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PRINCIPLES AND APPROACHES OF GENOME-EDITING IN PLANT

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ABSTRACT
Crop improvement as an innovation in plant breeding and genetics requires the deployment of new allelic variants. To achieve this, different types of genome modifications are recently in used, such as ZFN, TALEN, MN, and CRISPR/Cas genome editing systems. However, off-target mutations are the major concerned associated with the use of ZFNs for genome editing. As such, the creation of obligate heterodimeric ZFN architectures that rely on a charge-charge repulsion to prevent unwanted homodimerization of the FokI cleavage domain has been in used to enhance ZFNs specificity. TALENs offer distinct advantages for genome editing compared to ZFNs; it has higher specificity and reduced toxicity compared to some ZFNs and no selection or directed evolution is necessary to engineer TALE arrays. Compared with ZFNs and TALENs, the CRISPR/Cas system is characterized by its simplicity, efficiency, and low cost, and by its ability to target multiple genes. Due to these characteristic features, CRISPR/Cas9 has been rapidly exploited in plants and may be an effective solution to a variety of problems in plant breeding. Conclusively, the CRISPR/Cas9 system provides a valuable platform for generating mutants with high frequency in polyploid crops and very useful for post-transcriptional control of gene expression as well as the simultaneous editing of multiple target sites.

***Keywords*:** Interspaced short palindromic repeats (CRISPR); Transcription activator-like effector nucleases (TALENs); Zinc-finger nucleases (ZFNs); Sequence-specific nucleases (SSNs); Double-stranded breaks (DSBs).

INTRODUCTION

Conventional breeding is currently the most widely used approach in crop improvement, however, it is labor-intensive and takes several years to achieve the selection process (Zhang *et al.,* 2018). Moreover, genetically modified (GM) crops that have beneficial traits are produced but, their use is affected by largely unsubstantiated health and environmental safety concerns. As such, the advantages of GM traits have been restricted to a small number of cultivated crops. Moreover, a traditional breeding program that is conducted by mutagenesis using chemical compounds or irradiation, followed by screening for desired mutations, has several drawbacks (Mohanta *et al*., 2017). Methods using mutagenesis, intergeneric crosses, and translocation breeding are non-specific; and sometimes large parts of the genome are transferred instead of a single gene, or sometimes thousands of nucleotides are mutated instead of a single nucleotide. In the post-genomic era and, the availability of genome sequence data for multiple crop plants has revolutionized plant breeding programs. Whole-genome sequencing, transcriptome sequencing, identification of small nucleotide polymorphisms (SNPs) and other molecular markers have made it possible to create comprehensive genetic and linkage maps to determine potential quantitative trait loci (QTLs) of agronomic importance (Mohanta *et al*., 2017). Genomics is rapidly gaining importance in molecular breeding programs (Reference). Combinations of genomic tools with conventional breeding techniques have opened new doors in genome-based breeding programs. The desired agronomic traits need to be incorporated into the appropriate crop plants to maximize benefits. Therefore, "genome editing tools" is required to carry out the task of incorporating desired traits into crop genomes. It usually acts with precision, accuracy, and predictability, and do away with the messiness of inaccuracy (MacDonald & Deans, 2016). Nowadays, Several RNA, DNA, and protein-based tools have been developed to edit and incorporate suitable agronomic traits into the desired crops. Genome editing is defined as a collection of advanced molecular biology techniques that facilitate precise, efficient, and targeted modifications at genomic loci (Ji *et al*., 2015). Recently, the most commonly used to facilitate genome editing, shown in Figure (2a-d), are (1) clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9), (2) transcription activator-like effector nucleases (TALENs), (3) zinc-finger nucleases (ZFNs), and (4) homing endonucleases or meganucleases. The (CRISPR)-CRISPR-associated protein 9 (Cas9) provides simplicity and ease of targeted gene editing. All of these technologies use typical sequence-specific nucleases (SSNs) that can be induced to recognize specific DNA sequences and to generate double-stranded breaks (DSBs) (Fig. 1). The plant's endogenous repair systems fix the DSBs either by nonhomologous end joining (NHEJ), which can lead to the insertion or deletion of nucleotides thereby causing gene knockouts or by homologous recombination (HR), which can cause gene replacements and insertions (Fig.1)(Symington & Gautier, 2011). These DNA breaks then drive the activation of cellular DNA repair pathways and facilitate the introduction of site-specific genomic modifications (Ainley et al., 2013). This process is most often used to achieve gene knockout via random

base insertions and/or deletions that can be introduced by nonhomologous end joining (NHEJ) (Bibikova et al., 2001). Alternatively, in the presence of a donor template with homology to the targeted chromosomal site, gene integration, or base correction via homology-directed repair (HDR) can occur (HDR) (Urnov et al., 2005).

