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## *EDITOR'S CORNER*

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# Genome editing for crop improvement: Challenges and opportunities

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**ABSTRACT.** Genome or gene editing includes several new techniques to help scientists precisely modify genome sequences. The techniques also enables us to alter the regulation of gene expression patterns in a pre-determined region and facilitates novel insights into the functional genomics of an organism.

Emergence of genome editing has brought considerable excitement especially among agricultural scientists because of its simplicity, precision and power as it offers new opportunities to develop improved crop varieties with clear-cut addition of valuable traits or removal of undesirable traits. Research is underway to improve crop varieties with higher yields, strengthen stress tolerance, disease and pest resistance, decrease input costs, and increase nutritional value.

Genome editing encompasses a wide variety of tools using either a site-specific recombinase (SSR) or a site-specific nuclease (SSN) system. Both systems require recognition of a known sequence. The SSN system generates single or double strand DNA breaks and activates endogenous DNA repair pathways. SSR technology, such as Cre/loxP and FLP/FRT mediated systems, are able to knockdown or knock-in genes in the genome of eukaryotes, depending on the orientation of the specific sites (loxP, FLP, etc.) flanking the target site. There are 4 main classes of SSN developed to cleave genomic sequences, mega-nucleases (homing endonuclease), zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and the CRISPR/Cas nuclease system (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein). The recombinase mediated genome engineering depends on recombinase (sub-) family and target-site and induces high frequencies of homologous recombination.

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Improving crops with gene editing provides a range of options: by altering only a few nucleotides from billions found in the genomes of living cells, altering the full allele or by inserting a new gene in a targeted region of the genome. Due to its precision, gene editing is more precise than either conventional crop breeding methods or standard genetic engineering methods. Thus this technology is a very powerful tool that can be used toward securing the world's food supply. In addition to improving the nutritional value of crops, it is the most effective way to produce crops that can resist pests and thrive in tough climates.

There are 3 types of modifications produced by genome editing; Type I includes altering a few nucleotides, Type II involves replacing an allele with a pre-existing one and Type III allows for the insertion of new gene(s) in predetermined regions in the genome. Because most genome-editing techniques can leave behind traces of DNA alterations evident in a small number of nucleotides, crops created through gene editing could avoid the stringent regulation procedures commonly associated with GM crop development. For this reason many scientists believe plants improved with the more precise gene editing techniques will be more acceptable to the public than transgenic plants. With genome editing comes the promise of new crops being developed more rapidly with a very low risk of off-target effects. It can be performed in any laboratory with any crop, even those that have complex genomes and are not easily bred using conventional methods.

**KEYWORDS.** CRISPR/Cas, MegaN, SSN, SSR, ZFN and TALEN

## INTRODUCTION

Genetic engineering can speed up the development of improved crops and animals. First genetically modified (GM) crops were commercialized in 1996. Since then the cultivated area has increased 100 fold with 28 countries growing these crops (James, 2014). Nearly 2000+ studies have been published evaluating the safety of GM crops, and so far the results suggest that the impact of GM crops on food and environmental safety are not much different from conventionally bred crops. Nevertheless, there is continued skepticism toward this technology (James, 2014).

Generations of GM crops have so far relied on introducing new DNA sequences to the genome in a random location. One stated concern of this approach is that the introduced gene may impact or inactivate the activity of other important nearby genes. Moreover, public unease over GM crops is further intensified when speaking on introduction of 'foreign' genes from distantly related organisms as this is perceived as 'unnatural' despite emerging evidence to the contrary. For example, natural sweet potato varieties are now known to harbor T-genes from *Agrobacterium tumefaciens*

(Verma, 2013; Lucht, 2015). Developing GM crops is expensive and biosafety studies necessary to meet the regulatory requirements substantially add to this cost, estimated to be around \$120 M per trait (Lucht, 2015; Lusser et al., 2012). Regulatory requirements also delay product launches considerably. The regulatory costs, a skeptical public perception and restrictive government policies have limited the use of transgenesis for creating crops with valuable traits to a few commodity crops such as cotton, soybean and corn. Recent enhancements in DNA sequencing technology, along with their decreased cost, facilitate precise studies of plant, animal and human genomes driving an explosion in our knowledge of genomics. The challenge remains, however, to convert the enormous amount of genomic data into functional knowledge and subsequently to determine how genotype influences phenotype. Homologous recombination for targeting gene expression is a powerful method for providing information on gene function (Capecchi, 2005; Gaj et al., 2013). However, the low efficiency, long duration of studies, mutagenic effects and off-target effects have hampered the application of this technique. Although RNAi technology for targeted knocking-down gene expression

proved to be a rapid and inexpensive, compared to homologous recombination, hindering gene expression via RNAi is underutilized (Gaj et al., 2013; McManus and Sharp, 2002). Several methods are now being developed facilitating genomic DNA sequence manipulation, visualization and regulation of gene expression.

Genome editing uses more recent knowledge and technology to enable a specific area of the genome to be modified, thereby increasing the precision of the correction or insertion, preventing cell toxicity and offering perfect reproducibility (Voytas and Gao, 2014; Voytas, 2013). Genome editing or sometimes named genome engineering techniques are currently among the most promising technologies in terms of applied biological research and industrial innovation. Genome editing is a powerful technology that enables precise alterations to DNA, only a few nucleotides among the billions in the genome sequences in living cells. Scientists are using this technique to study the function of the genome and in an attempt to develop crops with pest resistance, enhanced nutritional value, and capable of growing on arid lands (Voytas, 2013; Padmaja, 1995). Genome engineering might prove to be more acceptable to the public than plants genetically engineered with foreign DNA in their genomes. It occurs also as a natural process without artificial genetic engineering. Viruses or subviral RNA-agents are used as vectoral agents to edit genetic sequences (Witzany, 2011).

Reverse genetics has been used for analyzing gene function and annotating DNA sequences. That includes knock-in and knockout using transposon-mediated modification, site-specific recombinase and RNA interference (RNAi) (Townsend et al., 2009; Tierney and Lamour, 2005). However, gene targeting has proved to be the most straightforward among the different forms of genetic modification techniques, for the exploration of gene function *in vivo*. Genetic modification using transposon will affect the level of expression of the induced gene produced by the random insertion positions of genes, while RNAi has temporary knockdown effects, unpredictable off-target influence and too much background noise (Chen et al., 2014; Martin and Caplen, 2007; Dietzl et al., 2007; Gonczy et al., 2000).

Genome editing could be conducted through site-specific recombinase or site-specific nuclease systems. However, There are several key considerations for gene editing; confirmatory genotyping strategies, off-target site analysis, modification expression and contamination (Chen et al., 2014).

