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Genome Editing for Speed Breeding of Horticultural Crops

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ABSTRACT

Climate change is one of the burdensome factors of agricultural productivity. Adverse biotic and abiotic stress impacts directly on plants resulting in poor productivity and yield loss. Modern protocols in genome editing using CRISPR, TALENs, ZFNs and Meganucleases enable editing at the precise site. The versatility of genome editing tools makes its application useful in fields like crop improvement, gene functional analyses, pathway research, studying animal models, genetic analyses, epigenetic research, drug development and biofuels research etc. The technological advancements in genome editing are adopted in both monocots and dicots for crop improvement. These technologies are precise, independent of breeding limitations like unexplored germplasm resources and reduce breeding cycles drastically from decades to years to meet the global requirements. The application of genome editing in agriculture proves to be a potential source for developing crops with biotic and abiotic stress, improved yield with better nutritional qualities.

Keywords: CRISPR, Crop improvement, Genome editing, Plant stress, Rapid breeding

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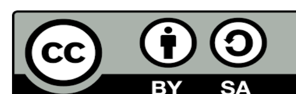

INTRODUCTION

CRISPR – The prominent tool for genome editing

CRISPR/*cas*, in genome editing has emerged recently as a tool of choice. This method is RNA-mediated nuclease defence based on the adaptive bacterial and archaeal immune system (He and Deem, 2010). Primitive research on CRISPR was made by Ishino *et al,* (1987) in *Escherichia coli* K12. They found a new class of 29 nucleotides interspaced short sequence repeats of undefined biological importance separated by variable 32 nucleotide spacer domains downstream of *iap* gene of *E.coli* K12 (Karimi *et al,* 2018). This initiative paved the path for further research on CRISPR. The association of the CRISPR regions with adaptive immunity in *Streptococcus thermophilus* was elucidated and at this point, new acquiring spacers were accompanied by the ability to impart phage resistance in a Cas-dependent manner (Barrangou *et al,* 2007). Few applications have been represented in fig. 1. Enabling CRISPR/CAS as a genome editing tool (He and Deem, 2010) which led subsequently to invent three different classes of CRISPR/CAS systems (Karimi *et al,* 2018).

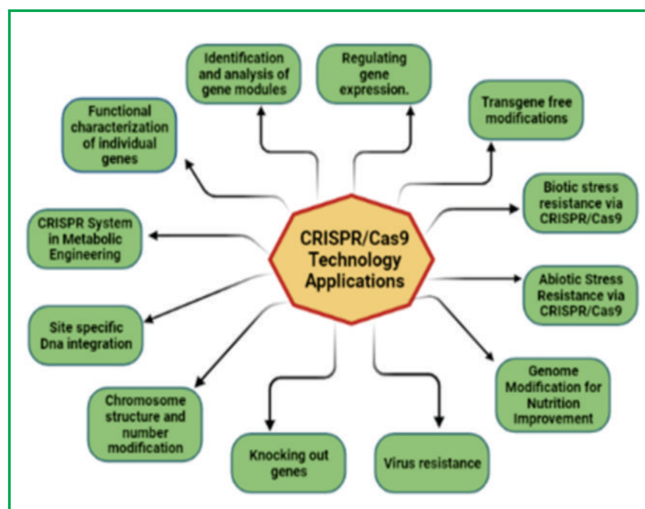

Fig. 1: Direct applications of CRISPR/Cas technology

Components of CRISPR

CRISPR loci contain three different elements; Direct repeat sequences; non-repetitive sequences (spacer); leader sequence which is absent at one end of the repeats. CRISPR components are shown in fig. 2. The upstream region of CRISPR is AT-rich leader sequence. The promoter to transcribe CRISPR is present in this leader sequence. Processed primary transcript produces crRNA with single spacer and flanking sequences derived from repeats. CAS acts as a regulatory element with the CRISPR region (Jansen *et al,* 2002). There are 45 documented Cas genes constituents of two classes grouped into the *Cas1- Cas6* core

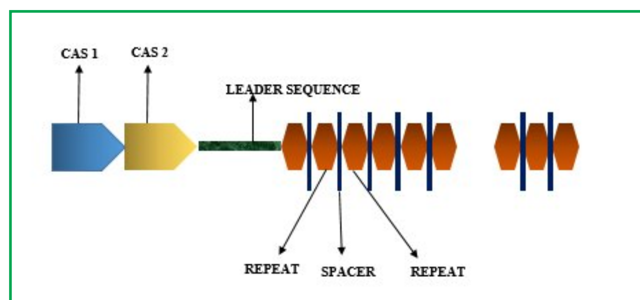

Fig. 2: Components of CRISPR showing the leader sequence, spacer and the repeat sequence