The risks involved in altering genomes through the use of genome-editing technology are significantly lower than those associated with GM crops because most edits alter only a few nucleotides, producing changes that are not unlike those found throughout naturally occurring populations (Voytas & Gao, 2014). Once the genomic-editing agents have segregated out, there is no way to distinguish between a 'naturally occurring' mutation and a gene edit. Thus, the introduction of genome editing into modern breeding programs should facilitate rapid and precise crop improvement. Here, the work reviewed key approaches, principles and some applications of genome editing in the plant to achieve advances throughout the Life Sciences for the betterment of mankind.

GENOME-EDITING METHODS

Zinc finger nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are targetable DNA cleavage proteins that are designed to cut DNA sequences at specific sites (Carroll, 2011). The targeted gene editing is facilitated through the DSBs in DNA for it to replace the gene by homologous recombination (Figure 2b). Each ZFN contains a DNA-cleaving domain consisting of a FokI nuclease domain and a DNA binding domain with a chain of two-finger modules, which recognizes a unique 6-bp hexamer in the DNA sequence (Gupta *et al*., 2012). The main function of ZFNs as dimers, with each monomer recognizing a specific "half-site" sequence—typically 9 to 18 base pairs (bps) of DNA—through the zinc-finger DNA-binding domain. This dimerization is mediated by the FokI cleavage domain, which cuts DNA within a five- to seven-bp spacer sequence that separates two flanking zinc-finger binding sites (Smith *et al*., 2000). Each ZFN is typically composed of three or four zinc-finger domains, with each individual domain composed of approximately 30 amino acid residues that are organized in a bba motif (Pavletich & Pabo, 1991). The residues that facilitate DNA recognition are located within the a-helical domain and typically interact with three bps of DNA, with occasional overlap from an adjacent domain (Wolfe *et al.,* 2000). However, off-target mutations are the major concerned associated with the use of ZFNs for genome editing (Pattanayak *et al.,* 2011). As such, creation of obligate heterodimeric ZFN architectures that rely on a charge-charge repulsion to prevent unwanted homodimerization of the FokI cleavage domain (Doyon *et al*., 2011) has been used as among approaches undertaken to enhance ZFNs specificity. Thereby minimizing the potential for ZFNs to dimerize at off-target sites. Additionally, protein- engineering methods have been used to enhance the cleavage efficiency of the FokI cleavage domain (Guo *et al.,* 2010). One particularly promising approach for improving ZFN specificity is to deliver them into cells as protein. Because of the intrinsic cell-penetrating activity of zinc-finger domains (Gaj *et al.,* 2014), ZFN proteins themselves are inherently cell-permeable and can facilitate gene editing with fewer off-target effects when applied directly onto cells as purified protein compared to when expressed within cells from nucleic acids (Gaj *et al.,* 2012). Modified ZFN proteins endowed with improved cell-penetrating activity have since been described (Liu *et al.,* 2015). In the absence of a DBS as reported by Wang *et al*. (2012) ZFNickases can facilitate gene correction. These enzymes, which consist of one catalytically inactivated ZFN monomer in combination with a second native ZFN monomer, can stimulate HDR by nicking or cleaving one strand of DNA and are derived from a concept first illustrated by Stoddard and colleagues using homing endonucleases (Smith *et al*., 2009). Rapid and randomly disrupt or integrate any genomic loci in the genome can be achieved using ZFNs. Mutations that are made through ZFNs are permanent and can be heritable. The selection of a ZNF strategy can be conducted by a bacterial two-hybrid system that uses ZFN-DNA interactions to activate the HS3 gene. A bacterial one-hybrid system can also be used to select ZFPs and to analyze sequence specificities in vivo (Durai *et al.,* 2006) Hitherto, maize, rapeseed, rice, soybean, Arabidopsis and apples among others are modified using ZFNs (Martínez-Fortún *et al.,* 2017; Ran *et al.,* 2017). However, ZFNs lack the targeting flexibility inherent to more recent genome- editing platforms, due to the difficulty associated with constructing zinc-finger arrays unlike that of TALENs and CRISPR-Cas9. Therefore it remains challenging to create zinc-finger domains that can effectively recognize all DNA triplets, especially those of the 5|-CNN-3| and 5|-TNN-3| variety.

