Genome Editing: Tools and Application in Plants

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Abstract

The emergence of genome editing methods promises a real revolution in genetic engineering. These technologies rely on engineered nucleases that cleave DNA in a sequence-specific manner because of the presence of a sequence-specific DNA-binding domain or RNA sequence. Genome editing by engineered nuclease have the potential to change the genomic architecture of a genome at precise locations, with desired accuracy. Several engineered nucleases, including zinc finger nucleases (ZFNs) and TAL effectors nucleases (TALENs) and CRISPR CAS 9 have been used in plants, promising to revolutionize conventional methods of genetic engineering. Targeted editing of the genomes of an organism used to improve productivity and quality of crops. Given the power of genome editing tools and the increasing number of researchers using and developing these tools, a revolutionary change is taking place in crop that resistance to various biotic and abiotic stresses to meet the increasing demand for food and ensure world food security in the future. The review highlights the broad applicability of engineered Nuclease (ZFN, TALEN, and CRISPR CAS 9) mediated targeted plant genome editing and their application for development of designer crops.

Keywords: Genome Editing; Talens; ZNF; CRSPR CAS9; Engineered Nucleas

Abbreviations: ZFNs: zinc finger nucleases; TALE: transcription activator-like effector; TALENs: TAL effectors nucleases; OMM: oligonucleotide-mediated mutagenesis; SDNs: site-directed nucleases DSBs: double-strand breaks; HDR: homologous Directed recombination; T-DNA: transfer DNA; RNAi: RNA interference; HR: homologous recombination; CRISPR: clustered regularly interspaced short palindromic repeats; PAM: protospacer adjacent motifs; FAD: Fatty acid desaturase

Introduction

Since the discovery of the DNA double helix in 1953, many basic biological concepts such as gene transcription and translation, genetic code and epigenetic modification, have been established by developing multiple experimental techniques. These include enzymes for in vitro DNA manipulations (such as polymerases, restriction endonucleases and DNA ligases), recombinant DNA technology, in vitro DNA synthesis, site-specific mutagenesis, and whole-genome sequencing. Nonetheless, site-specific modification within genomes has remained a major challenge [1].

The earliest method of genome editing in higher plants involved oligonucleotide-mediated mutagenesis (OMM) to cause site-specific gene targeting using chemically synthesized oligonucleotides with base replacement or addition caused by endogenous DNA-repair enzymes. The method differs from genome editing with engineered
Gene editing using programmable nucleases is a key component of the future in crop improvement and will significantly contribute to increasing crop yield without additional land, increase disease and herbicide resistance, addition of nutritional value to crops through advancing plant biology research and directly editing crops [7]. In this review, I briefly described recent method of genome editing systems and their application in plants and crop improvement.

**Genome Editing Tools**

The use of synthetic biology requires a complete understanding of the biological processes that need to be integrated into the genome. Several DNA, RNA, and protein-based tools have been developed to edit and incorporate suitable agronomic traits into the desired crops. Random integration of genes into the existing genomes of target organisms to obtain a transgene construct is one of the most common mechanisms for gene targeting [8,9]. Hence, plant biologists used transposons or retro-transposons to incorporate a transfer DNA (T-DNA) insertion mutant, resulting in random insertions [10]. Sometimes, the random insertion fails to completely knockout the open reading frame of a gene, leading to the increased possibility of obtaining mutant plants with partial functions, dominant-negative effects, or aberrant protein products. The introduction of single nucleotides into the genes (or amino acids into the proteins) cannot be completed using such methods. Hence, chemical mutagenesis methods and target-induced local lesions in genomes have been developed to overcome such problems [11,12].

With the beginning of the first transgenic experiments in the 1980s, strategies have been developed to establish new traits in crop plants by combinatorial use of strong or tissue-specific promoters fused to protein encoding genes. After realizing that certain transgenes and even similar endogenous genes were silenced, strategies were established for knocking down genes responsible for certain unwanted traits, which are based on RNA interference (RNAi) [13]. However, these approaches did not lead to complete gene knockouts in many cases and have not been widely adopted in plant breeding so far. Many efforts have been undertaken to develop homologous recombination (HR) in plants, which was widely used in bacteria, yeast and mouse for gene replacements or corrections, but could not be established in plants with a promising success rate [14]. A paradigm shift was established in the middle of the 1990s with the

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Gudeta D. Genome Editing: Tools and Application in Plants. J Microbiol Biotechnol, 2019, 4(1): 000135.

