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Chief Editor
Dr. SP Giri

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Chapter - 6

Genome Editing in Plants: CRISPR Cas Technology in a Future Perspective

Sijimol K and Minu S

Abstract

The generation of new plantlets with desired characters is a laborious and expensive technique. Genome-editing technology provides an advanced biotechnological tool that enables a precise and efficient targeted modification of an organism's genome. They have been utilized in a wide variety of plant species for the characterization gene as well as to improve desirable traits in agricultural crops, in particular. Among the gene editing tools, Clustered Regularly Interspaced Short Palindromic Repeats and associated protein 9 (CRISPR-Cas9) provides a technological breakthrough in targeted mutagenesis for the improvement of crops The application of CRISPR/Cas9 and related technologies in plant genome engineering will not only facilitate crop breeding but also accelerate the progress for generating transgene-free genome edited plants. The present paper highlighted the various aspects of CRISPR/Cas9 technology in plants and its applications in crop improvement.

Keywords: CRISPR/Cas9, genome editing, biotic stress, abiotic stress, crop improvement

1. Introduction

Precise editing and regulation of genomic information is essential for the better understanding of gene function in plants. The traditional method of plant breeding involves selection, crossing of plants for desired traits, and hence quite labor and time-intensive. During the few past decades, technological breakthroughs have made genome editing and their regulation became significantly easier. Gene editing allows targeting of gene and the modifications of respective DNA sequences through various technologies (Jaganathan *et al.* 2018). Initially, zinc finger nuclease (ZFN) and transcription activator like effector nuclease (TALEN) has been found to be the most flexible engineered nuclease employed in gene editing. Later on,

clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) has been developed (Osakabe *et al.* 2015) as an efficient technology. This is the most widely exploited tools due to its simplicity, higher efficiency, inexpensive, and provide easy experimental design when compared to the previous nucleases (Wang *et al.* 2016). They have been exploited in both monocots and dicots to enhance yield, quality, and nutritional value, to introduce or enhance tolerance to biotic/abiotic stresses, among others.

2. CRISPR/Cas9 Components

CRISPR belongs to group of DNA sequences, initially isolated through downstream processing of a gene (alkaline phosphatase isozyme gene) from *Escherichia coli* (Ishino *et al.* 1987). The natural role of CRISPR-Cas9 system is to provide adaptive antiviral immunity against viral DNA in bacteria and archaea (Jinek *et al.* 2012; Sternberg *et al.* 2014). The frequently used CRISPR-Cas9 system in plant genome editing is an adaptation of type II CRISPR-Cas system of *Streptococcus pyogenes,* gram-positive bacteria (Garneau *et al.* 2010). Type II CRISPR-Cas system consists of a Cas9 protein, which possess DNA endonuclease activity and an RNA transcript which forms short guide RNAs that direct Cas9 to the target DNA sequence (Lander 2016; Jiang & Doudna, 2017). In cell, Cas9 binds to guide RNA (gRNA), forms a binary complex, which have whole genome coverage for target DNA for their cleavage and specificity, determined by gRNA. Besides, Cas9 requires a specific proto-spacer adjacent motif (PAM) localized on the non-target DNA strand, directly towards downstream of target DNA sequence (Moundai *et al.* 2021). Cas9 proteins possess two nuclease domains, HNH and RuvC. HNH and RuvC-like nuclease domain cleaves the target DNA and non-target strand respectively, which is complementary to guide RNA sequence (Jinek *et al.* 2012; Gao *et al.* 2017). The activities of nuclease domains resulted in double stranded break (DSB) in DNA at the target site which can further utilized to introduce modifications by non homologous end joining (NHEJ) or homology-directed repair (HDR) (Symington & Gautier, 2011). In plants at higher taxonomic levels, occurrence of NHEJ is more frequent than HDR (Puchta, 2005) and they disrupt genes through the formations of small base pair indels (insertions/deletions) in the target genes at specific points. The HDR are employed to introduce specific point mutations and insert/replace desired sequences precisely into the target DNA (Li *et al.* 2013). The base editors had an advantage to induce point mutations in plants such as maize, rice and wheat without generating an excess of undesired products (Rees & Liu

2018). Thus, base editors will allow precise genome modifications to ensure plant stability, for the development of versatile DNA cassettes to co-express both gRNAs and Cas9 in same cells (Li *et al.* 2013). Presently, engineered Cas9 base editors are capable of editing a single base pair in the genome without the introduction of DSBs. The Cas9 base editors consist of a dead Cas9 domain fused to an enzyme, cytidine deaminase that are programmed with gRNA without inducing breaks in double stranded DNA (Komor *et al.* 2016; Gaudelli *et al.* 2017).