TALE Nucleases

TALENs comprised of an amino-terminal TALE DNA-binding domain fused to a carboxy-terminal FokI cleavage domain (Miller *et al*., 2011). Just, like ZFNs, dimerization of TALEN proteins is mediated by the FokI cleavage domain, which cuts within a 12- to 19-bp spacer sequence that separates each TALE binding site (Fig. 2c) (Miller *et al*., 2011). They are assembled and recognized between 12- to 20-bps of DNA, with more bases typically leading to higher genome-editing specificity (Guilinger *et al*., 2014a). The TALE binding domain consists of a series of repeat domains, each approximately 34 residues in length. Each repeat contacts DNA via the amino acid residues at positions 12 and 13, known as the repeat variable diresidues (RVDs) (Moscou & Bogdanove, 2009). Each TALE repeat recognizes only a single bp, with little to no target site overlap from adjacent domains (Mak *et al.,* 2012) this differs from that of ZFNs which recognize DNA triplets. The most commonly used RVDs for assembling synthetic TALE arrays are NG for thymine, NN or HN for guanine or adenine, NI for adenine and HD for cytosine (Streubel *et al., 2012*). The very suitable and straightforward approach used in the construction of TALE

DNA-binding domains is Golden Gate assembly (Cermak *et al*., 2011). Moreover, high-throughput TALE assembly methods have also been developed, such as; iterative capped assembly (Briggs *et al*., 2012), FLASH assembly (Reyon *et al*., 2012), and ligation independent cloning (Schmid-Burgk *et al.,* 2013), among others. Recently, advances in TALEN assembly were reached in initiating those methods that will enhanced their performance, such as; directed evolution as means to refine TALE specificity (Hubbard *et al*., 2015), specificity profiling to uncover nonconventional RVDs that improve TALEN activity (Miller *et al*., 2011), and even fusing TALE domains to homing endonuclease variants to generate chimeric nucleases with extended targeting specificity (Boissel *et al*., 2013).

TALENs uses DSBs in a manner similar to ZFNs. TALENs are similar to ZFNs that contain non-specific FokI endonucleases. However, the FokI domains of ZFNs fuse with specific DNA-binding domains of highly conserved repeats derived from transcription activator-like effectors (TALEs) (Joung & Sander, 2013). TALE proteins are found in Xanthomonas bacteria, which secrete TALEs to alter gene transcription in host plants (Boch & Bonas, 2010). The DNA-binding domains of TALEs contain up to 30 copies of 33–34 amino acid sequences that are highly conserved, except for 12th and 13th positions. The 12th and 13th positions are called the repeat-variable diresidue (RVD) and exhibit substantial correlation with specific nucleotide recognition. Each repeat can recognize a single base, and hence, new binding sites can be assembled for any DNA sequence. The FokI domain functions as a dimer, where the non-specific DNA cleavage domain of the FokI endonuclease can be used to design a hybrid nuclease (Wah *et al.*, 1998). The number of amino acid residues between the DNA-binding domain and the FokI cleavage domain and the number of bases between two separate TALEN-binding sites is important parameters that are affecting the activities of TALENs. The recognition of amino acids and DNA-binding TALE domains requires effective engineering of proteins. Upon the construction of TALENs, they are transferred to the plasmid vector and are then transformed into the target cells. Later, the gene product is expressed and enters the nucleus, where it carries out the necessary editing of the genome. Alternatively, the TALEN construct can be transformed into cells as mRNA, which eliminates the possibility of genomic integration of the TALEN. The mRNA-based approach increases the possibilities of homology-based repair (HDR) and leads to successful gene editing. TALEN technology has been successfully used in *Oryza sativa* (Li *et al*., 2012). The promoter region of the bacterial blight susceptible gene Os11N3 was targeted using TALEN technology, which led to the generation of disease-resistant rice (Li *et al*., 2012). Upon the construction of TALENs, they are transferred to the plasmid vector and are then transformed into the target cells. Later, the gene product is expressed and enters the nucleus, where it carries out the necessary editing of the genome. Alternatively, the TALEN construct can be transformed into cells as mRNA, which eliminates the possibility of genomic integration of the TALEN. The mRNA-based approach increases the possibilities of homology-based repair (HDR) and leads to successful gene editing. TALENs offer two distinct advantages for genome editing compared to ZFNs. First, TALENs have higher specificity and reduced toxicity compared to some ZFNs (Mussolino *et al*., 2014) due to their increased affinity for the target DNA (Meckler *et al.,* 2013). Second, no selection or directed evolution is necessary to engineer TALE arrays, this will definitely reduce the amount of time and experience needed to assemble a functional nuclease. A comparative study between ZFNs and TALENs to target particular genes showed the highest cleavage rates for the TALENs. This result was due to the ease of design and high cleavage activities of TALENs and the limitless range of targets that can be acted upon by TALENs (Joung & Sander, 2013). TALEN technology has been efficiently used to generate knockout mutants of Arabidopsis thaliana (Cermak *et al.*, 2011). Although TALEN technology is very efficient and is superior to ZFNs, the construction of engineered TALE repeats is challenging because it requires multiple identical repeat sequences (Joung & Sander, 2013). Although, methods are now in used to overcome this limitation as TALENs can be readily delivered into cells as mRNA (Mock *et al.*, 2015) and even protein (Liu *et al.*, 2014), although alternative codon usage and amino acid degeneracy can also be leveraged to express RVD arrays that might be less susceptible to recombination (Kim *et al*., 2013). In addition, adenoviral vectors have also proven particularly useful for mediating TALEN delivery to hard-to-transfect cell types (Maggio *et al*., 2016). Genome editing by TALENs has been demonstrated in a wide variety of plants including tomato, soybean, Arabidopsis, barley, potato, sugarcane, flax, rapeseed, rice, maize, and wheat (Martínez-Fortún *et al*., 2017; Ran *et al*., 2017).

