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Review Article

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Designer Nucleases for Genome Editing in Plants: Different Approaches and Applications

Amardeep[1*], Kisa Zaidi[2], Shalu Sharma[3] and Vikas Sharma[4]

[1, 4]Chaudhary Charan Singh University, Meerut-250 004, Uttar Pradesh, India

[2]Department of Biosciences, Shri Ram College, Muzaffarnagar, Uttar Pradesh, India

[3]College of Applied Education & Health Sciences (CAEHS), Meerut-250 001, Uttar Pradesh, India

*Corresponding Author E-mail: amar92home@gmail.com

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ABSTRACT

Targeted genome editing using artificial nucleases has the potential to accelerate basic research as well as plant breeding by providing the means to modify genomes rapidly in a precise and predictable manner. Zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) system comprise a powerful class of tools that are redefining the boundaries of biological research. These chimeric nucleases enable a broad range of genetic modifications by inducing DNA double-strand breaks that stimulate error prone nonhomologous end joining or homology-directed repair at specific genomic locations. In this review, recent approaches and progress of artificial nuclease technology in plants has been summarized and discussed.

Key words: *ZFNs, TALENs, CRISPR/Cas, NHEJ, HDR.*

INTRODUCTION

Recent progress in the development of molecular genetic methods enables the manipulation of genes in different organisms. Transgenic technology allows development of plants with specific qualities in shorter span of time as compared with the conventional breeding methods and allows the introduction of traits not achievable through plant breeding alone[31]. Given an increasing global population, there is a projected need to increase world food production by 40% in the next 20 years[53]. In addition to a growing population, climate change, degrading natural resources and changing food preferences have raised food and nutritional security to the level of the biggest challenge of the twenty-first century[5]. Genetically modified (GM) trait technology in the mid-1990s made a major impact in meeting the world food demand and there has been a rapid adoption of the technology. Recent advances in the area of gene manipulation methods favoured the development of genetically modified (GM) plants to improve agricultural productivity and decrease the use of fertilizers and pesticides. Genome editing with site-specific nucleases allows reverse genetics, Genome engineering and targeted transgene integration experiments to be carried out in an efficient and precise manner[6].

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In the past decade, a new approach has emerged that enables investigators to manipulate virtually any gene in a diverse range of cell types and organisms. 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[71, 9]. These chimeric nucleases enable efficient and precise genetic modifications by inducing targeted DNA double-strand breaks (DSBs) that stimulate the cellular DNA repair mechanisms, including error-prone non-homologous end joining (NHEJ) and homology directed repair (HDR) or homologous recombination (HR). In most cases, NHEJ causes random insertions or deletions (indels), which can result in frameshift mutations if they occur in the coding region of a gene, effectively creating a gene knockout. Alternatively, when the DSB generates overhangs, NHEJ can mediate the targeted introduction of a double-stranded DNA template with compatible overhangs[17, 49]. When a template with regions of homology to the sequence surrounding the DSB is available, the DNA damage can be repaired by HR, and this mechanism can be exploited to achieve precise gene modifications or gene insertions (Fig. 1).