3. CRIPSR Cas9 working mechanism

For the last few decades gene editing has been attempted in plants especially in vegetable crops through different tools. The accuracy of gene editing depends on several factors such as delivery methods, selection of vectors, of single guide RNA (sgRNA) and cas9 constructions, different transformation approaches, among others. CRSIPR/Cas9 undergoes DNA cleavages into two components, Cas9 and sgRNA. Cas9, a DNA endonuclease derived from different bacterial species used for cas9 isolation. Cas9 contains two domains such as HNH domain, Ruc V-like domain and both cleaves single and double stranded DNA. sgRNA, a synthetic RNA consists of 100 nucleotide and its 5' end possess 20 nucleotides which acts as guide sequence to identify the target sequence accompanied by protospacer adjacent motif (PAM) sequence, which is often the consensus NGG (Liu *et al.* 2017). Moreover, accuracy in each steps are crucial for the successful gene editing process.

3.1 Designing of delivery methods

With the advent of technologies, an efficient delivery system such as gene disruption, genomic deletion, gene knock out, multiplex genome editing etc. has been exploited. The continuous studies in the gene editing research allowed a precise and targeted delivery system of cas9 and its variant to the plant system (Wada *et al.* 2020)

3.2 Vectors for CRISPR/Cas9

Single vector and binary vector systems are usually employed in CRISPR mediated gene editing. Among these, binary vector systems are commonly utilised due to its accuracy and uniqueness in plant transformation. Vectors having both sgRNA and Cas9 protein cassettes are the highly promised one. Single polymerase II and dual polymerase II promoters are also employed to derive simultaneously the expression of both sgRNA and Cas9 proteins in a system.

3.3 CRISPR/Cas and single guide RNA expression

Expression of sgRNA consists of cloning the annealed products of two single-stranded fragments with a complimentary target-binding sequence on each strand between tRNA and gRNA sequences in a vector and assembling tRNA-gRNA units from several vectors with a binary vector containing expression cassette (Oh *et al.* 2020)

3.4 Cas9 expression cassettes construction

The expression levels of CRISPR-Cas9 are highly associated with the efficiency of the system. Besides, structure of each individual expression cassettes also had played a significant role in their efficiency. Thus, promoter selection to drive expressions of both cas9 and sgRNAs and the performance of cassettes expression in the overall expression system are vital for the precise and efficient genome editing method (Montecillo *et al.* 2020)

3.5 Delivery of vector through transformation approaches

The success of each delivery depend on types of tissues uses and their successive regeneration into whole plants. Different methods such as PEG mediated, Agrobacterium-mediated transformation, bombardment or biolistic transformation etc. has been exploited for the vector delivery in plants cell. Among these, Agrobacterium-mediated transformation was reported as a highly efficient method (Naim *et al.* 2018). Nevertheless, nanoparticle-mediated delivery as pollen magnetofection mediated has been suggested as new method for the delivery system to reduce time consumption and to omit the laborious tissue culture (Sandhya *et al.* 2020)

3.6 Mutant screening

The traditional way of screening CRISPR/Cas9-induced mutants from the initial samples through PCR is labor-intensive and tedious. This can be overcome through direct screening of Cas9-free transgenic lines from T1 seeds using a fluorescent microscope, greatly lessened the workload and timeframe for obtaining Cas9-free mutant lines (Zhang *et al.* 2020). Various cost-effective and sensitive screening techniques have been developed for the large-scale screening of CRISPR/Cas9-induced mutants (Guo *et al.* 2018; Wang &Wang 2019).

4. Gene editing for plant breeding

The application of CRISPR/Cas9 for the crop improvements in vegetables has been reported a few years back. Among vegetable crops, first CRISPR/Cas9-mediated genome editing was reported in tomato where

ARGONAUTE7 (*SlAGO7*) gene involved in leaf development was targeted (Brooks *et al.* 2014). Later on, developmental genes like SHORTROOT (root development; Ron *et al.* 2014), BLADE-ON-PETIOLE (for inflorescence development; Xu *et al.* 2016), and SELF PRUNING 5G (plant development; Soyk *et al.* 2017) was targeted for the functional validation in tomato. CRISPR/Cas9 mediated targeted gene responsible for the early development of plants has been reported in other vegetables like *Lactuca sativa* (Woo *et al.* 2015), *Brassica oleracea* (Lawrenson *et al.* 2015), among others. Genes (*Anthocyanin 1* (*ANT1*) involved in anthocyanin biosynthesis (Cermak *et al.* 2015), *Phytoene desaturase* (*SlPDS*), *Phytochrome interacting factor* (*SlPIF4*) (Pan *et al.* 2016), and *Phytoene synthase* (PSY1) (Hayut *et al.* 2017) involved in biosynthetic pathways for carotenoid biosynthesis in tomato were edited by CRISPR/Cas9. Hayut *et al.* (2017) reported the targeted recombination in heterozygote to for the stable expression of Cas9 and gRNA separately showed precise reshuffling of chromosomal segments between homologous chromosomes in somatic cells. The parthenocarpic tomato developed through gene editing technology had a greater demand among other vegetables. Sequence specific genomic editing of rice genes such as mitogen-activated protein kinase (*OsMPK2*), phytoene desaturase (*OsPDS*) and betaine aldehyde dehydrogenase (*OsBADH2*) are involved in regulation of abiotic stresses (Endo *et al.* 2020). Among the employed gene transfer methods, biolistic transformation is the most useful strategy for the effective delivery of larger template where mode of transfer through agrobacterium is not possible. The site-specific gene alterations and insertions were done in maize and soybean through biolistic transformation method (Svitashev *et al.* 2016; Li *et al.* 2015; 2016).