CRISPR-Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas is a family of DNA sequences that are commonly found in bacteria (Figure 2c). It contains fragments of DNA from viruses that have attacked the bacterium. These DNA fragments are used by the bacterium to recognize and destroy DNA from further attacks, and thereby protect themselves. CRISPR/Cas acts as a typical bacterial immune system that provides the bacteria with resistance to foreign genetic material. The type-II system provides protection against DNA from invading viruses and plasmids via RNA-guided DNA cleavage by Cas proteins (Sorek *et al.*, 2013). Short segments of foreign DNA are integrated within the CRISPR locus and transcribed into CRISPR RNA (crRNA), which then anneal to trans-activating crRNA (tracrRNA) to direct sequence-specific degradation of pathogenic DNA by the Cas9 protein (Jinek *et al*., 2012). The CRISPR system comprises CRISPR RNA (crRNA), trans-activating CRISPR RNA (tracrRNA), the Cas9 nuclease, and the protospacer adjacent motif (PAM) (Figure 5). Naturally, occurring CRISPR systems integrate the foreign DNA sequence into the CRISPR cluster (Sander & Joung, 2014).

Then, the CRISPR cluster that is harboring the foreign DNA produces crRNA (approximately 40nt long) containing the PAM region, which is complementary to the foreign DNA site. The crRNA hybridizes with the tracrRNA to form a guide RNA (gRNA). The gRNA activates the Cas9 system and binds to Cas9. Twenty nucleotides at the 5| end of the gRNA direct the Cas9 nuclease to the complementary base pair with the targeted DNA, leading to RNA-DNA complementary base-pairing (Sander & Joung, 2014). The prerequisite for cleavage is the presence of a PAM motif downstream of the target DNA; the PAM motif usually contains 5|-NGG-3| or 5|-NAG-3| (Hsu *et al*., 2013). Specificity is provided by the "seed sequence", which is present approximately 12 nucleotides upstream of the PAM motif and which should match between the RNA and target DNA (Bortesi & Fischer, 2015). Using this procedure, Cas9 nuclease activity can be directed to any DNA sequence (Sander & Joung, 2014). The Cas9 system induces DSBs, which are subsequently ligated by NHEJ or HDR. Some Cas9 variants cleave only at one site (nickase) of either the complementary or the non-complementary strands of the target DNA. The Cas9 nickase induces HDR with reduced levels of NHEJ indels (Mali *et al*., 2013). By using one Cas9 nuclease and multiple gRNA, more than one site can be targeted and altered simultaneously (Liu *et al*., 2014). This process is very useful when one gRNA is inefficient at disrupting a targeted gene or when altering more than one gene at the same time. Several studies have now shed light on the structural basis of DNA recognition by Cas9, revealing that the heteroduplex formed by the gRNA and its complementary strand of DNA is housed in a positively charged groove between the two nuclease domains (RuvC and HNH) within the Cas9 protein (Nishimasu *et al*., 2014), and that PAM recognition is mediated by an arginine-rich motif present in Cas9 (Anders *et al.,* 2014). Doudna and colleagues have since proposed that DNA strand displacement induces a structural rearrangement within the Cas9 protein that directs the nontarget DNA strand into the RuvC active site, which then positions the HNH domain near target DNA (Jiang *et al*., 2016), enabling Cas9-mediated cleavage of both DNA strands. The Cas9 nuclease and its gRNA can be delivered into cells for genome editing on the same or separate plasmids, and numerous resources have been developed to facilitate target site selection and gRNA construction, including E-CRISP (Heigwer *et al.,* 2014), among others. One of the major