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Genome Editing: Methods and Application in Plant Pathology

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A B S T R A C T

Genome manipulation technology is one of emerging field which brings real revolution in genetic engineering and biotechnology. Genome editing technique is consistent for improving average yield to achieve the growing demands of world's existing food famine. Because of their advantages such as simplicity, efficiency, high specificity and amenability to multiplexing, genome editing technologies are revolutionizing the way crop breeding is done and paving the way for next generation breeding. In different areas including plant research, new breeding techniques are of great concern such as plant pathogen resistance, developmental biology and abiotic stress tolerance. Meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) are the four types of nucleases used in genome editing (Jaganathan *et al.*, 2018). Homing endonuclease/meganuclease enzymes were the first among synthetic nucleases, to be used for genome editing purposes in plants, including *Arabidopsis* and maize. The recognition sites of homing endonucleases do not occur naturally in the plant genome, and this is the main limitation of these endonucleases as plant genome editing tools (Kumar & Jain, 2015). Chimeric restriction endonucleases were created as the first ZFNs and were shown to have *in vitro* activity. TALENs are similar to ZFNs and the DNA-binding domain is composed of highly conserved repeats derived from transcription activator-like effectors (TALEs), which are proteins secreted by *Xanthomonas* to alter transcription of genes in host plant cells. The type II CRISPR system is the most widely used from *Streptococcus pyogenes* (Amardeep *et al.*, 2017). Protection is provided in bacteria, the type-II CRISPR system against DNA from invading viruses and plasmids via RNA-guided DNA cleavage by Cas proteins. Indeed, these emerging technologies have the ability to manipulate and study model organisms and these technologies promise to expand our ability to explore and alter any genome and constitute a new and promising paradigm to understand and treat disease (Gaj *et al.*, 2013).

Background

Genetic engineering can accelerate the advancement of improved crops and animals. Firstly genetically modified (GM) crops were popularized in 1996. From that point forward the cultivated area has expanded 100 overlays with 28 nations growing these crops. About 2000 examinations have been distributed assessing the wellbeing of GM crops; thus far

the outcomes recommend that the effect of GM crops on nourishment and ecological security are very little not quite the same as expectedly conventional crops produced. Nevertheless, there is continued uncertainty toward this technology (James, 2014). The field of genome altering is encountering quick development as new techniques and advances keep on rising. Utilizing genome editing to increase agriculture productivity is required as the total population is relied upon to develop to 9.6 billion by 2050 while the area of arable land diminishes (Ray *et al.,* 2013). Besides potential for boosting crop yields, genome editing is now one of the best tools for carrying out reverse genetics and is emerging as an especially versatile tool for studying basic biology. Genetically modified plants are separated from regular transgenic plants as they may not join remote DNA.

In spite of the fact that genome editing can be utilized to bring outside DNA into the genome, it might just include changes of a couple of base combines in the plant's own DNA. This qualification makes genome altering a novel and amazing tool. Genome editing technique is performing outstandingly for increasing crop yield and proved to be important tool to fulfil the demand of the world's population and food famine and to become a realistic and environment friendly agriculture system, to more precise, fruitful, gainful approach. Moreover, public discomfort for utilizing GM crops is further intensified when speaking on introduction of 'foreign' genes from faintly related organisms as this is apparent as 'unnatural' despite emerging evidence to the contrary. For example, natural sweet potato varieties are now known to harbour T-genes from *Agrobacterium tumefaciens* (Verma, 2013; Lucht, 2015). These new and advanced strategies are shortly reviewed here and shown that how these are reliable tool for improving plants in desirable way.

Introduction

Plant breeding has been the most successful approach for developing new crop varieties since domestication occurred, making possible major advances in feeding the world and societal development. Crops are susceptible to a large set of pathogens including fungi, bacteria, and viruses, which cause important economic losses (FAO, 2017). Current crop improvement strategies include artificially mutating genes by chemical mutagenesis and ionizing radiation (Pathirana, 2011) or introducing new genes through *Agrobacterium tumefaciens*-mediated transformation (Gelvin, 2003) and direct gene transfer (Dunwell, 2014). The first strategy, known as 'classical mutagenesis', is limited by the fact that the genetic changes are induced randomly, so it is necessary to screen a large number of individuals to identify those carrying a mutation in the gene of interest and it then still remains unclear which alterations (if any) the other random induced mutations may cause. The latter transgenic approach also relies on random integration of transgene and faces many regulatory and public acceptance hurdles. With current breeding technologies, yield increases are still not currently projected to meet the demand of a growing population, diet changes and the use of bio fuels (Ray *et al.*, 2013).

