO concentration makes plant to produce more lateral and adventitious roots. In plant, nitric oxide synthase (NOS) catalyzes the synthesis of NO. Arginase competes with NOS for arginine (ARG) substrate. Over expression of arg significantly inhibits the NO accumulation in cotton root and then decreased the formation of lateral roots. Knockout of arg gene by CRISPR/Cas9 technology significantly enhanced root development in cotton (Wang *et al.*, 2017). The total number of lateral roots and the total root surface area were enhanced in both high and low nitrogen conditions in genome edited cotton plants as compared to the wild type cotton (Wang *et al.*, 2017). This suggests that knocking out of arg gene not only promotes root development but also enhances tolerance to nitrogen deficiency.

CRISPR/Cas9 genome editing is also employed to improve agronomically important traits in cotton. Mutations in the two genes Cloro plasto salterados 1 (GhCLA1) and vacuolar H+-pyrophosphatase (GhVP) were detected in cotton protoplasts and the mutations were nucleotide substitutions, with one nucleotide insertion and

one substitution in GhCLA1 and one deletion in GhVP, the mutation efficiencies were 47.6-81.8 pre cent in transgenic cotton plants. Evaluation using restriction enzyme PCR assay and sequence analysis detected no off target mutations (Chen *et al.*, 2017). CRISPR/Cas9 knockout of cotton gland formation 3(cgf3), transcription factor gene, resulted in the genome edited cotton plants with glandless phenotype (Janga *et al.*, 2019). Gene silencing of CGP1 gene (involves in gland formation) by CRISPR/Cas technology and the genome edited plants exhibited glandless-like phenotype (Gao *et al.*, 2020). It was also revealed that level of gossypols and associated terpenoids compounds were substantially depression in expression of genes involved in biosynthesis of gossypol was reported in the genome edited plants (Gao *et al.*, 2020). The calcium sensor CBL2 and its interacting kinase CIPK6 are involved in plant sugar homeostasis via interacting with tonoplast sugar transporter TST2. CRISPR/Cas9-mediated knockout of GhTST2 resulted in significantly decreased Glc content in cotton (Deng *et al.*, 2020).

CONCLUSION

The CRISPR/Cas9 technology is the ultimate molecular tool for genome editing. Genome edited plants are differentiated from conventional transgenic plants as they may not incorporate foreign DNA. Although genome editing can be used to introduce DNA insertion or deletion into the genome, it may simply involve changes of a few base pairs of the plant's own DNA. This distinction makes genome editing a novel and powerful breeding tool that has promising applications in Agriculture. Direct delivery of purified Cas9 protein with guide RNA into plant cells through plasmid mediated delivery, displays high efficiency and reduce off target effects. Following regeneration from edited cells, the ensuing plant is also likely to bypass genetically modified organism (GMO) legislation as the genome editing complex is degraded in the recipient cells. This CRISPR/Cas9 tool will provide unrivalled insights into plant molecular biology and enable us to improve crop plants with speed and accuracy through breeding. The plant genome editing is yet to reach a stage of commercialization, it is expected that in the 2030s we would see successful CRISPR/cas9-generated biotech cotton in fields. Most of these strategies could also be meant to target pests and disease resistant, agronomical and economically important traits of biotech cotton. The advent of CRISPR/Cas9 technology has expedited the approach to explore thecapacities of genome-editing tools.

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