### ***Genome Editing Mediated by Site-Specific Recombinase (SSRs)***

Site-specific recombinase (SSR) is a common genetic engineering strategy effecting a permanent modification of the target genome. Many site-specific recombinase systems, such as Cre/loxP and Flp/FRT (Wang et al., 2011; Allen and Weeks, 2005; Allen and Weeks, 2009; Araki et al., 1995), have been identified to perform DNA rearrangements. SSRs are useful tools for manipulation of genomes and for activating or de-activating gene expression in different organisms. The use of SSR for genomic engineering has entered its third decade since the initial discovery that recombinases function in heterologous systems (Wang et al., 2011). The use of site-specific recombination technology in plant genome manipulation has been demonstrated to effectively resolve complex transgene insertions to single copy, remove unwanted DNA, and precisely insert DNA into known genomic target sites. In addition, recombinases have also been demonstrated to be capable of site-specific recombination within non-nuclear targets, such as the plastid genome of tobacco. Wang et al. provided alternative strategies for the combined use of multiple site-specific recombinase systems for genome engineering to precisely insert transgenes into a pre-determined locus, and removal of unwanted selectable marker genes (Wang et al., 2011; Allen and Weeks, 2005; Allen and Weeks, 2009; Araki et al., 1995; Jia et al., 2006).

Recombinases recognize short target DNA sequences of approximately 30–40 nucleotides and catalyze directed DNA exchange reactions through recombination. Recombinases have been used to engineer genomes of higher organisms as the recognition sites for the recombinases are not commonly found in their genomes.

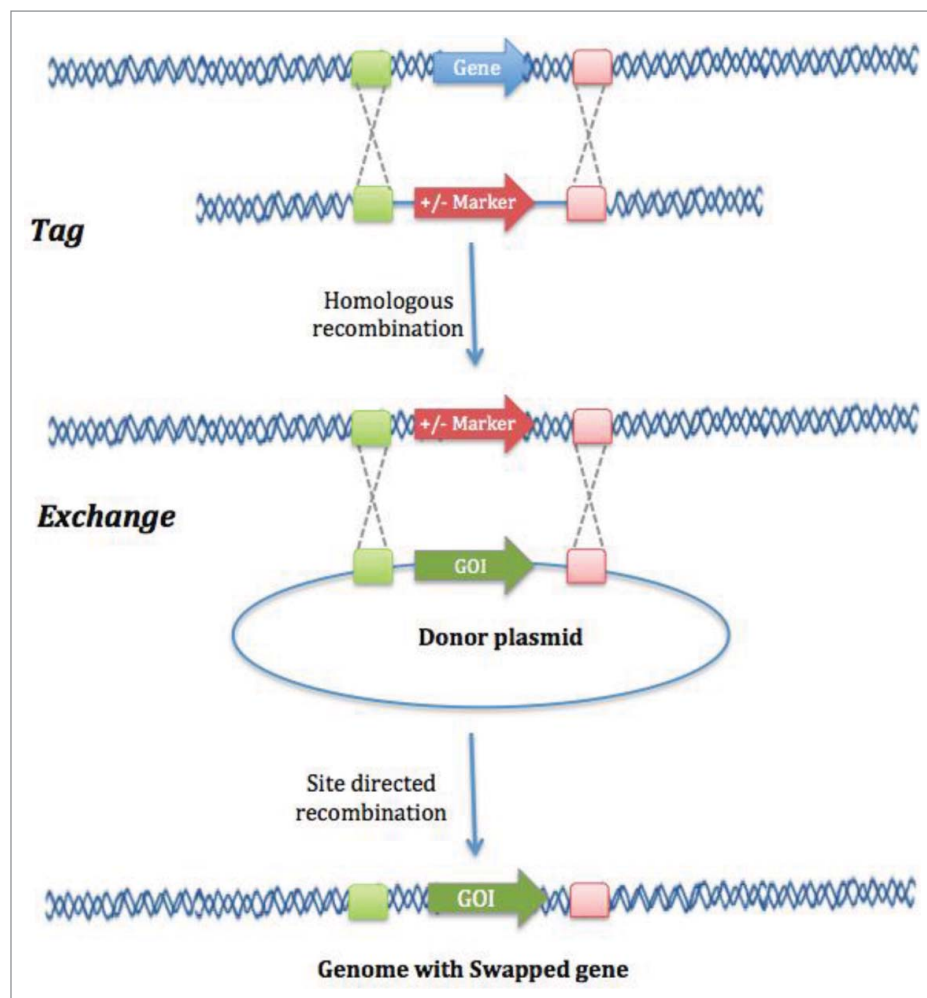
However, transient expression is an ideal method for recombinase expression as constitutive expression in a cell could result in toxicity and undesired off-target recombination.

All enzymes recombine at target sites, which are either identical (“*FRT*” in case of FLP-recombinase, “*loxP*” for Cre-recombinase) or different (“*attP*” and “*attB*” attachment sites on the phage and bacterial part, respectively) (Turan and Bode, 2011). The recombination patterns depend on recombinase (sub-) family and target-site orientation. Interaction of 2 identical substrate sites (*loxP* × *loxP* or *FRT* × *FRT*) leads to products of the same composition, whereas recombination of 2 non-identical sites

leads to 2 different hybrid sites (*attP* × *attB* → *attR* + *attL*). SSR could be used for gene swap based on a tag and exchange strategy (Fig. 1). The first step includes integration of site-specific recombinase cassette with marker gene in the target site using homologous recombination followed by exchange between the recombinase cassettes. The tag step requires a large homologous sequence while the difference between the exchange step requires short homology of ~50 bp for SSRs.

Recombinase has been used extensively to manipulate DNA in plants, bacteria, yeast and mammals either by creation of knockout or knock-in in the genome of the eukaryotes.

FIGURE 1. Gene swap strategy using Tag and Exchange strategy based on homologous recombination followed by site-specific recombination step.



One of the advantages of recombinases is the lack of dependence on the intracellular repair mechanisms. They also are capable of performing without the need for forming double-strand breaks. In addition, recombinases insert donor DNA into the genome, which simplifies detection of their activity. The disadvantage of these chimeric enzymes is a fairly high level of off-target effects and toxicity with high expression (Anastassiadis et al., 2009).

### ***Genome Editing Mediated by Site-Specific Nucleases (SSNs)***

SSNs rely on constructing endonucleases capable of cleaving DNA in a predetermined sequence in the genome. SSN has a DNA-binding domain or RNA sequence that binds to the target sequence (Carroll, 2014; Gaj et al., 2013). Cleaving the target sequence by the SSN is followed by cellular DNA repair mechanisms, leading to gene modification at the target sites. There are currently 4 families of engineered nucleases being used in genome editing: engineered meganuclease (MegaN), 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 system (Voytas, 2013; Čermák et al., 2015). These systems allowed us to easily, efficiently and cheaply modify the genome.

## ***MEGANUCLEASE-BASED ENGINEERING***

Meganucleases (MegaN) are naturally occurring endonucleases, which were discovered in the late 1980s. They belong to endonuclease family that can recognize and cut large DNA sequences (from 12 to 40 base pairs) unique or nearly-so in most genomes (Gallagher et al., 2014). In addition, they have the benefit of being less toxic in cells compared to other methods, such as ZFNs (**Fig. 2a**) (Smith et al., 2006). The large recognition sites make meganucleases perfect tools for genome

engineering, but the number of naturally occurring meganucleases is limited and not sufficient to cover all potentially interesting loci.