criticisms regarding the usefulness and specificity of the CRISPR/Cas9 technology is the relatively high frequencies of off-target mutations (Bortesi & Fischer, 2015). However, off-target mutations are rare in plants. Only 1.6% of off-target effects were predicted in rice (Xie & Yang, 2013). Though, considerable effort has been devoted to improving the specificity of this system, including using paired Cas9 (Ran *et al.,* 2013), which increase gene-editing specificity by requiring the induction of two sequential and adjacent nicking events for DSB formation, or truncated gRNA that are more sensitive to mismatches at the genomic target site than a full-length gRNA (Fu *et al.,* 2014). The mismatch was confined to position 11, which is present upstream of the PAM motif. It was considered that the 20nt gRNA sequence determines the specificity; however, it later found that only 8–12nt at the 3| ends (the seed sequence) is required for recognition of target sites (Jiang *et al.,* 2013), and multiple mismatches towards the PAM motif can be tolerated, depending upon the arrangement of the PAM motif (Fu *et al.,* 2013). DNA sequences that contain a missing base (Grna bulge) or an extra-base (DNA bulge) at various positions in the corresponding gRNA sequences induce off-target cleavage (Lin *et al*., 2014). Appropriate Grna design can greatly facilitate the reduction of off-target editing of the genome. Due to the Watson-Crick base-pairing of CRISPR/Cas9 with its target sequence, off-target sites can be easily predicted by using sequence analysis (Cho *et al.,* 2014). The CRISPR/Cas9 system can be reprogrammed to test off-target effects rapidly and in a cost-effective manner. Off-target cleavage has also been reduced by controlling the dosage of either the Cas9 protein or Grna within the cell (Hsu *et al*., 2013), or even by using Cas9 variants configured to enable conditional genome editing, such as a rapamycin-inducible split-Cas9 architecture (Zetsche *et al.,* 2015) or a Cas9 variant that contains a strategically placed small-molecule-responsive intein domain (Davis *et al.,* 2015). Nucleofection (Kim *et al*., 2014) or transient transfection (Zuris *et al*., 2015) of a preformed Cas9 ribonucleoprotein complex has also been shown to reduce off-target effects, enabling DNA-free gene editing in primary human T cells (Schumann *et al*., 2015), embryonic stem cells (Liu *et al.,* 2015). The incorporation of specific chemical modifications known to protect RNA from nuclease degradation and stabilize secondary structure can further enhance Cas9 ribonucleoprotein activity (Rahdar *et al*., 2015). Compared with ZFNs and TALENs, the CRISPR/Cas system is characterized by its simplicity, efficiency, and low cost, and by its ability to target multiple genes (Cong *et al*., 2013). Because of these characteristic features, CRISPR/Cas9 has been rapidly exploited in plants (Shan *et al*., 2013) and maybe an effective solution to a variety of problems in plant breeding (Zhang *et al*., 2018). In addition to genome editing, the CRISPR/Cas9 system can be used for the ectopic regulation of gene expression. Through the regulation of gene expression, we can understand the function of a gene, and can also engineer novel genetic regulatory circuits for synthetic biology (Bortesi & Fischer, 2015). The regulation of gene expression is mediated by inducible or repressible promoters, and disabled nucleases can be used to regulate gene expression (Bortesi & Fischer, 2015). To date, many crops such as soybean, sorghum, rice, maize, wheat, barley, potato, tomato, rapeseed, cotton, cucumber, lettuce, grapes, grapefruit, apple, oranges, and watermelon among others have been edited by this technique.