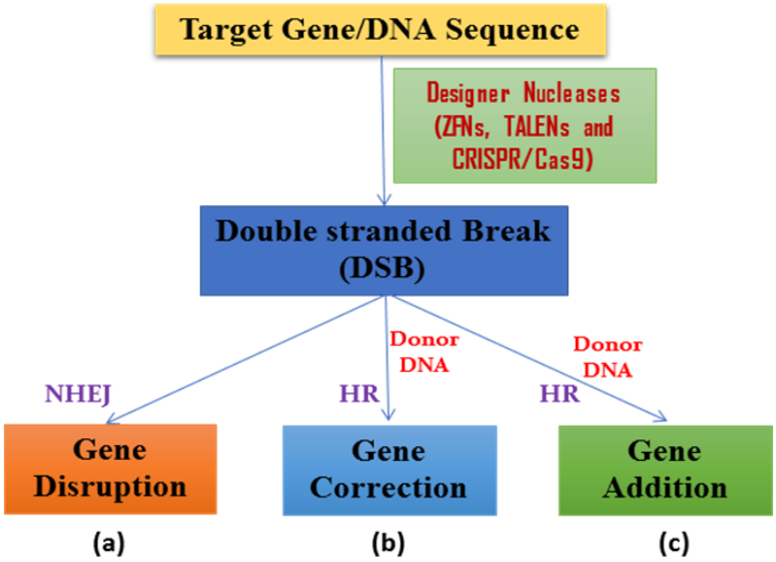

Fig. 1: Genome editing with designer nucleases. Double-strand breaks induced by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). (a) Repair by NHEJ usually results in the insertion or deletion of random base pairs, causing gene knockout by disruption. (b) HR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions or (c) to achieve gene insertion.

In this review, the development and use of the designer nucleases, zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs) and CRISPR/Cas9, for gene editing with prime focus on their application and to improving the productivity of important crop plants have been discussed.

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 [41]. 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[2, 56]. The creation of ZFNs that recognize and cleave any target sequence depends on the reliable creation of ZFPs that can specifically recognize that chosen target. 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.

The versatility of ZFNs arises from the ability to customize the DNA-binding domain to recognize virtually any sequence. An individual zinc-finger consists of approximately 30 amino acids in a conserved ββα configuration. Several amino acids on the surface of the α-helix typically contact 3 bp in the major groove of DNA, with varying levels of selectivity. The ZF motifs bind DNA by inserting the α-helix into the major groove of the DNA double helix. The α-helix residues have cross- recognition to adjacent elements, thus each motif must be chosen in the context of those around it[43].

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[62]. Commercially available ZFNs generally perform better than those designed using publicly available resources but they are much more expensive[58]. ZFNs 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. 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. If this faulty repair is in the coding region of a gene, it can disrupt the reading frame and create an inactive (knockout) gene. Alternatively, if a DNA fragment with strong homology to the disrupted is present, the new DNA fragment can bind and displace the original gene sequence by a process called homologous recombination and result in 'gene replacement' (Fig. 2). ZFNs can theoretically target any sequence but in practice the choice of targets is limited by the availability of modules based on the context dependent assembly platform[62]. A functional ZFN pair can be prepared for every ~100 bp of DNA sequence on average using publicly available libraries[40].

Successful use of ZFNs for gene editing in Arabidopsis was first reported in 2005[44] and demonstrated not only for targeted gene disruptions, but also for inheritance of the some of the mutant genes in the T1 generation. Zinc finger nucleases have also been utilized to demonstrate that several other gene editing and chromatin modification techniques are possible with designer nuclease technologies. Likewise, carefully designed pairs of ZFNs have been effective in allowing creation of both small[55] and large[57] deletions of chromosomal segments. ZFNs are chimeric proteins composed of a synthetic zinc finger-based DNA-binding domain and a DNA cleavage domain. ZFNs can be designed to cleave almost any long stretch of double-stranded DNA by modification of the zinc finger DNA-binding domain[22, 8].

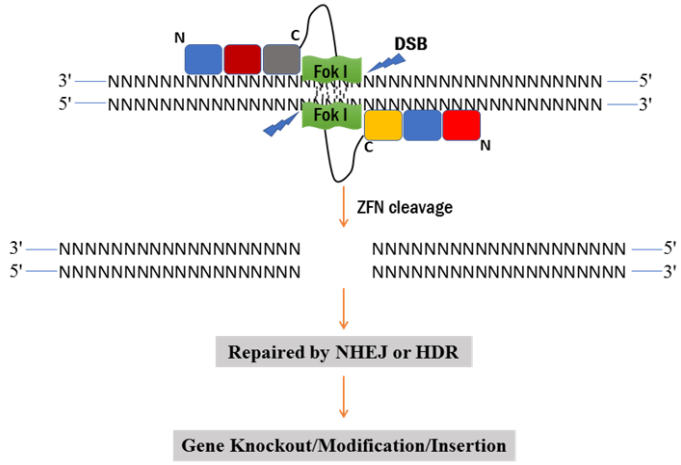

Fig. 2: Structure of Zinc Finger Nuclease. Zinc finger nucleases (ZFNs) bind to a target sequence, thereby dimerizing FokI nuclease. The DSB generated by ZFN cleavage induces DNA repair processes. Nuclease-induced double-strand breaks (DSBs) in a gene locus can be repaired by either NHEJ or HDR.