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family (Haft *et al,* 2005). *Cas1*, metal dependant DNA-specific endonuclease and *cas2*, an essential component for spacer acquisition are universal and form complex with all CRISPR-associated proteins (Karimi *et al,* 2018). The molecular mechanism of *cas* genes includes type I, according to *Cas3* existence; type II for the existence of *cas9* gene; and type III for *cas10* existence (Makarova *et al,* 2011). The main functional domain of CAS protein is RNA Recognition Motif (RRM) (Karimi *et al,* 2018).

Mechanism of action

The CRISPR bacterial immune system consists of a three-stage protection mechanism: adaptation, crRNA processing and interference (Newsom *et al,* 2021). The mechanism of CRISPR action against the foreign DNA and destruction of invading DNA by CAS protein is shown in fig. 3. Adaptation adds repeats from foreign DNA into the CRISPR region. Upon invasion of foreign DNA into the bacterial cell, a repeat region is identified and inserted between the spacer and the first repeat of the CRISPR region (Abdallah *et al,* 2015). In crRNA processing, the CRISPR array is transcribed into pre-crRNA. The associated *cas1* and *cas2* along with short crRNAs of 39 to 45 nucleotides crRNA is having helicase and nuclease activity to degrade the invading DNA (Haft *et al,* 2005). The interference stage involves ternary of Cas-crRNA-tracrRNA at the side of the 3' end of a duplex structure which binds with CAS protein and crRNA to recognize the protospacer sequence when the CAS mediated DNA interference is carried out by tracrRNA and CAS protein will cut the foreign DNA (*Abdallah et al,* 2015).

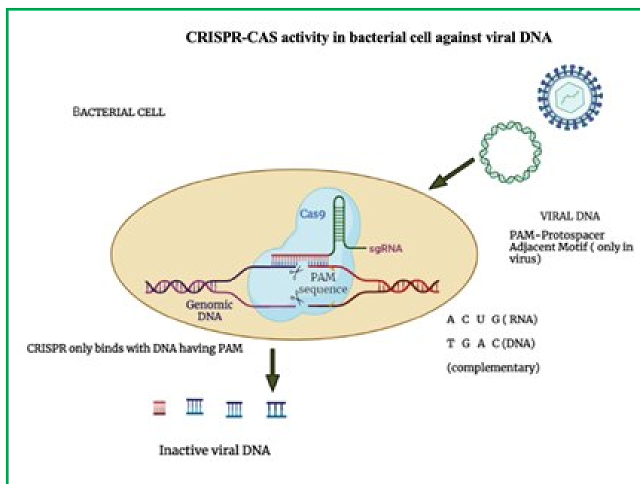

Fig. 3: CRISPR-CAS activity in bacterial cells against viral DNA

The Bacterial RNA-mediated immune system (CRISPR-CAS) will undergo adaptation, crRNA processing, and interference mechanism then finally, CAS protein will cleave the foreign DNA. Once the CRISPR-CAS system degrades the foreign DNA, it will become inactive, thereby bacterial cells are protecting themselves from the viral attacks.

However, the CRISPR/CAS system must differentiate the foreign and self DNA to avoid the self-targeting and autoimmune effect of the existence of PAM sequence in invading foreign DNA for type I, II, and V systems and the presence of PFS (protospacer flanking sequence) in type VI (Gleditzsch *et al,* 2019). The CRISPR system is having a high sequence specificity because of its RNA-DNA binding nature compared to MegN, ZFNs, and TALENs which are dependent on protein–DNA binding action. Cleavage efficiency is high in CRISPR/Cas compared to ZFNs, and TALENs and probably there are no more off-target effects in CRISPR (Xu *et al,* 2019). More intriguingly, reconstructed key components of the CRISPR/Cas9 system can introduce DSBs in a site-specific way. The fig. 4 represents the double-strand breaks and two possible mechanisms of homologous end joining and non-homologous end joining mechanism suggesting the potential use of this programmable RNA-guided (sgRNA) CRISPR/Cas9 system for genome editing in organisms other than bacteria (Gasiunas *et al,* 2012). This possibility was soon demonstrated in plants (Nekrasov *et al,* 2013).