Meganucleases were modified for use in genome editing by different companies. The areas responsible for DNA cleavage and the areas that interact with specific DNA sites in meganucleases were modified by scientists from Collectis Company to interact with different DNA sequences from those of the original meganucleases, while retaining their ability to cut the DNA and their high degree of specificity. Another approach was developed by Precision Biosciences, Inc. where they developed a fully rational design process called the directed nuclease editor (DNE), capable of creating highly specific engineered meganucleases that successfully target and modify a user-defined location in a genome (Ashworth et al., 2010). Once the DNA has been cut, cells go through natural DNA repair mechanisms, with homologous recombination enabling the incorporation of a modified sequence or a new gene.

A disadvantage of meganuclease is that the construction of sequence specific enzymes for all possible sequences is costly and time consuming compared to other SSN systems. Each new genome-engineering target therefore requires an initial protein engineering stage to produce a custom meganuclease. Therefore, meganucleases proved technically challenging to work with and is also hindered by patent disputes (Smith et al., 2011).

### ***Zinc Finger Nuclease-Based Engineering***

Zinc finger motifs occur in several transcription factors. The C-terminal part of each finger is responsible for the specific recognition of the DNA sequence. The recognized sequences are short, made up of around 3 base pairs, so a protein could be engineered for binding a specific DNA sequence in the genome of around 20 base pairs by combining 6 to 8 zinc fingers with characterized recognition sites. Several approaches are used to design specific zinc finger nucleases for the chosen sequences. These synthetic proteins could be used in editing of a specific gene



site-specific genome editing tools. The method generally adopted for this involves associating 2 constructed proteins – each containing specifically chosen zinc fingers (binding domains) – with the catalytic domain of the FokI endonuclease (**Fig. 2b**). The two proteins recognize 2 DNA sequences that are a few nucleotides apart. That allows the 2 endonucleases to associate together forming a dimer to effect double stranded DNA cleavage (Kim et al., 2007).

However, there are several disadvantages for using ZFN-based technology, including the complexity and high cost of protein domains construction for each particular genome locus and the probability of inaccurate cleavage of target DNA (off-target effects) due to single nucleotide substitutions or inappropriate interaction between domains (Puchta and Hohn, 2010). Several optimizations need to be made in order to improve editing plant genomes using ZFN-mediated targeting, including the reduction of toxicity of the nucleases, the appropriate choice of the plant tissue for targeting, the introduction of enzyme activity, the lack of off-target mutagenesis, and a reliable detection of mutated cases (Puchta and Hohn, 2010).

### *TALEN Genome Editing System*

In 2011, another method was developed for increasing efficiency, safety and accessibility of genome editing – called TALEN (Transcription Activator-Like Effector Nucleases) system. The TALEN system developed from the transcription activator-like effectors (TALES) produced by the phytopathogenic bacteria *Xanthomonas* genus (Boch and Bonas, 2010; Urnov et al., 2010). The effector proteins belong to a DNA binding protein family and can be used to activate the expression of their target genes just like the transcription factors of eukaryotic genomes.

The DNA binding domain of the TALE proteins consist of tandem repeats of 34 amino acid residues monomers, each of them binds one nucleotide in the target nucleotide sequence. Two amino acids of the monomers located at positions 12 and 13 are highly variable (repeat variable di-residue, RVD), and are responsible for the recognition of a specific nucleotide. The last tandem repeat that binds a

nucleotide at the 3'-end of the recognition site consists only of 20 amino acid residues instead of 34 and is called a half-repeat. The 5'-end of the target DNA molecule always contains the nucleotide thymidine and it affects the binding efficiency (Miller et al., 2015). Studies of the RVDs revealed that di-residues Asn-Ile, Asn-Gly, 2 2-Asn and His-Asp (HD) bind to A, T, G & C nucleotides, respectively.

TALENs are artificial restriction enzymes generated by combining the catalytic domain of restriction endonuclease FokI with suitable monomers of the DNA-binding domain to construct artificial nucleases that could target any nucleotide sequences in the genome (**Fig. 2c**). The only limitation to the selection of TALEN nuclease sites is the need for T before the 5'-end of the target sequences.

The final constructed TALEN nuclease consists of nuclear localization signal, an artificial DNA-binding domain and the FokI catalytic domain. TALENs work as pairs and their bindings sites are chosen so that they are located on opposite DNA strands and are separated by a small fragment (12–25 bp) of spacer sequence. Once in the nucleus, artificial nucleases bind to target sites: the FokI domains located at the c-termini of a chimeric protein dimerize to cause a double-strand break in a spacer sequence.

The big obstacle in applying TALEN system is in constructing the vector with suitable monomers for binding the target DNA in the genome. Several techniques have been conducted for constructing TALE DNA-binding domains consisting of 20–30 or even more monomers. One of the strategies is based on standard DNA cloning using DNA restriction endonucleases and ligation monomers as first step to generate a dimers library, as a second step the Golden Gate reaction is used (Weber et al., 2011; Engler et al., 2009), which is a simultaneous ligation of several dimers in the same reaction mixture.

In order to reduce the time needed to develop genetic constructs expressing TALEN, several companies have developed simple, efficient and accessible techniques for the construction of TALENs such as the Addgene Depository kit (<http://www.addgene.org/TALEN/>), commercial platform from Cellectis Bioresearch which enables one to generate up

to 7,200 of these constructs annually and the Fast Ligation-based Automatable Solid-phase High-throughput (FLASH) platform as a rapid and cost-effective method (Reyon et al., 2012).

Methods to modify plant genomes that do not require DNA delivery would have value in both commercial and academic settings. Luo et al. (Luo et al., 2015) demonstrated non-transgenic plant genome engineering by introducing sequence-specific nucleases as purified protein. This approach enabled targeted mutagenesis of endogenous sequences within plant cells, while avoiding integration of foreign DNA into the genome (Luo et al., 2015).

### *CRISPR/CAS Genome Editing System*

A novel genome editing system, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has been developed in 2013. CRISPRs are genetic elements that gave immunity to bacteria to protect them against viruses. The protection mechanism includes 3 main stages: adaptation, transcription and interference. At the adaptation stage, when foreign DNA entered bacterial cells, a small fragment is inserted into the CRISPR locus of the host genome in specialized repeat structures separated from each other by short palindromic repeats and therefore they received the name CRISPR. The *Cas* genes (CRISPR associated), are located close to the CRISPR cassette express protein and have helicase and nuclease activity (Haft et al., 2005). At the transcription stage, the entire CRISPR locus is transcribed into a long pre-crRNA (poly-spacer precursor crRNA) and produce short crRNAs (CRISPR RNA) of 39–45 nucleotides containing one spacer sequence (**Fig. 2d**). The interference stage includes assembled ternary Cas9-crRNA-tracrRNA complex at the 3' side of the crRNA-tracrRNA duplex structure and binds to Cas-proteins. The crRNA recognizes complement with the protospacer sequence, while tracrRNA is required for Cas-mediated DNA interference and Cas-proteins to cut matching foreign DNA sequences causing DNA degradation.