Transcription activator like effector nucleases (TALENs)

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 **(TALE**s**),** which are proteins secreted by *Xanthomonas* bacteria to alter transcription of genes in host plant cells[3]. 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 defenses[39]. Further investigation of the effector protein action mechanisms revealed that they are capable of DNA binding and activating the expression of their target genes via mimicking the eukaryotic transcription factors. TALE proteins are composed of a central domain responsible for DNA binding, a nuclear localization signal, and a domain that activates the target gene transcription[64].

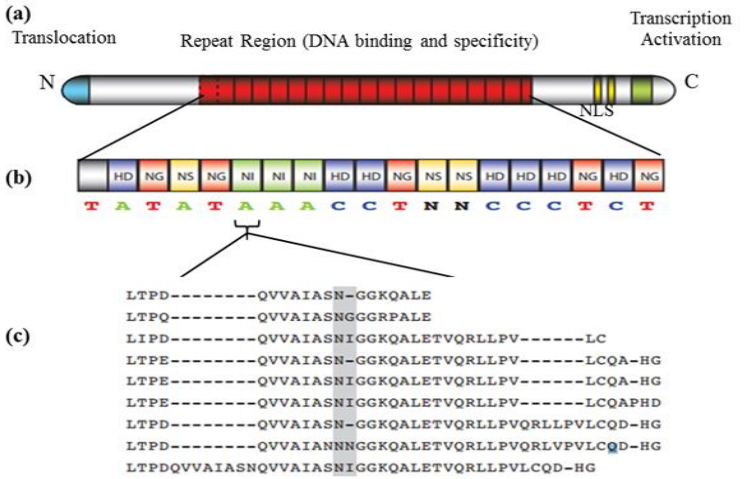

Fig. 3: Functional domains of *Xanthomonas* TAL effectors and hypervariable region. (a) The N-terminal region contains the type III translocation signal. The C-terminal region contributes to DNA binding and is indicated with dashed lines. NLS, nuclear localization signal; transcription activation domain that is required for transcriptional activation. Repeats of different length in known TAL effectors. (b) TALE-derived DNA-binding domain aligned with its target DNA sequence. Note the matching of repeat domains to single bases in the target site according to the TALE code. (c) The hypervariable amino acids at positions 12 and 13 that confer DNA target specificity are shaded in gray.

TALEN uses a set of modular DNA recognition domains, each of the 30-35 amino acid repeat domains recognizes one base pair of DNA. The repeats are located centrally in the protein between N-terminal sequences required for bacterial type III secretion and C-terminal sequences required for nuclear localization and activation of transcription. Target recognition specificity of TALEs repeat is determined by the presence of repeat variable di-residue (RVD) at 12 and 13 positions of each repeat[50]. More than 20 unique RVD sequences have been observed in TAL effectors, but just seven – HD, NG, NI, NN, NS, "N*" (which corresponds to a 33 residue repeat in which the RVD appears to be missing its second residue), and HG – account for nearly 90% of all repeats[3] and respectively specify C, T, A, G/A, A/C/T/G, C/T, and T[4, 50]. These relationships enable prediction of targets for existing TAL effectors, and engineering of artificial TAL effectors that bind DNA sequences of choice. Nearly all engineered TALE repeat arrays published to date use four domains with the hypervariable residues NN, NI, HD and NG, for the recognition of G, A, C and T, respectively. Engineered TALE repeats with desired specificities can be created using the code. 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[47, 21] (Fig. 3). Sets of domains linked together to identify a desired sequence can be fused in-frame with FokI endonuclease to form genome-specific restriction enzymes. Each repeat in the DNA-binding domain of a TALEN recognizes one nucleotide in the target in a largely context-independent fashion, making the engineering of nucleases with new DNA sequence specificities easier and more reliable than ZFNs[60]. TALENs enable a broad range of genetic modifications by inducing DNA DSBs that stimulate error-prone non-homologous end joining or homology-directed repair at specific genomic locations[24] (Fig. 4).