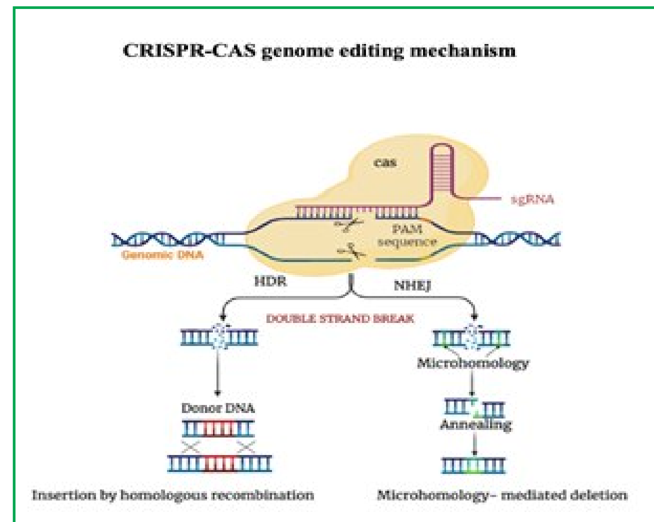

Fig. 4: CRISPR-CAS genome editing mechanism

The presence of Protospacer Adjacent Motif (PAM) will help avoid self-targeting effects, particularly for type I, II and V systems. After the DSB (Double Strand Break) subsequently NHEJ (Non-Homologous End Joining) or HDR (Homologous Directed Repair) can be performed for desired traits.

Genome editing system

There are four families of engineered nucleases familiarly being used in genome editing: Engineered Meganuclease (MegN), Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and the Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated protein 9 (CRISPR/Cas9) nuclease systems. MegN is the genetic engineering tool used for DBS-induced genome manipulation that is found naturally in Yeast (Epinat *et al,* 2003). In these enzymes, the binding site and restriction site occur within the same unit hence difficult to modify and comparatively less toxic than the other technologies (Gao *et al,* 2010). It is capable of recognizing 12 to 40 bp and cutting the DNA double-strand in a site-specific manner (Smith *et al,* 2006). Because of the limitation of the recognizable sites MegNs have not been used widely. ZFNs or TALENs are generated by fusing the DNA cleavage domain of the endonuclease Fok I with zinc fingers (ZFs) or with transcriptional activator-like effectors (TALEs) (Xu *et al,* 2019).

ZFNs were first discovered from the (TFIIIA) transcription factor of *Xenopus laevis (African* clawed toad) (Klug, 2010). ZFNs typically exhibit an array of 3 or 4-finger domains, which can recognize 18–24 bp sequences (Kamburova *et al,* 2017). ZFNs consist of a binding domain and cleaving domain (two half-fok I – nonspecific type II endonuclease) which can cleave at dimerization (Kim *et al,* 1996). The repairing mechanism of ZFNs-induced double-strand breaks is Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) (Jia *et al,* 2014). ZFNs are efficiently used in several organisms including plants; high-frequency modification of tobacco gene (Townsend *et al,* 2009), and Targeted inactivation of an endogenous gene in Arabidopsis (Zhang *et al,* 2010).

TALENs (Transcription Activator-Like) Effectors are proteins secreted by *Xanthomonas* sp. bacteria via their type III secretion system when they infect various plant species, unlike ZFNs, TALENs contain single nucleotides instead of 3 or 4 domains (Joung and Sander, 2013). In TALENs host specificity and DNA binding actions are carried out by the central repeat domain which consists of 34 amino acid repeats that bind to one nucleotide in the target nucleotide sequence. An amino acid repeat of positions 12 and 13 are highly variable (Repeat Variable Diresidues) in the central repeat domain, these RVD are responsible for the recognition of specific nucleotides in the target nucleotide sequence (Kamburova *et al,* 2017). These three domains and their role in the recognition of specific nucleotides are shown in fig. 5. TALEN binds to a single nucleotide that it makes easier to construct TALENs as compared to ZFN, the main advantages of TALENs are DNA binding specificity is higher, off-target effects are lower, and construction of DNA-binding domains is easier (Kamburova *et al,* 2017). The activity of ZFNs and TALENs are compared in fig. 6.