CRISPR has been adapted to create RNA directed genome engineering tools by introducing some modifications, including codon-

optimization for the Cas9 nuclease for adequate transcription in higher eukaryotic cells, fused to nuclear localization signals (NLS). In addition, a single chimeric guide sgRNA (crRNA-tracrRNA duplex) is constructed under the control of promoter type III instead of expressing 2 non-coding RNAs (tracrRNA and pre-crRNA) (**Fig. 2d**).

The CRISPR element consists a complex of non-coding RNAs and Cas proteins (CRISPR associated), which have nuclease activity. In contrast to the chimeric TALEN proteins, recognition by the CRISPR/Cas system is carried out *via* the complementary interaction between a non-coding RNA and the target site DNA.

Basic plasmid constructs used for this system contain the elements necessary for CRISPR/Cas9 activity: Cas9 nuclease/nickase, CRISPR mRNA, and tracrRNA. To change the target sequence, this construct only needs cutting off the original 30 nucleotide guide sequence flanked by unique restriction sites and replacing it with an artificially synthesized one (Nemudryi et al., 2014).

Both wild-type Cas9, which creates a double stranded break at the target site, and Cas9 Nickase, which creates a single stranded break, could be used in genome editing. Nickase is mutated Cas9 that cut only single stranded DNA, Nickase HNH cut the binding strand while Nickase RuvC cut the complementary strand. For genome editing using Cas9 Nickase 2 guide RNAs are required for each type and that will reduce off target events.

The CASPR-Cas platform is a cost-effective and easy-to-use technology to precisely and efficiently target, edit, modify, regulate, and mark genomic loci of a wide array of cells and organisms. CRISPR-Cas9 has triggered a revolution in which laboratories around the world are using the technology for innovative applications in biology.

### *Comparison Between Different Types of Site-Specific Synthetic Nuclease for Plant Genome Editing*

The development of the SSN systems is an important step in the achievement of modern genomic engineering. Engineered site specific



nucleases with higher specificity will have less unintended modifications and cellular toxicity (no off-target activities) (Mahfouz and Li, 2011). To date, there is no definitive answer to the question of which, if any, of the systems is preferred. A detailed comparison of the different SSN platforms is presented in **Table 1**. For each particular case, it will be necessary to test different variants and to choose among them those that are most appropriate to the research goals. The MegaN, ZFN and TALEN are based on protein-DNA interactions, CRISPR/Cas9 system depends on RNA DNA pairing there for it is able to modify gene targets that other systems are not able to modify (Feng et al., 2014; Li et al., 2013; Nekrasov et al., 2013; Zhang et al., 2014; Bortesi and Fischer, 2015).

#### *How Do Targeted SSNs Enable Precise Genome Modifications?*

Programmable SSNs enable precise genome editing by translocating to the nucleus and introducing DNA double-strand breaks (DSBs) at predetermined endogenous genomic loci. Breaks achieved with different SSN platforms initiate a variety of DNA-repair mechanisms in the target cell, ranging from DNA deletions to the insertion of large arrays of transgenes (**Fig. 3**). One mechanism for break repair is non-homologous end-joining (NHEJ), when the 2 DNA ends ligate together (no template is present), causing small deletions or more rarely insertions (InDel) at the junction of the newly rejoined chromosomal DNA (Gorbunova and Levy, 1997; Salomon and Puchta, 1998). If NHEJ causes a small InDel producing frameshift mutation or altering key amino acid residues in the target gene product, a knockout (loss of function) mutation can be created. Another mechanism for repair is homologous recombination (HR), which could be achieved by introducing both a SSN and a DNA repair template with sequence similarity to the break site. Broken DNA ends are joined to the exogenous template via homology-directed repair (HDR). The capture of heterologous DNA sequences can be used for manipulating plant genomes via targeted gene knock-in (targeted insertion). The frequency of genome editing

depends on many factors including the species, stage of cell cycle and tissue type and the kind of platform used for editing.

#### **IMPORTANT CONSIDERATIONS: IDENTIFYING TARGET SEQUENCES AND DECREASING OFF-TARGET EFFECTS**

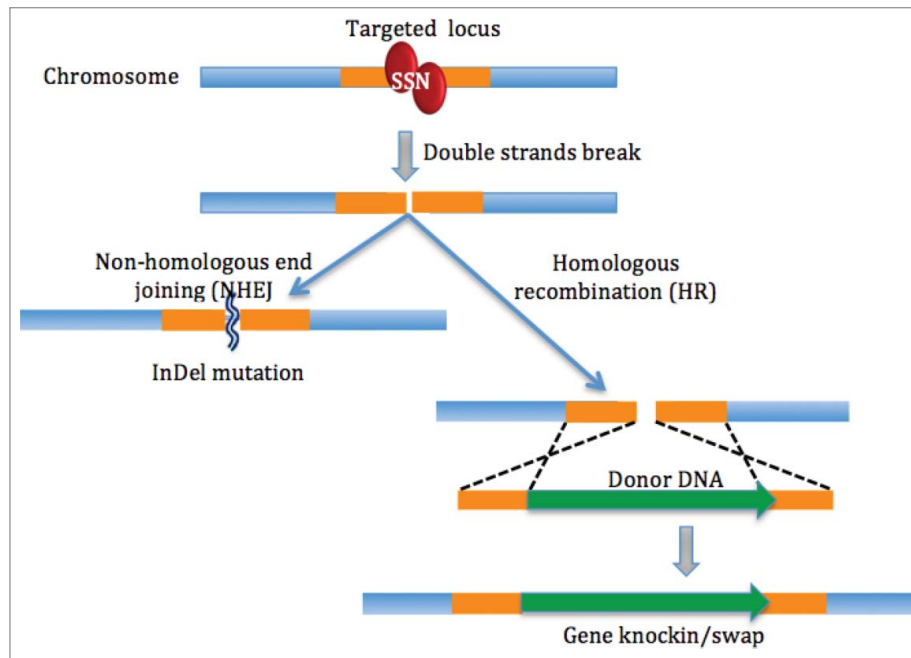
SSNs are designed to cut target sites efficiently in the genome, but can also create unwanted cleavages at off-target sites with high sequence homology to on-target sites, thus inducing off-target mutations (Koo et al., 2015; Bae et al., 2014). Therefore, before beginning work with any genome editing system, the target sites should be selected carefully using suitable bioinformatic analytical software. These programs are designed to identify repeated and homologous regions so they can be avoided.. Different bioinformatics programs have been developed: zinc-finger nucleases (ZFNs) (Bibikova et al., 2003; Kim et al., 2009; Urnov et al., 2010), transcription activator-like effector nucleases (TALENs) (Kim et al., 2013a; Kim et al., 2013b; Miller et al., 2011), and RNA-guided engineered nucleases (RGENs) repurposed from the type II clustered, regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) system (Cho et al., 2013a; Cho et al., 2013b; Cong et al., 2013; Mali et al., 2013).