The first study on genome editing in fruits has been reported in citrus by mutating gene, *Phytoene desaturase* (*CsPDS*) involved in carotenoid biosynthesis pathway (Jia and Wang, 2014). The knock-out of susceptibility gene *CsLOB1* has been adopted for developing citrus canker resistant plants through CRISPR Cas9 method (Jia *et al.* 2017). Different alleles of *CsLOB1* gene present in citrus contain the effector-binding element (EBEPthA4), which is recognized by main effector PthA4 of Xcc to activate *CsLOB1* expression. A different strategy has also adopted to develop citrus canker resistance by targeting this effector-binding element in promoter of *CsLOB1* (Peng *et al.* 2017). Besides, targeted editing of *Phytoene desaturase* by CRISPR/Cas9 has been reported in watermelon and grape (Nakajima *et al.* 2017; Tian *et al.* 2018).

4.1 Approaches to increase biotic stress resistance

Current crop production systems are prone to increasing pathogen attacks. A proper understanding of molecular plant-pathogen interactions, the availability of genomic information for crops and pathogens will provide a novel approach for disease resistance in crops which will further utilised for the breeding programmes. The most common approach in gene editing focuses on the "simple" knockout by NHEJ repair of plant susceptibility factors required for the colonization of an efficient host (Schenke and Cai 2020). However, genome re-writing via homology-directed repair or base editing can also prevent host manipulation by changing the targets of pathogen-derived effectors or molecules beyond recognition, which also decreases plant susceptibility. The phenotypic expressions of various traits for biotic/ abiotic stress resistance through gene editing demonstrated the efficiency of CRISPR based technology in plants (Zafar *et al.* 2020). In fact, multiple genes responsible for abiotic stress responses are involved in metabolism, regulatory pathways, etc. in plants (Biswas *et al.* 2021). Single or multiple genes can be targeted through CRISPR/Cas9 system to increase the capability of plants to adapt in various stress environment. The multiple targeted genes are responsible for various biotic stresses in Arabidopsis thaliana (Paixio *et al.* 2019; Osakabe & Osakabe, 2017), rice (Alfatih *et al.* 2020, Miao *et al.* 2018). In rice, the expression of ethylene responsive factor OsERF922 and OsSWEET13 has well established for blast and blight resistance respectively (Jaganathan *et al.* 2021).

CRISPR/Cas9 is an important tool for developing crops that is resistant to various pathogens. Plant genome possesses R gene (resistance) as well as genes conferring susceptibility to particular disease. The targeted knockout of the susceptibility gene will make the plant more resistant to specific disease. In wheat, knockout of gene responsible for powdery mildew has been employed to develop disease resistance in wheat (Wang *et al.* 2014). The eukaryotic translation initiation factor *eIF4E* is one of the major host factors required for multiplication of RNA viruses in plant cells. Silencing of *eIF4E* gene in tomato and melon develop a wide range of disease resistance to RNA virus (Mazier *et al.* 2011; Rodríguez Hernández *et al.* 2012). Similarly, knock-out of *eIF4E* gene through CRISPR/Cas9 using two gRNAs targeted at two different sites of *eIF4E* gene has been attempted in Cucumber for a broad spectrum resistance against viruses from potyviridae family (Chandrasekaran *et al.*, 2016). The knockout of *mildew resistant locus o* (Sl*Mlo1*) gene conferring susceptibility to fungi *Oidium neolycopersici* causing powdery mildew disease has been reported in tomato

(Nekrasov *et al.* 2017). Another strategy has been employed to develop multiple virus resistance by introducing single *Cas9* gene along with multiple gRNAs targeting each virus through mimicking the CRISPR/Cas9 system in plants.