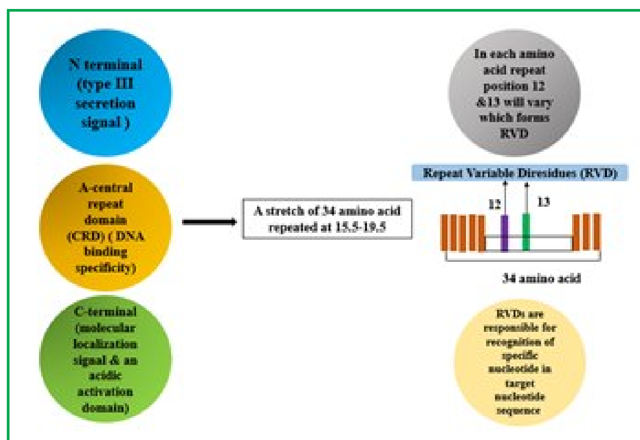

Fig. 5: TALENs are organized into 3 domains; N-terminal, A-central, C-terminal

Only the 12 and 13 position of amino acid stretch varies (Repeat Variable Diresidues) which are playing the main role in the recognition of specific nucleotides in the targeted sequence.

Genome editing for Horticultural crop improvement

Horticultural crops contribute significantly to a country's economy by increasing rural income. Genome editing of

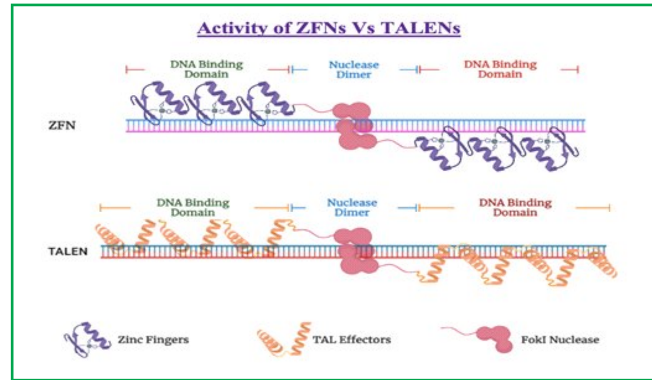

Fig. 6: TALENs consist of a single nucleotide which helps for higher specificity in targeted sequences compared to zinc finger nuclease containing 3 or 4 domains for binding.

horticultural crops would be highly beneficial as they are the key exportable goods in many countries. The first evidence of genome editing in a horticulture crop was performed with TALENs in *Brassica oleracea* by targeting the FRIGIDA gene (Sun *et al,* 2013). TALENs to knock out the *VInv* gene (which is responsible for encoding a protein involved in the glucose and fructose breakdown from sucrose) within the commercial potato variety Ranger Russet, to prevent reducing sugar accumulation in cold temperatures, hence improving cold storage (Clasen *et al,* 2016). SbRFP1 may act as a negative regulator of BAM1 and StvacINV1 in the potato Cold-induced sweetening process, slowing down the buildup of reducing sugars (Zhang *et al,* 2013). The genes (*INVINH1* and *SlVPE5*) which prevent the soluble sugar accumulation in tomatoes were knocked out (Wang *et al,* 2021) using CRISPR/Cas9 technology which results in enhanced tomato sweetness and can be used for tomato sauce production. SLORRM4 mutants were created using CRISPR/Cas9 gene editing technology which facilitates delay in fruit ripening (Yang *et al,* 2017). Early flowering in annual herbaceous species and perennial woody plants is induced by overexpression of an apple *FT* (FLOWERING LOCUS T) gene (Tränkner *et al,* 2010).

The LCY (lycopene epsilon-cyclase) gene was edited using a CRISPR/Cas9-based technique to generate the β-carotene-rich Cavendish banana cultivar (cv.) Grand Naine (Kaur *et al,* 2020). The weak lodging and significant damage caused by typhoons and storms are two problems that commercial banana types face. To address this issue, CRISPR/Cas9 was employed to alter the *MaGA20ox2* gene and semi-dwarf banana crops were produced (Shao *et al,* 2020). CRISPR/Cas9 technology using *Agrobacterium tumefaciens* mediated Genome Editing Increases Lycopene content about 5.1 fold in Tomato Fruit (Li *et al,* 2018).

CONCLUSION

In recent years, crop breeding for trait improvement is switching from conventional breeding to genome engineering for its precise editing and effectiveness. Apart from this precise DNA editing, scientists are using this site-specific binding enzyme as a programmable precise tool for error-prone and highly efficient editing. Each of the key nucleases

that are used to cut and edit the genome has benefits and drawbacks, and the best gene-editing technique depends on the circumstances. Besides the advances in editing a plant's blueprint, the concern in decisions making and regulatory issues draw wide attention. However, after assessment and arguments, the Indian Ministry of Environment, Forest and Climate Change has given exemptions to SDN1 and SDN2 edited plants from being considered as transgenic plants. We must think about the greatest applications for the technology, and the modifications required to make it safe and successful.

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