ZFNs and TALENs consist of a common nuclease domain derived from FokI, (type IIS restriction enzyme) and distinct DNA-binding domains. DNA-binding domains of ZFNs and TALENs can be engineered to target pre-selected DNA sequences. The FokI nuclease domain must dimerize to cleave DNA (Bitinaite et al., 1998), therefore they accentuate their high specificities. Typically, a ZFN pair recognizes an 18-to 36-bp DNA sequence, and a TALEN pair recognizes a 30- to 40-bp DNA sequence, surpassing the complexity of the genome. ZFNs can induce off-target mutations (the binding site recognizes 3-bp sub-sites) and can be cytotoxic (Kim et al., 2009), which may arise from their off-target effects. Constructed ZFNs with more specificity could be made by

TABLE 1. Comparison between the different systems of site-specific nuclease used for genome editing

	MegaN	ZFN	TALEN	Cas9
Recognition site	Between 14 and 40 bp	Typically 9–18 bp per ZFN monomer; 18–36 bp per ZFN pair	Typically 14–20 bp per TALEN monomer; 28–40 bp per TALEN pair	22 bp (20-bp guide sequence + 2-bp protospacer adjacent motif (PAM) for Cas9; up to 44 bp for double nicking)
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting	Targeting novel sequences often results in low efficiency	Difficult to target non-G-rich sequences	5' targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM
Cleavage efficiency Off-target effects	Low efficiency Possible off-target activities	Low efficiency Possible off-target activities	Efficient Limited off-target activities, not fully studied in plants	Highly efficient No off-targeted activities reported in plants, but high off-target levels reported in other systems.
Mechanism of action	Introduction of double-strand breaks (DSBs) in target DNA	Introduction of double-strand breaks (DSBs) in target DNA	Introduction of double-strand breaks (DSBs) in target DNA	Introduction of DSBs in target DNA by wtCas9 or single strand nicks by Cas9 nickase
Cleavage efficiency Affordability	Efficient Limited	Efficient Limited	Efficient Affordable but resource intensive	High efficient Highly affordable
Programmable Structure	Highly difficult Monomer	Highly difficult Dimer	Difficult Dimer	Easy Monomer

FIGURE 3. Repair mechanisms of double stranded breaks induced by genome editing through non-homologous end joining (left) and homologous recombination (left).



different companies but are expensive. In addition ZFNs prefer guanine-rich target sequences, limiting targetable sites.

TALENs can be designed to target almost any DNA sequence, a critical advantage over ZFNs and RGENs. TAL effector modules recognize single bases, for each base, specific modules are used to make TALENs. TAL effector arrays often consist of up to 20 modules, making it time-consuming and labor-intensive to prepare plasmids that encode TALENs. In general, TALENs are not cytotoxic, but can induce off-target mutations (Mussolino et al., 2011). Fortunately, TALEN off-target effects can be avoided by choosing unique target sequences that differ by at least 7 nucleotides from any other site in the genome (Kim et al., 2013a).

CRISPR/Cas-derived RGENs belong to another class of programmable nucleases and function as monomers. The specificity of an RGEN is determined by both the gRNA, which hybridizes with a 20-bp target DNA sequence, and Cas9, which recognizes the 5'-NGG-3' sequence known as the protospacer-adjacent motif

(PAM). New RGENs with desired specificity could be prepared by replacing *in vitro* transcribed gRNA making these nucleases affordable and scalable.

#### *Delivery of Constructs into Target Organism*

Delivering constructs into target cells or organisms is the bottleneck for genome engineering. Plants have cells with a thick wall, therefore delivery systems for plants includes transient and stable transformation using protoplast-plasmid transformation in cell cultures (Shan et al., 2013; Mao et al., 2013), *Agrobacterium*-mediated transformation, gene gun and viral vectors (transient expression by protoplast transformation) and agro-infiltration (Nekrasov et al., 2013; Shan et al., 2013; Li et al., 2013). Gene knockouts and precise modification have been produced in plants, such as Arabidopsis, wheat, rice, and tobacco (Feng et al., 2014; Nekrasov et al., 2013; Shan et al., 2013; Mao et al., 2013; McManus

and Sharp, 2002; Voytas and Gao, 2014; Voytas, 2013; Padmaja, 1995; Witzany, 2011; Townsend et al., 2009; Tierney and Lamour, 2005; Chen et al., 2014; Martin and Caplen, 2007; Dietzl et al., 2007; Gonczy et al., 2000; Wang et al., 2011; Allen and Weeks, 2005; Allen and Weeks, 2009; Araki et al., 1995; Jia et al., 2006; Turan and Bode, 2011; Turan and Bode, 2011; Anastasiadis et al., 2009; Carroll, 2014; Gaj et al., 2013; Čermák et al., 2015; Gallagher et al., 2014; Smith et al., 2006; Ashworth et al., 2010; Smith et al., 2011; Rebar et al., 2002; Kim et al., 2007; Puchta and Hohn, 2010; Boch and Bonas, 2010; Urnov et al., 2010; Miller et al., 2015; Weber et al., 2011; Engler et al., 2009; Reyon et al., 2012; Luo et al., 2015; Haft et al., 2005; Nemudryi et al., 2014; Mahfouz and Li, 2011; Feng et al., 2014; Li et al., 2013; Nekrasov et al., 2013; Zhang et al., 2014; Bortesi and Fischer, 2015; Gorbunova and Levy, 1997; Salomon and Puchta, 1998; Koo et al., 2015; Bae et al., 2014; Bibikova et al., 2003; Kim et al., 2009; Urnov et al., 2010; Kim et al., 2013; Kim et al., 2013; Miller et al., 2011; Cho et al., 2013a; Cho et al., 2013b; Cong et al., 2013; Mali et al., 2013; Bitinaite et al., 1998; Mussolino et al., 2011; van der Hoorn et al., 2000; Shan et al., 2013; Mao et al., 2013; Li et al., 2013; Feng et al., 2013; Xie and Yang, 2013; Miao et al., 2013; Jiang et al., 2013; Upadhyay et al., 2013). One of the primary advantages of virus-mediated delivery is its amenability to multiplexing (Feng et al., 2013). In the case of CRISPR, Cas9 nuclease could be intergating with multiple gRNAs to introduce mutations in several genes simultaneously.

Viruses can serve as a vehicle to deliver genome engineering reagents to all plant parts, they do not need transformation and/or tissue culture for delivering and mutated seeds could easily recovered. There are some features relevant to using viruses as a vector for genome editing; they must systematically infect a large number of plant species, they are easily introduced into plants via *Agrobacterium*; they have small genomic size good for facilitating cloning and agroinfections; and the virus genome does not integrate into plant genomes.

The genome of the Tobacco rattle virus (TRV) has been developed for use in virus-mediated genome editing. The persistence of the TRV-

mediated Cas9 activity for up to 30 d post-agroinfection. Further, data indicate that TRV-mediated genome editing exhibited no off-target activities at potential off-targets indicating the precision of the system for plant genome engineering. Taken together, the data establish the feasibility and exciting possibilities of using virus-mediated CRISPR/Cas9 for targeted engineering of plant genomes (Ali et al., 2015).

Transient expression of gRNA libraries can be delivered via a virus-based system in a monoallelic overexpression line for Cas9, and it is possible to recover plants carrying only the desired mutations and lacking any CRISPR/Cas9 system components (Marton et al., 2010). Furthermore, a CRISPR/Cas9 genome regulation platform can be built by generating an overexpression line of catalytically inactive dCas9.