However, conventional genetic engineering strategy has several issues and limitations, one of which is the complexity associated with the manipulation of large genomes of higher plants (Nemudryi *et al.,* 2014). Currently, several tools that help to solve the problems of precise genome editing of plants are at scientists' disposal. In 1996, for the first time, it was shown that protein domains such as "Zinc fingers" coupled with *Fok*I endonuclease domains act as site-specific nucleases (zinc finger nucleases (ZFNs)), which cleave the DNA in vitro in strictly

defined regions (Kim *et al.,* 1996). Such a chimeric protein has a modular structure, because each of the "Zinc finger" domains recognizes one triplet of nucleotides. This method became the basis for the editing of cultured cells, including model and nonmodel plants (Gaj *et al.,* 2013). 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). However, the low efficiency, long duration of studies, mutagenic effects and off-target effects has troubled 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 (McManus and Sharp, 2002). 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 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). 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). Alternative strategies were provided 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).

Mechanisms of genome editing systems

This core technology – commonly referred to as 'genome editing' – is based on the use of engineered nucleases composed of sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module (Urnov *et al.,* 2010; Carroll, 2011).

Novel genome editing tools, also referred to as genome editing with engineered nuclease (GEEN) technologies, allow cleavage and rejoining of DNA molecules in specified sites to successfully modify the hereditary material of cells. To this end, special enzymes such as restriction endonucleases and ligase can be used for cleaving and rejoining of DNA molecules in small genomes like bacterial and viral genomes. However, using restriction endonucleases and ligases, it is extremely difficult to manipulate large and complex genomes of higher organisms, including plant genomes.

The problem is that the restriction endonucleases can only "target" relatively short DNA sequences. While such specificity is enough for short DNA viruses and bacteria, it is not sufficient to work with large plant genomes. The first efforts to create methods for the editing of complex genomes were associated with the designing of "artificial enzymes" as oligonucleotides (short nucleotide sequences) that could selectively bind to specific sequences in the structure of the target DNA and have chemical groups capable of cleaving DNA (Knorre and Vlasov, 1985). Moreover, many studies have

used physical, chemical, or biological (e.g., T-DNA/ transposon insertion) mutagenesis to identify mutants and construct mutant libraries corresponding to tens of thousands of genes in model plants, such as Arabidopsis (Kuromori *et al.,* 2006) and rice (Wu *et al.,* 2003; Yang *et al.,* 2013). The emergence of programmable sequence-specific nucleases (SSNs) provided a breakthrough in genome manipulation. SSNs can induce double-stranded breaks (DSBs) in specific chromosomal sites. The resulting DSBs can be repaired by the error-prone non-homologous end joining (NHEJ) pathway, often producing nucleotide insertions, deletions, and substitutions. Another independent pathway, homology-directed repair (HDR), also can repair the DSBs if homologous donor templates are present at the time of DSB formation (Symington and Gautier, 2011) (Fig. 1–4).

Meganucleases

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). The concept of gene editing with programmable nucleases began with meganucleases and has been developed over the past two decade. Meganucleases are homing endonucleases that recognize a large DNA target sequence and make a double-stranded break. Multiple families of homing endonucleases exist but the LAGLIDADG family is the most common one for genome engineering. These function as homodimers and cleave the DNA using two compact active sites (Jurica *et al.,* 1998). Direct interactions between the DNA and protein side chains recognize up to 18 bp of target DNA and changing the amino acid sequence of endonucleases alters their specificity (Seligman *et al.,* 2002). Two endonucleases fused together recognize a longer chimeric DNA sequence (Chevalier *et al.,*, 2002) and they can be engineered to recognize entirely novel sequences (Smith *et al.,* 2006). In practice meganucleases are difficult to engineer because the DNA-binding and endonuclease activities reside on the same domain, and their development has stalled compared to other programmable nucleases. 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).