Meganucleases (MN)

Meganucleases are the proteins in the LAGLIDADG family, for conserved amino acid sequence. They are characterized by their capacity to recognize and cut large DNA sequences (from 14 to

40) base pair's enzymes. They are found commonly in microbial species (Fig. 2a). These enzymes make extensive sequence-specific contacts with their DNA substrate (Stoddard, 2011), and thus typically show exquisite specificity. However, unlike ZFNs and TALENs, the binding and cleavage domains in homing endonucleases are not modular. This overlap in form and function make their repurposing challenging and limits their utility for more routine applications of genome editing. However, have the benefit of causing less toxicity in cells than methods such as Zinc finger nuclease (ZFN), likely because of more stringent DNA sequence recognition, however, the construction of sequence-specific enzymes for all possible sequences is costly and time-consuming, as one is not benefiting from combinatorial possibilities that methods such as ZFNs and TALEN-based fusions utilize. More recently megaTALs—fusions of a rare-cleaving homing endonuclease to a TALE-binding domain—have been reported to induce highly specific gene modifications (Lin *et al*., 2014). These enzymes have enabled integration of antitumor and anti-HIV factors into the human CCR5 gene in both primary T cells and hematopoietic stem/progenitor cells (Sather *et al*., 2015), as well as disruption of endogenous T-cell receptor elements in T cells (Osborn *et al*., 2016), indicating their potential for enabling and enhancing immunotherapies.

APPLICATIONS

Gene knockouts are the must predominant application used in CRISPR/Cas9, usually achieved by the introduction of small indels that result in frame-shift mutations or by introducing premature stop codons (Fig. 2d). The unique ability of the CRISPR/Cas9 system to selectively bind to specific DNA sites has helped to regulate gene activity (Lowder *et al*., 2016). For this purpose, proteins activating or repressing the activity of promoters that control the gene function can be attached to the catalytically inactive mutant Cas9 protein. A tiller-spreading phenotype as an example was generated by knocked out LAZY1 gene in rice using CRISPR/Cas9 which is a higher-yielding variety (Miao *et al*., 2013). Additionally, it was shown that complex binding to the target DNA can inhibit or stimulate the function of the target gene (Lowder *et al*., 2016). Furthermore, using the CRISPR/Cas9 system, several genetic constructs targeted to different genome sites can simultaneously be introduced into cells (Wang *et al*., 2013). CRISPR/Cas9 system was used by Li *et al*. (2016) to mutate the Gn1a, DEP1, and GS3 genes of the rice cultivar Zhonghua11, producing mutants with enhanced grain number, dense erect panicles, and larger grain size, respectively. Grain Weight 2 (GW2) gene was also disrupted to increases the grain weight and protein content in wheat (Zhang *et al*., 2018). genome editing system in plant breeding has been used (1) for gene pyramiding and knockout, (2) to make small modifications to gene function (Mao *et al*., 2013), (3) to insert point mutations similar to natural SNPs (Xu *et al*., 2017), (4) for integration of foreign genes (5) for the repression or activation of gene expression, as well as (6) in epigenetic editing (Kumar & Jain, 2014). For example, the use of ZFN in *Arabidopsis thaliana* (Osakabe *et al.*, 2010) and *Zea mays* (Shukla *et al.*, 2009) has led to the successful development of Herbicide-tolerant genotypes was successfully developed through insertion of herbicide resistance genes into targeted sites in the genome (Shukla *et al*., 2009) using ZFN in Arabidopsis (Townsend *et al*., 2009) and Zea mays (Shukla *et al*., 2009), an example ZmIPK1 gene was disrupted by insertion of PAT gene cassettes, and this resulted in herbicide tolerance and alteration of the inositol phosphate profile of developing maize seeds (Shukla *et al*., 2009). ZFN was also used for the targeted modification of endogenous malate dehydrogenase (MDH) gene in plants and the plants containing modified MDH have shown increased yield (Shukla *et al*., 2009). Moreover, Trait stacking in maize was also successfully achieved using ZFN-mediated targeted transgene (Ainley *et al*., 2013). Additionally, a major area of application of genome editing approaches in plant breeding is to create varieties resistant to various pathogens and/or pests. This is achieved by modifying: genes regulating the interaction between the effector and target, resistance genes (R-genes), susceptibility genes (S-genes), the genes regulating plant hormonal balance and susceptibility genes (S-genes) (Andolfo *et al.*, 2016). For example in rice, the bacterial blight susceptibility gene OsSWEET14 was disrupted and the resulting mutant rice was found to be resistant to bacterial blight (Li *et al*., 2012). Three TaMLO homoeologs genes were also knockout in wheat using TALENs in order to create powdery mildew-resistant (Wang *et al*., 2014). By knocking out the maize GL2 gene, (Char *et al*., 2015) obtained mutants with the glossy phenotype, with reduced epicuticular wax in the leaves and the potential to be surface manured. In sugarcane, cell wall composition and saccharification efficiency have been improved by TALEN-mediated mutagenesis (Kannan *et al.*, 2018). The CRISPR/Cas9 system has been investigated for its efficacy in providing interference against geminiviruses by using a transient transformation system such that *N. benthamiana* degradation/suppression of curly top virus genome by single-guide RNA/Cas9 (sgRNA/Cas9) has been demonstrated (Ji *et al*., 2015). In other efforts, where sgRNAs specific for tomato yellow leaf curl virus (TYLCV) or bean yellow dwarf virus (BeYDV) sequences were introduced into *N. benthamiana* plants expressing Cas9 endonuclease and challenged with the corresponding viruses, it was demonstrated that the CRISPR/Cas9 system not only targeted viruses for degradation but also introduced mutations at the target sequences (Ali *et al*., 2015) due to interference with the copy number of freely replicating viruses (Andolfo *et al*., 2016). A TALEN-mediated knockout in soybean to improve the shelf life and heat stability of soybean oil (Haun *et al*., 2014), CRISPR/Cas9 technology has been used to target FAD2 to improve oleic acid content while decreasing polyunsaturated fatty acids in the emerging oilseed plant *Camelina sativa* (Jiang *et al*., 2017). In rice, Sun *et al*. (2017) used CRISPR/Cas9 technology to generate targeted mutations in SBEIIb, leading to