Geminivirus-based VIGE could be a powerful tool in plant genome editing because they have small (~3 kb), circular DNA genomes that replicate in the nucleus, and can move from cell to cell through plasmodesmata. Geminivirus has been used as virus-based guide RNA (gRNA) delivery system for CRISPR/Cas9 mediated plant genome editing (VIGE) to precisely target genome locations and thus cause mutations. VIGE is performed by using a modified Cabbage Leaf Curl virus (CaLCuV) vector to express gRNAs in stable transgenic plants expressing Cas9 (Yin et al., 2015). Recently, a geminivirus genome has been developed to deliver different nucleases platform (including ZFN, TALENs, and the CRISPR/Cas system) and repair template for homologous repair of a double-stranded break. Baltes et al. (2014) removed the coat protein and movement protein genes from bean yellow dwarf virus, leaving only the sequences required for replication and used it as a vector for genome editing. One of the primary advantages of virus-mediated delivery of gRNA molecules is its amenability to multiplexing.

### *Beyond Genome Editing of SSNs (Other Applications of TALE and CRISPR)*

One of the major challenges in biotechnology is the ability to regulate the transcription of endogenous genes. Defective SSNs with

lost nuclease activity were used to generate artificial DNA-binding domains to construct chimeric proteins capable of affecting gene regulation directly on the genome (**Fig. 4**). These chimeric proteins are composed of SSN DNA-binding and effector domains (Beerli et al., 1998; Perez-Pinera et al., 2013; Li et al., 2012; Mahfouz et al., 2012). The effector domains could activate or suppress gene expression domains, histone methyltransferase, and histone deacetylase domains affects histone organization in the target site. These chimeric proteins have enormous prospects for application both in applied and in fundamental science. Modification of DNA-binding domains of the TALE or CRISPR/dCas9 (defective Cas9) systems, take place followed by attaching to effector domain (repressor/activator domains) for controlling targeted gene expression according to tissue type and development stage. In addition, the fusion between the synthetic protein and methylases/demethylases or chromatin modifiers proteins could regulate the transcription of the targeted gene and help to understand epigenetics. The ability to modulate gene expression will help in the development of synthetic biology by identifying gene functions and gene networks (Maeder et al., 2013).

### ***OPPORTUNITIES FOR CROP IMPROVEMENT***

Although genome engineering for crop improvement is in its early stage, some crop species have been developed using this technology. Most modifications were through NHEJ produced by SSNs causing gene knockout. NHEJ are used to study gene function and for trait development even for polyploidy plants (Curtin et al., 2013; Qi et al., 2013). Gene knockout using SSNs has been used to elucidate plant gene of important genes that could be used for crop improvement in diverse plant species, including several important crops, such as barley, soybean, maize, rice, wheat, and sorghum (Voytas and Gao, 2014; Curtin et al., 2013; Qi et al., 2013; Shan et al., 2013;

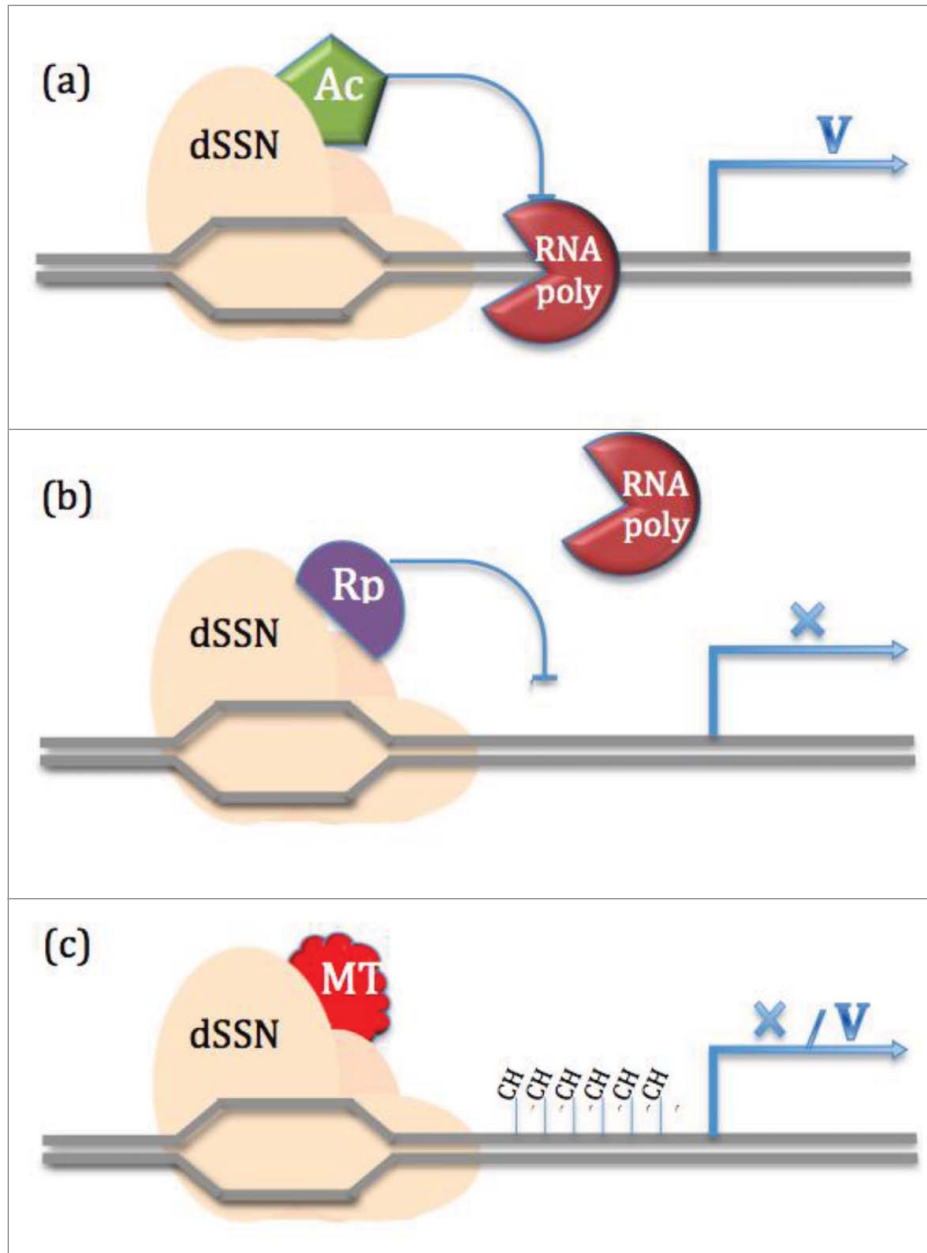
Wendt et al., 2013; D'Halluin et al., 2008; D'Halluin et al., 2008; Gao et al., 2010; Lyznik et al., 1991; Yang et al., 2009; Shukla et al., 2009; Liang et al., 2013; Shan et al., 2013; Jiang et al., 2013; Shan et al., 2013; Li et al., 2012; Wang et al., 2013; Mao et al., 2013; Miao et al., 2013; Xie and Yang, 2013).