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 are also hindered by patent disputes (Smith *et al.,* 2011).

Zinc Finger Nuclease (ZFNs)

ZFNs are fusion proteins consisting of "zinc finger" domains obtained from transcription factors attached to the endonuclease domain from the bacterial Fok I restriction enzyme. Zinc fingers (ZF) are proteins composed of conjugated Cys^2His^2 motifs that each recognizes a specific nucleotide triplet based on the residues in their α-helix. These are capable of sequence-specific DNA binding, fused to a nuclease domain for DNA cleavage. Each zinc finger domain recognizes a 3- base pair DNA sequence, and tandem domains can potentially bind to an extended

nucleotide sequence that is unique to a genome. The first ZFNs were created as chimeric restriction endonucleases and were shown to have *in vitro* activity (Kim *et al.*, 1996). 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 by fusing it with the catalytic domain of the FokI endonuclease in order to induce a targeted DNA break, and therefore to use these proteins as genome engineering tools (Rebar *et al.*, 2002). The DNA-binding domain of ZFNs contains several ZF motifs whose number can be changed. Three ZF motifs are believed to be the minimum to achieve the adequate specificity and affinity. Although adding more ZF motifs may enhance the binding specificity, it also increases the difficulty of ZFP gene synthesis and searching for an appropriate site. Three or four ZF motifs have been used wildly and successfully for strictly cleavage in genome (Bibikova, 2003). The identification of ZF motifs that specifically recognize each of the 64 possible DNA triplets is a key step towards the construction of "artificial" DNA-binding proteins that recognize any pre-determined target sequence within a plant or mammalian genome (Porteus, 2006). The design of ZFNs is considered difficult due to the complex nature of the interaction between zinc fingers and DNA and further limitations imposed by context-dependent specificity. The Fok I nuclease domain requires dimerization to cleave DNA and therefore two ZFNs with their C-terminal regions are needed to bind opposite DNA strands of the cleavage site (separated by 5–7 bp). The FokI domain has been crucial to the success of ZFNs, as it possesses several characteristics that support the goal of targeted cleavage within complex genomes. The ZFN monomer can cut the target site if the two ZF-binding sites are palindromic. This spacing allows the two inactive FokI nuclease domains to dimerize, become active as a nuclease and create a double-stranded DNA break (DSB) in the middle of the spacer region between the two ZFNs. The DSB is often repaired by the NHEJ DNA repair mechanism that is error-prone. That is, during the repair process, usually small number of nucleotides can be deleted or added at the cleavage site (Sander, 2011). 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).

Transcription activator like effector nucleases (TALENs)

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). Transcription activator like effector nucleases (TALENs) have rapidly emerged as an alternative to ZFNs for genome editing and introducing targeted DSBs. TALENs are similar to ZFNs and comprise a non-specific Fok I nuclease domain fused to a customizable DNA-binding domain. The DNA-binding domain is composed of highly conserved repeats derived from transcription activator-like effectors (TALEs), which are proteins secreted by *Xanthomonas* bacteria to alter transcription of genes in host plant cells (Boch *et al.*, 2010). These bacteria are pathogens of crop plants, such as rice, pepper, and tomato; and they cause significant economic damage to agriculture, which was

the motivation for their thorough study. The bacteria were found to secrete effector proteins (TALEs) to the cytoplasm of plant cells, there they enter the nucleus, bind to effector-specific promoter sequences, and activate the expression of individual plant genes, which can either benefit the bacterium or trigger host defences (Kay *et al.*, 2007). Co-crystal structures of TALE DNA-binding domains bound to their cognate sites have shown that individual repeats comprise two-helix v-shaped bundles that stack to form a superhelix around the DNA and the hypervariable residues at positions 12 and 13 are positioned in the DNA major-groove. The residues at position 8 and position 12 within the same repeat make a contact with each other that may stabilize the structure of the domain while the residues at position 13 can make base-specific contacts with the DNA (Mak *et al.*, 2012).

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 Cellestis 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.*, (2015) demonstrate 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. In the short time since the first TALENs were reported, they have proven powerful reagents for reverse genetics in multiple experimental systems. They are rapidly being employed to ameliorate genetic diseases through gene therapy and to solve challenges in agriculture.