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introduction of double strand breaks (DSBs) by mega nucleases that have a recognition site of 18 bp and were first identified in yeast mitochondria. With induced DSBs by mega nucleases, much higher numbers of HR events could be observed [15] and finally DSBs have also lead to induced mutations by incorrect repair mechanisms. With the use of mega nucleases, the position for a HR or a putative mutation was exactly predictable [16].

However, recognition sites are randomly scattered in the genome and a redesign of recognition motifs for specific target genes is very laborious. With the invention of zinc finger nucleases (ZFNs), for the first time every gene could be targeted. The discovery of transcription activator-like effector (TALE) proteins, which are channeled to plant cells by bacterial pathogens of the genus Xanthomonas to activate plant genes and, thereby, increasing the virulence of the pathogen allowed an even easier targeting of genome editing sites [17]. In recent years, we have the fastest spread of a method in biology ever that achieves genome editing. It relies on an adaptive immune response of bacteria and archae bacteria and is based on clustered regularly interspaced short palindromic repeats (CRISPR). This allows bacteria to identify invading DNA by a small RNA encoded by the so-called spacer, which was taken up by the species during a historical or more recent attack of virus or plasmids [18].

**Zinc Finger Nucleases**

ZFNs are chimeric molecules with three to four zinc finger DNA binding domains, from which each recognizes a triplet of nucleotides by binding, and a FokI nuclease [19]. Zinc finger nucleases are proteins bearing multiple zinc finger domains that are capable of recognizing a specific sequence of six to nine consecutive base pairs within the genome of a particular organism. To the C-terminal end of this DNA recognition molecule is added a nonspecific nuclease domain from the restriction enzyme FokI to create one-half of a ZFN pair. The second half of the pair has a similar structure and designed to recognize and bind to a DNA sequence on the opposite DNA strand approximately 6 nucleotides away from the first ZFN [20].

The DSB is often repaired by the nonhomologous end joining (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 gene (but not the exact same sequence) 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’ [21,22].

Successful use of ZFNs for gene editing in Arabidopsis was first reported in 2005 [23]. Then reported in tobacco 2005 [24]. An important step forward in making gene replacement through HR more facile was the recent design of plasmids containing DNA replication origins from Gemini viruses. These modified viruses allowed delivery of ZFNs (or Cas9/sgRNA genes) and, simultaneously, delivery of fragments of DNA that were homologous to target gene sequences and that contained a desired gene mutation [25]. Zinc finger nucleases have also been utilized to demonstrate that several other gene editing and chromatin modification techniques are possible with designer nuclease technologies [26]. For example, cell- and tissue-specific gene expression is possible, as shown by the ability to localize gene expression to the egg cell in Arabidopsis [27]. A designed pairs of ZFNs have been been effective in allowing creation of both small and large [28,29] (Figure 1). Deletions of chromosomal segments [30] (Table 1).
Organism | Method | References
--- | --- | ---
*Danio rerio* | Embryo Injection Zygote | [31]
*Hemicentrotus pulcherrimus* | Injection | [32]
*Hemichrodonchus pulcherrimus* | Embryo, injection | [33]
*Rattus norvegicus* | Zygote, injection | [34]
*Mus musculus* | Zygote, injection | [35]
*A. thaliana* | DNA transformation | [36]
*Nicotiana sp.* | Cell culture | [37]
*Zea mays* | Viral delivery | [38]
*Homo sapiens* | Viral delivery | [39]
*M. musculus* | DNA transformation | [40]
*Cricetulus griseus* | DNA transformation | [41]
*Sus domestica* | DNA transformation | [42]

Table 1: Reported instances of successful ZFN-induced gene target

**Transcription Activator-Like Effectors Nucleases (Talens)**

Transcription activator-like effectors nucleases are engineered from fusing a TAL effectors DNA-binding domain to a DNA cleavage domain (a nuclease which cuts DNA strands). Transcription activator-like effectors (TALEs) can be engineered to bind to practically any desired DNA sequence, so when combined with a nuclease, DNA can be cut at specific locations [43]. The restriction enzymes can be introduced into cells, for use in genome editing with engineered nucleases (Figure 2).