The goals of plant breeding are common across species, for example, increasing the concentrations of nutrients and some unique secondary metabolites; extending the shelf life of fruits, vegetables, and cut flowers; improving yield potential; and enhancing tolerance to abiotic stresses and resistance to pest and disease.

Anthocyanins may be beneficial to human health, as they are reported to inhibit certain cancers and degenerative diseases (Seeram et al., 2004; Joseph et al., 1999; Renaud and de Lorgeril, 1992; Hou et al., 2004). In addition, anthocyanins are responsible for the coloration of flowers, fruits and vegetables (Allan et al., 2008). Studies in model plants have revealed that the activity of MYB-bHLH-WD repeat (MBW) complexes regulate the transcription of anthocyanin genes (Albert et al., 2014), conserved anthocyanin biosynthetic and regulatory pathway. Central to this process is the activity of MYB-bHLH-WD repeat (MBW) complexes that regulate the transcription of anthocyanin genes (Albert et al., 2014). Further studies showed that the MYB factors in the complex are the key regulators that determine the patterning and spatial localization of anthocyanins. In petunia, RNAi was used to reduce the expression of the MYB27 which function as anthocyanin synthesis repressors (Albert et al., 2014). Genome-editing technologies could be used to modify some of repressor genes to obtain plants with high concentrations of anthocyanin.

Gibberellin (GA) affects plant height, while strigolactone (SL) influences shoot branching. Semi-dwarf phenotypes or more branches could be generated could be obtained by disrupting the function of the genes in the GA or SL biosynthetic or signaling transduction (D'Halluin et al., 2008). Producing semi-dwarf statured fruit/nut trees could have the potential to increase

FIGURE 4. Targeted genome regulation mediated by defected SSN-based factors. The dSSN fusion to (a) activator potentially enhance gene expression by recruitment of transcription factors and RNA polymerase II, (b) potent repressors domains could either prevent the RNA polymerase from binding to the promoter region of specific gene or (c) methylases/demethylases or chromatin modifiers could regulate the transcription of the targeted gene.



productivity via higher density plantings and reduced labor cost, resulting in reduced land, water, pesticide, and fertilizer use (Hollender and Dardick, 2015).

Genome editing could also be used to prevent the accumulation of unwanted metabolites that have a negative impact in food and feed quality and processing. Mustard and cabbage

produce glucosinolates and have toxic effects in high doses (Hannoufa et al., 2014). Some plants like cassava produces cyanide, which cause acute cyanide intoxication, goiters, and even ataxia or partial paralysis (Padmaja, 1995). Also, phytate is an anti-nutrient that limits mineral absorption and also contributes to environmental pollution through the waste stream. Genome editing was used to mutate the *IPK1* gene, which catalyze the final step in phytate biosynthesis in maize (Shukla et al., 2009).

Combinations of mutant *FAD2* and *FAD3* genes in soybean were used to produce high oleic acid and low linolenic acid soybean oil allowing the accumulation of monounsaturated fats and reduce the level of linolenic acid in seeds, producing healthier and improved shelf-life oil (Pham et al., 2012).

In rice, the protein produced by the *OsSWEET14* gene contributes to pathogen survival and virulence of the bacterial pathogen, *Xanthomonas oryzae* (Li et al., 2012). Genome editing using TALEN was used to create a mutation in the effector-binding site in the promoter of *OsSWEET14*, thereby eliminating the protein transcription and consequently reducing the pathogen's virulence.

Targeted gene insertion through HR has been demonstrated with different SSN platforms in several plant species, including tobacco, maize, and rice (Townsend et al., 2009). HR was used to introduce amino acid changes in a plant gene encoding acetolactate synthase (ALS)—an enzyme involved in branched-chain amino acid biosynthesis (Townsend et al., 2009), ALS is the target of several herbicides, including imidazolinones and sulfonylureas, and amino acid substitutions were introduced into the ALS gene that prevent inhibition of the enzyme by these herbicides. The modified plants could grow in the presence of the herbicide.

Plants encode EPSPS—the target of the herbicide glyphosate—so genome editing could be used to modify the native gene to make it tolerate glyphosate while maintaining its normal enzymatic functions. The use of HR to create herbicide tolerance provides an alternative and more precise way to replace transgenesis in developing glyphosate-tolerant plants. In the

future, HR could be more effective in crop improvement by creating traits that cannot be achieved through traditional breeding or transgenesis.

## PERSPECTIVES

Plants are used as sources of food, feed, fiber, medicines and biofuels. For many years, conventional breeding was used to improve the properties of domestic crops. The presence of different alleles in nature allows the improvement of crops, either by traditional breeding, genetic engineering or genome editing. In addition, mutation has been used to develop new alleles, but as they are random and could affect many non-targeted alleles, they must be subjected to substantial screening and backcrosses. Genome editing, however, targets a specific position in the genome, so less screening or backcrossing is required. In addition multiple alleles could be modified simultaneously using CRISPR/Cas9 system.

Genome editing targets modifications to the genome easily and efficiently, its utilities help to improve food and feed, drug development, animal models, genetic variation, materials, fuel, gene surgery, in addition to investigating chromosome structure and functional dynamics.

The modifications produced via genome editing could be divided into 3 different types. Type-1 produced from NHEJ which acts as simple mutagen causing SNPs or INDELS causing switch off a specific gene. In that situation, remnants of transgenes can be eliminated by selfing or backcrossing. The modifications produced by this type are indistinguishable from the mutations that occur spontaneously or during mutation breeding, which is significantly more genetically disruptive.

Genome editing in the type-2 mode is the most challenging for regulators and ironically will probably be the most useful for plant breeding because it enables editing of existing alleles to redefine their function or new ones to be 'knocked-in'. The editing could be designed either to retain the function from existing useful alleles already in the gene pool but where