The CRISPR/Cas9 system

Until 2013, the dominant genome editing tools were zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Christian *et al.*, 2010). Distant arrays of short repeats interspaced with unique spacers (CRISPR loci) have been observed in bacterial and archaeal genomes for a long time. Three research groups independently reported the homology of hyper variable spacer sequences with viral genome and plasmid sequences (Bolotin *et al.*, 2005; Mojica *et al.*, 2005; Pourcel *et al.*, 2005). These studies hypothesized that CRISPR loci and Cas proteins could play a role in imparting immunity against transmissible genetic elements. Recently, the unique ability of the CRISPR–Cas system to degrade the genetic material of invading foreign DNA is being exploited as a genome editing tool. The CRISPR–Cas system is present in most archaeal (90%) and many bacterial (48%) genomes (Rousseau *et al.*, 2009). This system has the ability to incorporate short sequences of non-self genetic material (spacers) at specific locations

within the CRISPRs in the genome (Bhaya *et al.,* 2011; Wiedenheft *et al.,* 2012). Recently, the bacterial type II clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system has attracted attention due to its ability to induce sequence specific genome editing. In bacteria, the CRISPR system provides acquired immunity against invading foreign DNA via RNA-guided DNA cleavage. The latest ground-breaking technology for genome editing is based on RNA-guided engineered nucleases, which already hold great promise due to their simplicity, efficiency and versatility. The most widely used system is the type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR-associated) system from *Streptococcus pyogenes.* CRISPR/Cas systems are part of the adaptive immune system of bacteria and archaea, protecting them against invading nucleic acids such as viruses by cleaving the foreign DNA in a sequence-dependent manner. A prerequisite for cleavage of the target DNA is the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5′-NGG-3′ but less frequently NAG (Jinek *et al.,* 2012). Different variants of Cas9, such as native Cas9, Cas9 nickase, and dCas9 (nuclease-deficient Cas9), can be employed for different applications. Wild-type humanized Cas9 (hCas9) has been used in mammalian cells to generate gene knockouts (Cho *et al.,* 2013; Cong *et al.,* 2013; Mali *et al.,* 2013).

Till today, genome-editing protocols have adopted three different types of Cas9 nuclease. The first Cas9 type can cut DNA site-specifically and results in the activation of DSB repair. Cellular NHEJ mechanism is used to repair DSBs (Hsu *et al.,* 2013).

Schaeffer and Nakata, (2015) concluded that, as a consequence, insertions/deletions (indels) take place that interrupt the targeted loci. Otherwise, if any similarity between donor template and target locus is witnessed, the DSB may be mended by HDR pathway allowing exact substitute mutations to be prepared. It cuts single strand of DNA without activation of NHEJ. As an alternative, DNA repairs took place via the HDR pathway only. Hence it produces less indel mutations (Jinek *et al.,* 2012). Mutations in the HNH domain and RuvC domain discharge cleavage activity, but do not prevent DNA binding. Therefore, this particular variant can be utilized in sequence-specific targeting of any genome regardless of cleavage. This situation can result in edited plants exempted from current GMO regulations. So we can hope for widespread application of RNA-guided genome editing in agriculture and plant biotechnology (Amardeep *et al.,* 2017).

A comparison of CRISPR/Cas9, ZFNs and TALENs

ZFNs and TALENs function as dimers and only protein components are required. Sequence specificity is conferred by the DNA-binding domain of each polypeptide and cleavage is carried out by the FokI nuclease domain. In contrast, the CRISPR/Cas9 system consists of a single monomeric protein and a chimeric RNA. Sequence specificity is conferred by a 20-nt sequence in the gRNA and cleavage is mediated by the Cas9 protein. The design of ZFNs is considered difficult due to the complex nature of the interaction between zinc fingers and DNA and further limitations imposed by context-dependent specificity. Table 1 is given below for comparison (Shah *et al.,* 2017). In comparison, gRNA-based cleavage relies on a simple Watson–Crick base pairing with the target DNA sequence, so sophisticated protein engineering for each target is unnecessary and only 20 nt in the gRNA need to be modified to recognize a different target. ZFNs and TALENs both carry the catalytic domain of the restriction

endonuclease Fok I, which generates a DSB with cohesive overhangs varying in length depending on the linker and spacer. Cas9 has two cleavage domains known as RuvC and HNH, which cleave the target DNA three nucleotides upstream of the PAM leaving blunt ends (Jinek *et al.,* 2012).