5. CRISPR/Cas mediated cis engineering

The advent of CRISPR/Cas-mediated *cis*-regulatory region engineering (*cis*-engineering) provides a more accurate method for modulating the gene expression in specific manner, which leads to phenotypic diversity of the species, which have advantages in crop improvement. In fact, the *cis*-regulatory regions (CRR) are non-coding DNA sequences controls the gene transcription, consist of combinations of cis regulatory elements (CREs) that affects the level of gene expression often in a spatiotemporal manner (Bulger and Grounidne 2011; Wittkopp *et al.* 2012). The common natural variations in *cis*-regulatory regions are Single-nucleotide polymorphisms (SNPs), insertions, deletions, inversions, and epigenetic variations, which are associated with domestication of crops. The modulation of gene expression through CRISPR/Cas-mediated *cis*-engineering is more likely to benefit crop improvement with less adverse pleiotropic effects when compared to coding regions (Wolter *et al.* 2019; Pandiarajan and Grover 2018). Several strategies was employed for the application of CRISPR/Cas mediated cis-engineering for the improvement of horticultural crops. With the available genomic and transcriptomic informations, the identification of novel CREs will be possible in horticultural crops using bioinformatics pipelines and other experimental approaches (Mehrotra *et al.* 2011; Chen *et al.* 2019). The de novo discovery of CREs are mainly based on the conservation of sequences that exists among group of co-expressed genes or among orthologs of multiple species (Gruel *et al.* 2011, Mehrotra *et al.* 2011). Thus CRISPR/Cas-mediated *cis*-engineering is found to be an important tool for generating horticultural crops in order to better adapt to climate change and could provide food for providing food for an increasing world population.

6. Biosafety and regulation for CRISPR edited plants

There are several biosafety and social concerns regarding the genome editing plants related to target gene site selection, designing of guide RNA, effects of off-target, and the delivery method. The major concern is the risk factor by generating genetic changes in plants due to the off-target mutations (Liang *et al.* 2018). The integration of transgenes and possible risk of off target mutations can be prevented through delivery of *in vitro* pre-assembled CRISPR-Cas9 ribonucleoproteins (Svitashev *et al.* 2016; Liang *et al.* 2018).

Eventhough these methods has been adopted in several crop species, there are some drawbacks in its application such as they are expensive, possess low stability, need higher technical requirements (Malnoy *et al.* 2016; Subburaj *et al.* 2016; Murovec *et al.* 2018). Other concerns are Cas9specificity and the limited number of sites that can be targeted due to the involvement of PAM (Spencer and Zhang, 2017). This can be tackled through engineered protein, which leads to the identification of Cas9 mutations through alteration of PAM recognition, thereby enhance its fidelity, and recognize other motifs (Kleinstiver *et al.* 2016; Leenay & Beisel, 2017). Moreover, still there are several concerns in the society about distinction between transgenic plants, genetically modified plants, and genome edited plants (Eckerstorfer *et al.* 2019). Transgene free edited plants will be possible through CRISPR cas9 technology by transgene carrying CRISPR-Cas9 cassette might be removed by gene segregation (Mounadi *et al.* 2020). Once the transgene is removed, genome edited plants will be known as non-transgenic. Creating awareness to the public on principles of genome editing might help to create misconceptions among them (Garcia Ruiz *et al.* 2018; Eckerstorfer *et al.* 2019)

7. Conclusions

Generally, genome editing technology and CRISPR-Cas9 in particular is an emerged tool with a greater potential for the transformation of agriculture sectors through the development of plants which are resistant to both biotic and abiotic stresses. Through this technology, nutritional value and yield of the plants become higher when compared to traditional breeding approaches. In fact, these technologies are necessary to effectively exploit the production of transgene free plantlets. However, there is a need to screen policy regulations of gene-edited plants and proper awareness should be provided to the public concerning the pros and cons of gene editing technology.

8. Future implications

CRISPR has been widely adopted to introduce traits of interest for economically important crops for their breeding programmes. Several studies have demonstrated CRISPR-mediated engineered disease resistance against multiple pathogens (Xu *et al.* 2019; Oliva *et al.* 2019). The edited genes in commercial crop varieties will be made available in near future, which showed the potential application for CRISPR technology. In addition to regulatory and policy improvements, various technological interventions are also required for the development and testing of crops developed though CRISPR technology. Moreover, extensive field trials are also needed to

assess the performance of these crops, for resistance to various biotic/abiotic stresses and for their productivity.

The advent of gene editing technology had a significant impact to understand the possibility to explore the genome modifications through genetic engineering techniques. The development of Cas proteins and their variants and different methods of transfer has broaden the methodology for precise gene expression. Eventhough, gene-editing technology is well established in crops; these approaches never replace the traditional breeding methods but found to be an alternative to produce plants with trait of our interests. Hence gene edited plantlets can be exploited to improve genetic variability in germplasm for the production of stable varieties in wide range of crops. Through gene editing technology, domestication of wild crop varieties can be increased in future for growing plants in adverse extreme conditions without affecting its yield.

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