TABLE 2. Comparison between different genome improving technologies

Mutagen	Chemical (EMS) / Physical (gamma, X-ray or fast neutron radiation)	Biological – Transgenics (e.g., Agro or gene gun)	Biological – Editing (ZFNs, TALENs or CRISPR/Cas)
Characteristics of genetic variation	<ul style="list-style-type: none"> <li>-Substitutions, deletions and chromosomal mutations</li> <li>-Loss of function mutations mainly</li> <li>-Recessive traits mainly</li> <li>-Unnecessary of knowing gene functions or sequences</li> <li>-Easy production of random mutations</li> </ul>	<ul style="list-style-type: none"> <li>-Insertion</li> <li>-Loss of function and gain of function mutations</li> <li>-Dominant traits</li> <li>-Insertion of genes into host plant genome randomly</li> <li>-Easy production of random mutations</li> <li>-Unlimited application potential</li> </ul>	<ul style="list-style-type: none"> <li>-Substitutions, deletions, insertions and insertion</li> <li>-Loss of function and gain of function mutations</li> <li>-Recessive and dominant traits</li> <li>-Gene specific mutations</li> <li>-Efficient creation of plants with desirable traits</li> <li>-Necessity of knowing gene functions and sequences</li> <li>-Prerequisite of efficient genetic transformation</li> <li>-Limited application</li> <li>-Transgenic process but non-transgenic traits</li> </ul>
Advantages	<ul style="list-style-type: none"> <li>-Unnecessary of knowing gene functions or sequences</li> <li>-Easy production of random mutations</li> </ul>	<ul style="list-style-type: none"> <li>-Insertion of genes into host plant genome randomly</li> <li>-Easy production of random mutations</li> <li>-Unlimited application potential</li> </ul>	<ul style="list-style-type: none"> <li>-Efficient creation of plants with desirable traits</li> <li>-Necessity of knowing gene functions and sequences</li> <li>-Prerequisite of efficient genetic transformation</li> </ul>
Disadvantages	<ul style="list-style-type: none"> <li>-Inefficient screening of desirable traits</li> <li>-Non-specific mutations</li> <li>-Limited application potential</li> <li>-Non-transgenic process and traits</li> </ul>	<ul style="list-style-type: none"> <li>-Necessity of knowing gene functions and sequences</li> <li>-Prerequisite of efficient genetic transformation</li> <li>-Unlimited application</li> <li>-Transgenic process and traits</li> </ul>	<ul style="list-style-type: none"> <li>-Prerequisite of efficient genetic transformation</li> <li>-Limited application</li> <li>-Transgenic process but non-transgenic traits</li> </ul>
Other features	<ul style="list-style-type: none"> <li>-Limited application potential</li> <li>-Non-transgenic process and traits</li> </ul>	<ul style="list-style-type: none"> <li>-Unlimited application</li> <li>-Transgenic process and traits</li> </ul>	<ul style="list-style-type: none"> <li>-Limited application</li> <li>-Transgenic process but non-transgenic traits</li> </ul>
Regulatory frameworks	<ul style="list-style-type: none"> <li>excluded from the scope of GMO regulatory frameworks</li> </ul>	<ul style="list-style-type: none"> <li>Restricted by GMO regulatory frameworks</li> </ul>	<ul style="list-style-type: none"> <li>Partially excluded from the scope of GMO regulatory frameworks</li> </ul>

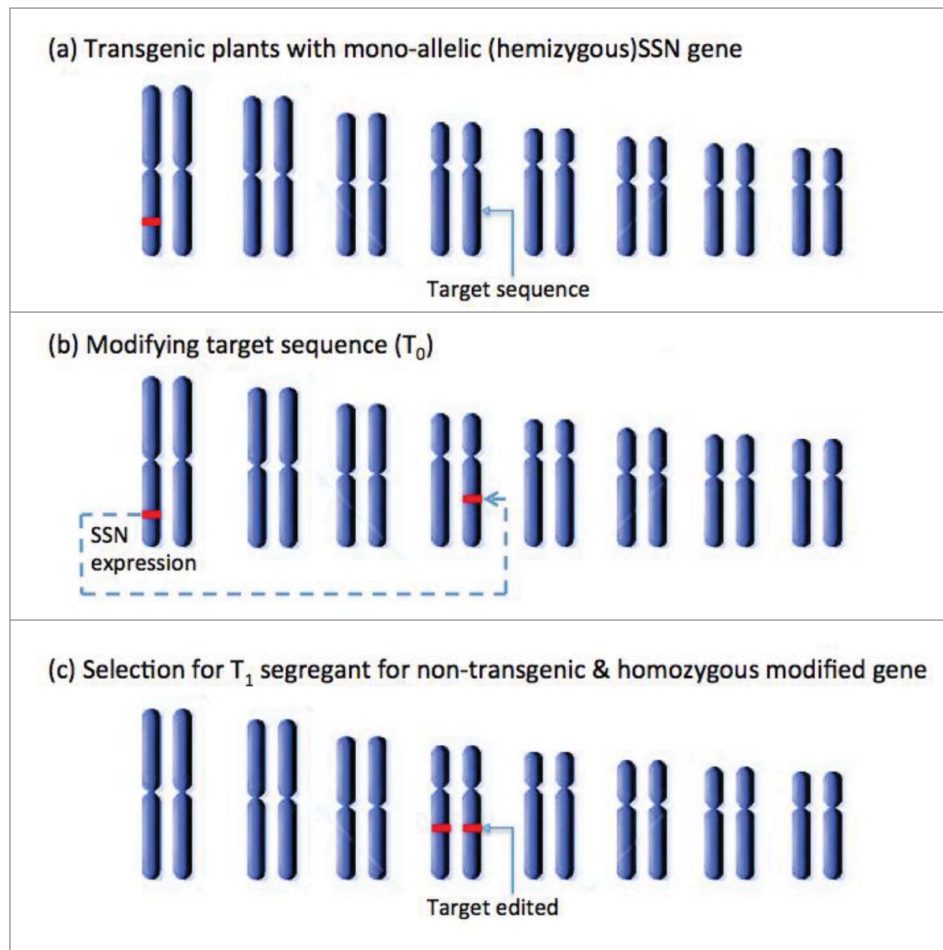


modified or lost its function during conventional breeding to development of commercial germplasm, or from redesigning the allele to create novel traits. In that case an HR repair system, like Genome Repair Oligonucleotide technology, is required (Jones, 2015a; Jones, 2015b; Jones, this issue).

Type-3 editing provides the introduction of a complete functional transgene targeted to a pre-determined ‘safe harbor site’ in the genome. It is useful compared to GMO products, as the insertion site in the genome could be predetermined, avoiding adverse position effects (i.e. disruption of native genes).

For over 100 years, scientists have devoted their efforts to develop and refine the techniques for improving the quality and quantity of crops starting from using conventional breeding to modern biotechnology techniques. Genome-editing technologies provide many advantages compared with traditional breeding and transgenic methods (Table 2). Although traditional breeding technologies have provided improved traits for crops, the long breeding cycles, high heterozygosities, lack of various degrees of precision in hybridization, and low frequencies of desirable mutations have made new varietal development highly resource-demanding. Trans-

FIGURE 5. Developing of edited plants using SSN genome insertion followed by selection for non transgenic T1 segregant lines.



genic technology has a wide applications in improving crops as it can overcome the incompatibility barriers between species. However, this technique faces a number of obstacles including regulatory obstacles, public acceptance, time and cost of risk assessments required before commercialization. Now-a-days, using the new gene editing technology are time saving, more precise and efficient for improving traits. Crops developed by genome editing are expected to be more acceptable to consumers as they are considered non-transgenic. Combining all the 3 modification technologies will help even more in improving crop traits. Judicious application of genome editing techniques will provide more food, feed and fiber, produced in a more sustainable manner, thus contributing to a more food secure future globally. In addition, Transgene-free plants could be modified using SSN platforms (**Fig. 5**) via transient expression of the nuclease components using agroinfiltration or viral vectors, the delivery of the components directly as functional gRNA and Cas9 protein or developing mono-allele transgenic plants with Cas9 followed by incorporating the gRNA in a separate chromosome to the targeted locus so that they can be removed by segregation.

### ***DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST***

No potential conflicts of interest were disclosed.

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