Applications of genome editing in plants

For functional genomics: - Genome modification of different types can be achieved through use of TALEN, CRISPR/Cas genome editing systems, ZFN and ODM. Through these several modifications can be created such as new gene insertion in specific locations, substitution or correction of gene fragments and individual genetic elements, point mutations and deletion of large regions of the nucleotide sequences (Zhang *et al.,* 2016).

In crop improvement: -

Blast resistance in rice: - Several genome editing techniques such as CRISPR/CAS system and TALENS are frequently applied to achieve disease resistance in a crop like rice. Interaction between the TAL effectors of targeted host infection vulnerability genes and bacterial parasite *Xanthomonas oryzae* pv. *Oryzae* cause rice blast disease (Shah *et al.*, 2018)

Aroma in rice: - Aromatic rice has primary fragrance compound.

Powdery mildew resistant wheat: - *Blumeria graminis* f. sp. *tritici* causes powdery mildew, which is one of severe wheat crop disease, it drastically reduce yield specifically in temperate zones.

Declining of phytic acid in maize: - Through the use of genome engineering technologies, significant reduction in phytic acid concentration can be achieved. In 2009, a ZFN was engineered to modify IPK1 gene, which is involved in regulation of bioagents of phytic acid.

Improved oleic level in soyabean oil:- TALENs have been utilized to slow down the two fatty acids desaturase genes activity in soyabean i.e., FAD2 and FAD3, which are responsible for converting oleic acid to linolenic acid. This technology increased oleic acid content in plants (Haun *et al.,* 2014) (Table 2–4).

Herbicide-resistant crops: - Genome editing technologies have achieved the target to generate herbicide resistant crops. ZFN mediated genome editing alter function of ACETOLACTATE SYNTHASE (ALS) gene by inducing point mutation at specific locus as this gene is specially targeted by imidazolinone (IMI) and sulfonylurea (SU) herbicide (Townsend *et al.,* 2009).

Limitations and risk

Unfortunately, because of low affinity and low specificity, gene editing with ZFNs has displayed high frequencies of off-target edits and high toxicity. It is difficult, however, to construct the nuclease protein and a new TALEN protein must be generated for each DNA target site, which increases time and costs for development. However, a crucial current concern for the CRISPR/Cas9 system is the potential for higher off-target effects than with TALENs. When the sgRNA sequence recognizes partial mismatches outside the seed sequence instead of on-target sites, then off-target edits will be produced. Researchers need to consider the ecological implications of unanticipated downstream effects when genome editing is used for plant improvement. Plant genome editing represents a wide variety of potential reagents and methodologies with potential outcomes for which off-target effects may be consequential (Zhao and Wolt, 2017).

Table.1 Comparison between different platforms of genome editing

Platforms				
Points	**ZFNs**	**TALENs**	**CRISPR/Cas9**	**Reference**
Components	Zinc finger domain Nonspecific FokI nuclease domain.	TALE DNA- binding domains Nonspecific FokI nuclease domain	crRNA/sgRNA	Kumar and Jain, 2015; Sauer *et al.*, 2016
Structural proteins	Dimeric protein	Dimeric protein	Monomeric protein	Sauer *et al.*, 2016
Catalytic domain	Restriction endonuclease FokI	Restriction endonuclease FokI	DSBs in target DNA or single strand DNA nicks	Chen *et al.*, 2016
Length of target sequence (bp)	24-36	24-59	20-22	Chen *et al.*, 2016
Protein engineering steps	Required	Required	Should not be complex to test gRNA	Sauer *et al.*,, 2016
Cloning	Necessary	Necessary	Not necessary	Weeks *et al.*, 2016
gRNA production	Not applicable	Not applicable	Easy to produce	Norman *et al.*, 2016
Mode of action	Double-strand breaks in target DNA	Double-strand breaks in target DNA	Double-strand breaks or single-strand nicks in target DNA	Sauer *et al.*, 2016
Target recognition efficiency	High	High	High	Kumar and Jain, 2015; Sauer *et al.*, 2016
Mutation rate	High	Middle	Low	Gaj *et al.*, 2013
Creation of large scale libraries	Impossible	Technically difficult	Possible	Sauer *et al.*, 2016
Multiplexing	Difficult	Difficult	Possible	Norman *et al.*, 2016