a higher proportion of long chains in amylopectin, which improved the fine structure and nutritional properties of the starch (Sun *et al*., 2017).

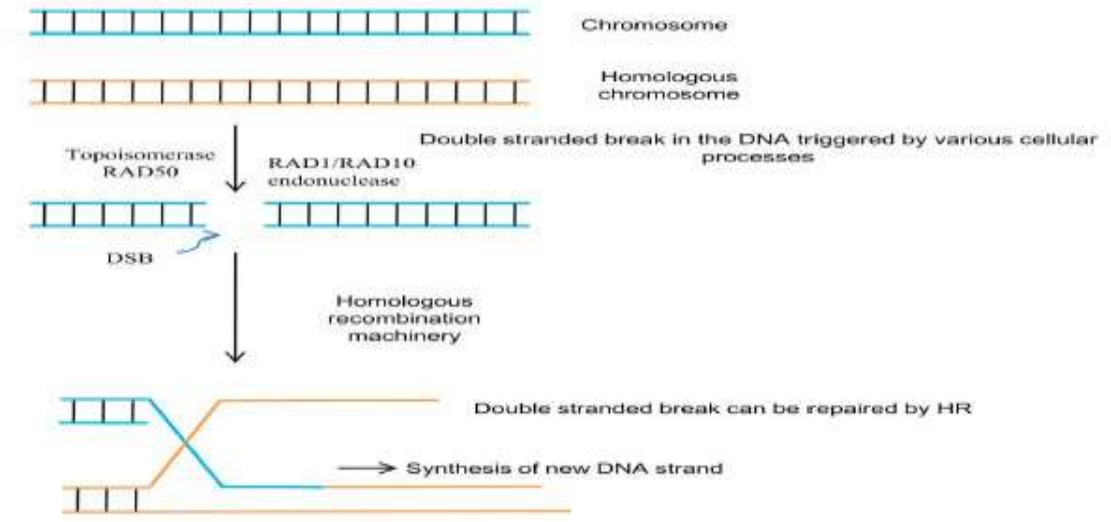
CONCLUSIONS

Crop improvement as an innovation in plant breeding and genetics requires the deployment of new allelic variants. For precise genome editing in plants, ZFNs and DSBs can be used which have a huge impact in functional genomics studies, especially for novel trait discovery in plants. However, precise genome editing at specific sites can be achieved using targeted-mutation-related breeding methods, rather than random mutations, and these methods will reduce the possibility of undesired side effects. As such, the CRISPR/Cas9 system provides a valuable platform for generating mutants with high frequency in polyploid crops.