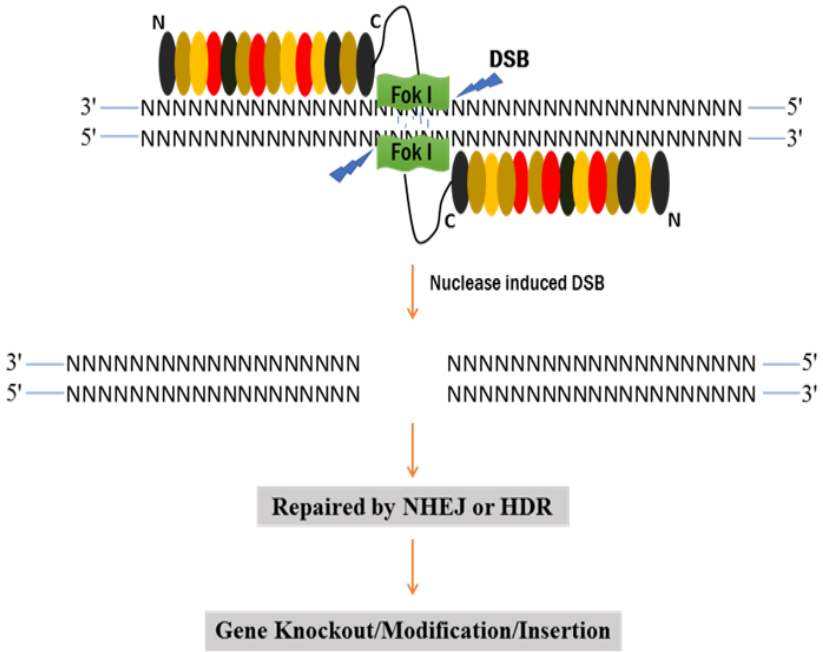

Fig. 4: TALENs bind and cleave as dimers on a target DNA site. Note that the TALE-derived amino- and carboxy-terminal domains flanking the repeats may make some contacts to the DNA. Cleavage by the FokI domains occurs in the "spacer" sequence that lies between the two regions of the DNA bound by the two TALEN monomers.Nuclease-induced double-strand breaks (DSBs) in a gene locus can be repaired by either NHEJ or HDR.

TALENs can be used to increase the overall number of bases that are specifically recognized in the target DNA[48]. 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[41] (ZFNs) and transcription activator-like effector nucleases (TALENs)[14]. 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*[38]. 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. The immunity is acquired by the integration of short fragments of the invading DNA known as spacers between two adjacent repeats at the proximal end of a CRISPR locus. The CRISPR arrays, including the spacers, are transcribed during subsequent encounters with invasive DNA and are processed into small interfering CRISPR RNAs (crRNAs) approximately 40 nt in length, which combine with the transactivating CRISPR RNA (tracrRNA) to activate and guide the Cas9 nuclease[1]. This cleaves homologous double-stranded DNA sequences known as protospacers in the invading DNA[1]. 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′[27, 38] but less frequently NAG[30]. Although a 20 bp recognition sequence in the single-guide RNA (sgRNA) was initially considered necessary to determine specificity, it was later shown that a perfect match between the 7–12 bp at the 3′ end of the sgRNA (called the seed region) and the equivalent region of the target DNA confers target site recognition and cleavage (Fig. 5), whereas multiple mismatches in the PAM-distal region are generally tolerated[30, 23, 37, 54]. Moreover, some initial *in vitro* and *in vivo* evidence suggested that Cas9 endonuclease activity is not affected by DNA CpG methylation[30].

Type II system, comprises of crRNA and trRNA that combine into one sgRNA (single guide RNA)[38]. Amazingly, the sgRNA programmed Cas9 appeared more effective in targeted gene modifications rather than individual trRNA and crRNA. 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[30]. As a consequence, insertions/deletions (indels) takes 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[29, 63].