Table.2 Successful examples of genome editing in plants using ZFNs

S. No.	Plant Name	Nuclease Type	Targeted Gene	References
1	Arabidopsis	ZFN	ADH1, TT4	Zhang *et al.*, 2010
2	Soya bean	ZFN	DCL4a, DCL4b	Curtin *et al.*, 2013 Curtin *et al.*, 2011
3	Maize	ZFN	IPK1, Zein protein 15	Shukla *et al.*, 2009
4	Arabidopsis	ZFN	ABI4, KU80 and ADH1, TT4	Osakabe *et al.*, 2010 Zhang *et al.*, 2010
5	Tobacco	ZFN	SuRA, SuRB (Acetolactate synthase genes)	Townsend *et al.*, 2009
6	Cotton	ZFN	hppd, epsps	D˝Halluin *et al.*, 2013

Table.3 Successful examples of genome editing in plants using TALENs

S. No	Plant Name	Nuclease Type	Targeted Gene	References
1	Arabidopsis	TALEN	ADH1, TT4, MAPKKK1, DSK2B, NATA2, GLL22a, GLL22b	Cermak *et al.*, 2011 Char *et al.*, 2017
2	Barley	TALEN	GFP (transgene)	Gurushidze *et al.*, 2014
3	Maize	TALEN	GL2	Char *et al.*, 2015
4	Tomato	TALEN	PROCERA	Lor *et al.*, 2014
5	Rice	TALEN	11N3, DEP1, BADH2, CKX2, SD1, OsSWEET14	Li *et al.*, 2012 Shan *et al.*, 2013
6	Wheat	TALEN	MLO	Wang *et al.*, 2016
7	Potato	TALEN	ALS	Nicolia *et al.*, 2015

Table.4 Successful examples of genome editing in plants using Cas9/sg RNA

S. No	Plant Name	Nuclease Type	Targeted Gene	References
1	Soya bean	Cas9/sgRNA	GFP (transgene), miR1514, miR1509	Jacobs *et al.*, 2015
2	Sorghum	Cas9/sgRNA	DsRED2	Jiang *et al.*, 2013
3	Sweet orange	Cas9/sgRNA	PDS	Jia and Wang, 2014
4	Cotton	Cas9/sgRNA	CLA1, VP	Chen *et al.*, 2017
5	Tomato	Cas9/sgRNA	SHR, GFP (transgene), AGO, 08g041770, 07g021170, 12g044760, RIN, SIIAA9	Brooks *et al.*, 2014 Ito *et al.*, 2015 Ron *et al.*, 2014 Ueta *et al.*, 2017
6	Wheat (Durum)	Cas9/sgRNA	GASR7	Zhang *et al.*, 2016
7	Populus	Cas9/sgRNA	4CL1, 4CL2, 4CL5	Zhou *et al.*, 2015
8	Arabidopsis	Cas9/sgRNA	FT, SPL4, ABP1	Hyun *et al.*, 2015 Gao *et al.*, 2015
9	*N. tabacum*	Cas9/sgRNA	PDS, PDR6	Gao *et al.*, 2015
10	Rice	Cas9/sgRNA	MPK1, MPK2, MPK5, MPK6, PDS, SWEET11, BEL	Ma *et al.*, 2017 Xu *et al.*, 2014 Xie *et al.*, 2015
11	Wheat (common)	Cas9/sgRNA	GASR7, GW2, DEP1, NAC2, PIN1, LOX2,	Zhang *et al.*, 2016
12	Grape	Cas9/sgRNA	IdnDH	Ren *et al.*, 2016
13	*Lotus japonicus*	Cas9/sgRNA	SYMRK, LjLb1, LjLb2, LjLb3	Wang *et al.*, 2016
14	Petunia	Cas9/sgRNA	NR	Subburaj *et al.*, 2016
15	Maize	Cas9/sgRNA	ARGOS8	Shi *et al.*, 2017 Char *et al.*, 2017