**Crispr/Cas9 Technology**

The search for an evolutionary genome editing approach results in the advent of a system called clustered Regularly Interspaced Short Palindromic Repeat (CRISPR/Cas9). When compared with previous genome editing systems (TALENs and ZFNs), CRISPR/Cas9’s simplicity, efficiency, specificity, minimal off-target effects, and amenability to multiplexing has brought hasty genetic manipulation in almost all tested eukaryotes CRISPR/Cas9 Technology. CRISPR/Cas9 system was first discovered by Japanese scientist in bacteria as an adaptive immune system by which enables the bacteria to defend against invading foreign DNA, like bacteriophage. Later on they were found in 40 % of sequenced bacterial genomes and 90% of the archaea [46].

The CRISPR system is composed of CRISPR loci in the genome and a Cas9 protein. The engineered CRISPR/Cas9 system contains the following three main components: the CRISPR associated protein 9 (Cas9), and two noncoding CRISPR RNAs (crRNAs). A trans-activating crRNA (tracrRNA) and a precursor crRNA (precr RNA). Cas9 contains an HNH nuclease domain and a RuvC-like nuclease domain that involved in the crRNA maturation process and crRNA-guided DNA cleavage. The tracrRNA is
a small trans encoded RNA complementarity to the repeats within the pre-crRNA. The pre-crRNAs are transcribed from CRISPR loci. The palindromic repeats (usually between 23 and 47 bp) are typically identical in length and sequence within a CRISPR locus. The spacers (typically 21-72 bp) are derived from invading viral DNA and guide Cas9 to cleave an invading protospacer. The pre-crRNA encompasses much of the CRISPR repeat-spacer array and is transcribed together with the tracrRNA. Subsequently, the tracrRNA hybridizes with the pre-crRNA to form an RNA duplex and associates with Cas9. The mature crRNA:tracrRNA duplex directs Cas9 to the DNA target sequence consisting of protospacer adjacent motifs (PAM) and complementary protospacer sequence. Finally, the Cas9 RuvC-like nuclease domain cleaves the DNA strand that is complementary to the RNA guide while the RuvC-like nuclease domain cleaves the DNA strand that is non-complementary to the target to create a DSB within the protospacer about 3-4 nucleotides upstream of the PAM [47-49]. A Protospacer Adjacent Motif (PAM) downstream of the gRNA binding region is required for Cas9 recognition and cleavage as illustrated in (Figure 3) [50]. Cas9/gRNA cuts both strands of the target DNA, triggering endogenous Double Strand Break (DSB) repair. For a knockout experiment, the DSB is repaired via the efficient but error-prone Non Homologues End Joining (NHEJ) pathway, which introduces an indel at the DSB site that knocks out gene function. In a knock-in experiment, the DSB is repaired by Homology Repair (HR) using the donor template present, resulting in the donor DNA sequence integrating into the DSB site.

**NHEJ based genome editing by CRISPR-Cas9**

Due to ease of engineering, CRISPR-Cas9 has been widely adopted for genome editing in plants (Table 2). CRISPR-Cas9 quickly moved beyond proof-of-concept; promoting a reverse genetics revolution in plant research and creating many desirable traits in major crops. Using rice as an example, multiple yield-related genes have been targeted in rice [50].

CRISPR-Cas9 has been widely used for functional study on rice genes. In addition, environment induced male sterility has been engineered to facilitate hybrid-based breeding [51]. Disease resistance traits have been developed by knocking out host genes in rice and Arabidopsis [52]. The intrinsic property of CRISPR-Cas9 for targeting viral DNA for cleavage makes it a great tool to increase plant immunity against DNA viruses (Table 3).

| Plant species | Target gene            | Modification | Reference |
|---------------|------------------------|--------------|-----------|
| Arabidopsis   | PDS3, FLS2, RACK1b,    | NHEJ         | [53]      |
| Arabidopsis   | BR11, GAL1, JAZ1       | NHEJ         | [54]      |
| Arabidopsis   | CHLI1, CHLI2, TT4      | NHEJ         | [55]      |
| Arabidopsis   | AP1                    | NHEJ         | [56]      |
| Barley        | GFP (transgene)        | NHEJ         | [57]      |
| Cabbage       | HvPM19                 | NHEJ         | [57]      |
| Camelina      | BoiC. GA4.a            | NHEJ         | [58]      |
| C. reinhardtii| FAD2                   | NHEJ         | [59]      |
| Cotton        | CpFTSY, ZEP            | NHEJ         | [60]      |
| Cotton        | GFP (transgene)        | NHEJ         | [61]      |
| Cotton        | MYB25-like A, MYB25    | NHEJ         | [58]      |
| Dandelion     | DPT 5                  | NHEJ         | [62]      |
| Flax          | CLA1, VP               | NHEJ, HDR    | [63]      |
| Grape         | 1-FFT                  | NHEJ         | [64]      |
| Lettuce       | EPSPS, BFP (transgene) | NHEJ         | [65]      |
| Liverwort     | Rt3                    | NHEJ         | [66]      |
| Lotus         | IDN DH, BIN2, ARF1     | NHEJ         | [67]      |