FUTURE PERSPECTIVES

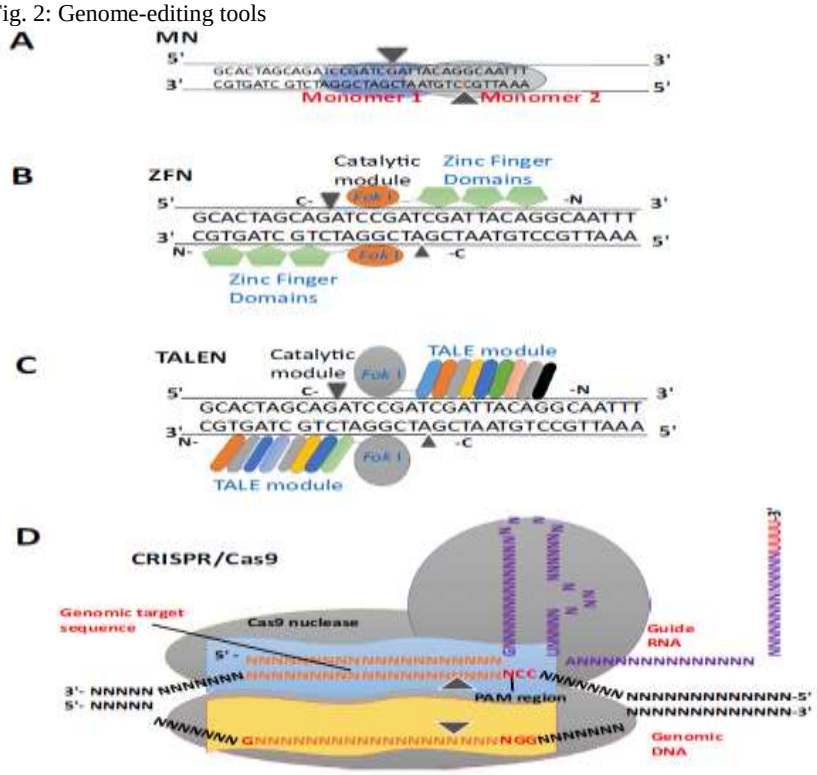
Additionally, genome editing techniques have great potential to facilitate whole-genome functional studies leading to applications in polyploid crops. Generally, these techniques give hope for improving crops to achieve future food security. However, the biggest limitation for target prediction in different genome editing tools are insufficient genetic data set to address the sequence specificities, and a larger effort is necessary to address this problem. Though, the presence of the DNA-binding domain is enough to predict the target to a little extent. The CRISPR/Cas9 system can be very useful for post-transcriptional control of gene expression. Hitherto, the complete genomes of many crops have been sequenced, the challenge of the post-genomic era is to analyze the functions of all crop genes systematically, as most have unknown functions. As such this can be achieved by Gene knockout as an effective strategy for identifying gene functions; henceforth, large-scale mutant libraries at the whole-genome level are needed for functional genomics studies. CRISPR systems have an advantage over other genome-editing methods in multiplexing, the simultaneous editing of multiple target sites. Several groups have assembled multiple sgRNAs into single Cas9/sgRNA expression vectors using Golden Gate cloning or the Gibson Assembly method, in which multiple sgRNAs are driven by separate promoters. Genome editing tools can also be used to regulate some genes, it involves the repression and activation of genes and is often achieved by fusing transcriptional repressors or activators to the DNA-binding domains of genome-editing constructs, thereby targeting the regulatory regions of endogenous genes. CRISPR/Cas9 editing efficiency has varied dramatically, especially in polyploid crops. The presence of paralogs and orthologs with functional redundancy requires the difficult simultaneously knockout of all copies of genes with the same function. However, optimization of Cas9 codon, promoters and target sequence composition (GC content) may directly affect mutagenic efficiency in polyploid crops. Therefore, to knock out homologous genes (paralogs and orthologs) simultaneously, it is necessary to design sgRNA from a conserved region that can target all gene copies. For genetically modified organisms (GMOs) perspective, an efficient A. tumefaciens-mediated approach has been established in many polyploid species such as the one reported by Li *et al*. (2018) and Gao *et al*. (2017) in rapeseed and cotton respectively, using simple and efficient methods of transforming CRISPR/Cas9 reagents into plant genomes. However, introducing foreign DNA fragment is unavoidable and this increases the chance of off-target mutation and genome contamination.

Figure 1. Homologous recombination induced repair of double-stranded breaks (DBS). HR: homologous recombination.



Source: Mohanta *et al*. (2017)

Fig. 2: Genome-editing tools



Source: Zaman *et al.* (2018)

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