Fig.1 Genome editing with designer nucleases. Specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (Amardeep *et al.*, 2017)

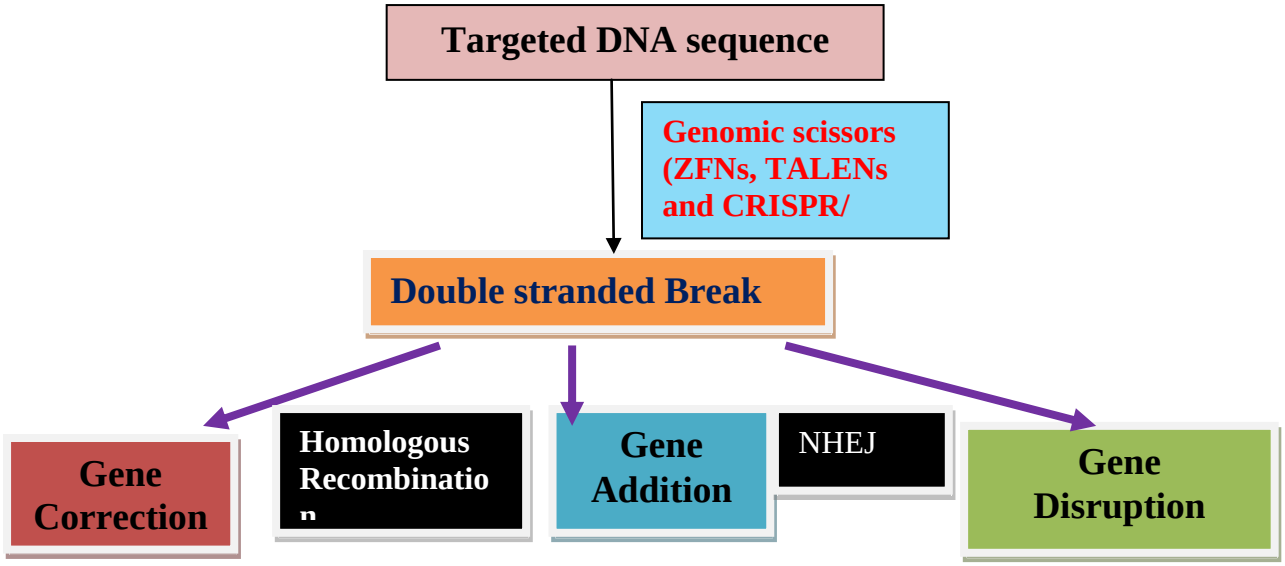


Fig.2 Structure of zinc finger nuclease and mechanism of gene editing (Amardeep *et al.*, 2017)

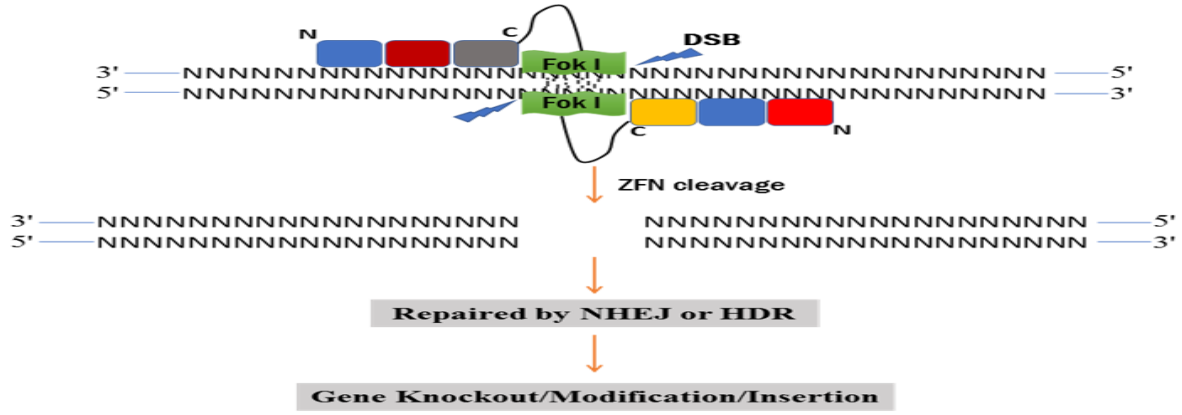


Fig.3 TALENs bind and cleave as dimers on a target DNA site (Amardeep *et al.*, 2017)