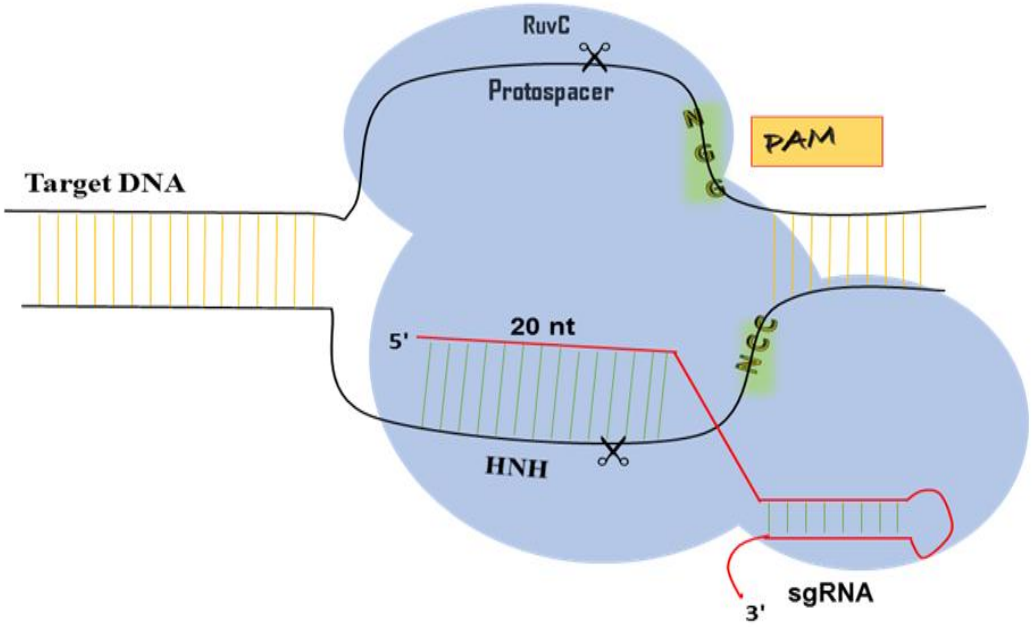

Fig. 5: RNA-guided DNA cleavage by Cas9. Cas9 can be reprogrammed to cleave DNA by a single guide RNA molecule (sgRNA), which contains a 20-nt segment determining target specificity which activates Cas9 to cleave the target DNA (protospacer). The presence of a protospacer-adjacent motif (PAM), i.e., an NGG (or less frequently NAG) sequence directly downstream from the target DNA, is a prerequisite for DNA cleavage by Cas9. Among the 20 RNA nucleotides determining target specificity, the so-called seed sequence of approximately 12 nt upstream of the PAM is thought to be particularly important for the pairing between RNA and target DNA

Cong et al.[16] introduced advanced Cas9-D10A, a mutant form having more précised nickase activity. 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[38, 16]. Mutations in the HNH domain and RuvC domain discharge cleavage activity, but do not prevent DNA binding[27]. 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.

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 (Table 1). 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[62]. 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 (Fig. 4), which cleave the target DNA three nucleotides upstream of the PAM leaving blunt ends[38].

Table 1: Comparison of ZFN, TALEN and CRISPR/Cas9

	Zinc Finger Nucleases (ZFNs)	Transcription factor like effector nucleases (TALENs)	CRISPR/Cas9
DNA binding Determinant	Zinc finger protein	Transcription- activator -like effector protein	crRNA/sgRNA
Endonuclease	FokI	FokI	Cas9
Mode of Action	DSBs in target DNA	DSBs in target DNA	DSBs in target DNA or single-strand DNA nicks (Cas9 nickase).
Target site length (bp)	18-36	30-40	20-22
Binding site	3 nucleotides	1 nucleotide	1:1 nucleotide pairing
Binding Specificity	High	High	Low
Structural proteins	CRISPR consists of single monomeric protein and chimeric RNA	ZFNs work as dimeric and only protein component required	TALENs also work as dimeric and require protein component
Catalytic domain	two cleavage domains called RuvC and HNH	catalytic domain of restriction endonuclease FokI which generates a DSB	catalytic domain of restriction endonuclease FokI which generate a DSB