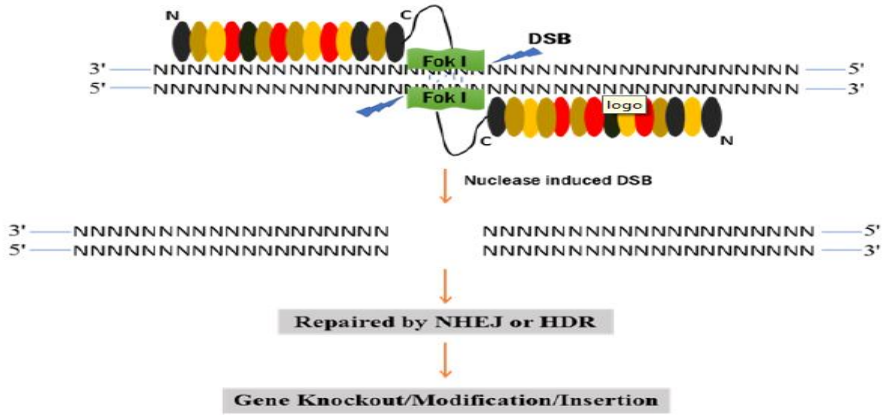
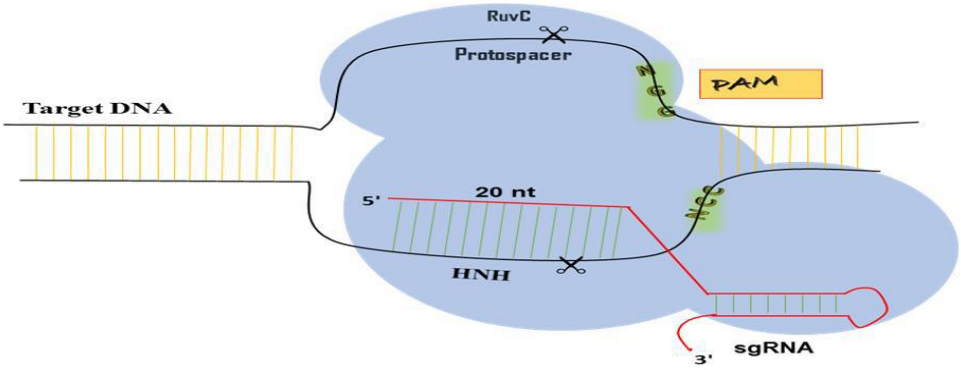


Fig.4 RNA-guided DNA cleavage by Cas9 (Amardeep *et al.*, 2017)



Conclusion and future thrust are as follows:

Genome editing tools have proved to be beneficial for functional genomics as well as crop improvement. Although, there are several limitation and considerations for genome editing technologies. High efficiency in genome editing has been translated into the quantity or screened plants in order to get the desired modification. Among various Genome Editing Systems including TALEN's, ZFN's and CRISPR-cas9, CRISPR-cas9 based platforms have proved to be more competent and less expensive and the studies about plants are made in more significant way with the development of new and improved techniques. These technologies assure to amplify and change any genome. From more fruitful research in future the understanding of multiple CRISPR cas9 system should be explored. The simplicity, flexibility, versatility, and efficiency, of CRISPR/Cas9 genome editing system will help to overstep the potential of previous genome editing systems. For crop improvement these tools are becoming more popular molecular tools of choice. These editing systems are being harnessed for unprecedented understanding of plant biology and crop yield improvement through rapid and targeted mutagenesis and associated breeding (Belhaj *et al.*, 2015; Huang *et al.*, 2016).

No ethical issue is involved with genome editing in plants compared with clinical and medical research, and thus it is more suitable for applied research. To minimize the off-target effects and to make delivery methods more efficient efforts can be made further. A key question is there that the products of genome modifications made by editing will get greater public acceptance as compared to earlier GMOs.

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