Applications of genome editing in plants

Plants provide us with food, animal feed, medicines, chemicals, renewable materials and biofuels. The domestication of plants has involved the development of strategies to improve the performance of crops and tailor their properties. The availability of beneficial alleles in nature therefore limits what can be achieved using this approach. New alleles can be introduced by random mutagenesis, but this must be followed by the time consuming screening of large populations to identify mutants with desirable properties. Table 2, 3 and 4 contain successful examples of genome editing in plants using designer nucleases. Genes encoding CRISPR-Cas9 components have successfully been expressed both stably and transiently in plants. Multiple targets can be edited simultaneously when several gRNAs are expressed in one cell. Genes encoding CRISPR-Cas9 components have successfully been expressed both stably and transiently in plants. Multiple targets can be edited simultaneously when several gRNAs are expressed in one cell. Considering that redundant genes are common in plant genomes, CRISPR-Cas9-mediated multiplexed genome editing could yield higher order mutants with relative ease compared with conventional crossing methods. Genome editing can therefore accelerate plant breeding by allowing the introduction of precise and predictable modifications directly in an elite background, and the CRISPR/Cas9 system is particularly beneficial because multiple traits can be modified simultaneously.

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

S. No.	Plant Name	Nuclease Type	Targeted Gene	Reference
1	Arabidopsis	ZFN	ADH1, TT4	76
2	Soya bean	ZFN	DCL4a, DCL4b	18,19
3	Maize	ZFN	IPK1, Zein protein 15	67
4	Arabidopsis	ZFN	ABI4, KU80 and ADH1, TT4	52,76
5	Tobacco	ZFN	SuRA, SuRB (Acetolactate synthase genes)	69
6	Cotton	ZFN	hppd, epsps	20

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

S. No	Plant Name	Nuclease Type	Targeted Gene	Reference
1	Arabidopsis	TALEN	ADH1, TT4, MAPKKK1, DSK2B, NATA2, GLL22a, GLL22b	10, 15
2	Barley	TALEN	GFP (transgene)	28
3	Maize	TALEN	GL2	12
4	Tomato	TALEN	PROCERA	45
5	Rice	TALEN	11N3, DEP1, BADH2, CKX2, SD1, OsSWEET14	42, 65
6	Wheat	TALEN	MLO	73
7	Potato	TALEN	ALS	51

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

S. No	Plant Name	Nuclease Type	Targeted Gene	Reference
1	Soya bean	Cas9/sgRNA	GFP (transgene), miR1514, miR1509	34
2	Sorghum	Cas9/sgRNA	DsRED2	36
3	Sweet orange	Cas9/sgRNA	PDS	35
4	Cotton	Cas9/sgRNA	CLA1, VP	13
5	Tomato	Cas9/sgRNA	SHR, GFP (transgene), AGO, 08g041770, 07g021170, 12g044760, RIN, SIIAA9	7, 61, 33, 70
6	Wheat (Durum)	Cas9/sgRNA	GASR7	77
7	Populus	Cas9/sgRNA	4CL1, 4CL2, 4CL5	78
8	Arabidopsis	Cas9/sgRNA	FT, SPL4, ABP1	32 26
9	*N. tabacum*	Cas9/sgRNA	PDS, PDR6	25
10	Rice	Cas9/sgRNA	MPK1, MPK2, MPK5, MPK6, PDS, SWEET11, BEL	74, 46, 75
11	Wheat (common)	Cas9/sgRNA	GASR7, GW2, DEP1, NAC2, PIN1, LOX2,	77
12	Grape	Cas9/sgRNA	IdnDH	59
13	*Lotus japonicus*	Cas9/sgRNA	SYMRK, LjLb1, LjLb2, LjLb3	72
14	Petunia	Cas9/sgRNA	NR	68
15	Maize	Cas9/sgRNA	ARGOS8	66, 11

CONCLUSION

In summary, progress in the field of genome editing has been remarkable, and there is growing evidence to indicate that the delivery of genome-editing materials can be achieved in vitro and in vivo in a range of tissues. ZFNs, TALENs and especially, Cas9/sgRNA systems for gene editing and the rapid development of new and improved techniques opening significant opportunities for studying plant growth, development and productivity. The structural analysis of Cas9 and its interaction with gRNA and target DNA will facilitate the development of engineered nucleases with greater efficiency and specificity. CRISPR/Cas9 and other gene editing technologies now have the same kind of potential to allow, at modest cost, the same kind of accelerated progress not only with model plants, but also with crop plants. These technologies promise to expand our ability to explore and alter any genome.

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