Table 2: CRISPR-Cas9 mediated genome editing in plants.
Table 3: Comparison of plant genome editing techniques.

Application of Genome Editing in Plants

Gene Knockout

At present, the most widely used and important application of genome editing is to knockout target genes. In plants, NHEJ is the main pathway used to repair DSBs, and the process can introduce small deletions or insertions (indels), typically smaller than 100 bp. Introduction of indels in a coding region mostly leads to frame shift mutations resulting in the loss of gene function. Most importantly, the mutations are stable and heritable in future generations. Due to its simplicity and high efficiency, CRISPR/Cas9 is now the dominant tool for knocking out genes [72].

Genome editing has been used for simultaneous targeting of multiple genes in many plant species, including Arabidopsis, rice, maize, soybean and tobacco. Multiplex gene editing is not only useful for functional genomics research, such as the study of redundant gene families and functionally related genes but is also important for crop improvement, allowing fast pyramiding of multiple traits. For example, CRISPR/Cas9 was used to simultaneously knockout three negative regulators of grain size in rice, GW2, GW5, and TGW6 and the new varieties exhibited 20%-30% increases in grain size and weight compared to the wild type [73].

When two DSBs are introduced on the same chromosome at a certain distance, the two sites may connect through the NHEJ pathway resulting in the deletion of the intervening sequence. Relatively large deletions are useful for some purposes in research and crop improvement, such as the study of gene clusters and non-coding RNAs. TALEN and CRISPR have been used to produce large deletions in species such as rice, Arabidopsis, and tobacco [74]. In rice, up to 245 kb has been removed from the genome with a high frequency using CRISPR/Cas9 and our group successfully deleted a large genomic fragment in Arabidopsis containing the CBF1, CBF2 and CBF3 genes [75].

Gene Targeting

Gene targeting refers to the use of genetic engineering methods to produce a one-for-one substitution of a DNA fragment (gene replacement) or the insertion of a new sequence in a specific genomic locus (gene knock in). Gene targeting has many applications in functional genomics research, such as precise gene modifications and epitope tagging of endogenous proteins. Gene targeting has been the focus of research for a long time, mostly based on homologous recombination, but the low frequency of targeted integration limited its use to a very few species such as tobacco and rice [76]. For many years, ZFN, TALEN and CRISPR/Cas9 have been successfully used for gene targeting in tobacco, maize, Arabidopsis, tomato, rice, barley, flax, moss soybean and wheat. One drawback of HDR technique is low efficiency, but theoretically possible to increase HDR-mediated gene targeting efficiency by suppression of the NHEJ pathway. Another way to improve HDR-mediated gene targeting is to deliver large amounts of repair template, donor DNA, to the plant nucleus. Particle bombardment can provide multiple copies of donor DNA and has been employed for genome editing-assisted gene targeting in multiple plants [63,77]. Gemini virus system is also another method to
deliver abundant donor DNA which has the property of excising a fragment of its genomic DNA once inside a cell to produce a self-replicating plasmid.

Application of Genome Editing Systems in Crop Improvement

In the last several years, genome editing has been used to produce new crop varieties with improved traits, including increased yield, enhanced disease resistance, improved food quality and higher stress tolerance (Table 4).

Improved Yield

Grain yield is mainly determined by grain number, size and weight, all of which are typical quantitative traits, and many genes affecting crop yield have been characterized. Knockout of genes known to negatively affect yield, such as GS3, DEP1, GS5, GW2, Gn1a, and TGW6 in rice, is a simple and direct way to improve crops. GS3, DEP1 and Gn1a have been individually mutated using CRISPR/Cas9, and some of the predicted phenotypes were observed [50]. Simultaneous knockout of GW2, GW5, and TGW6 in rice resulted in a 29.8% increase in thousand-grain weight in the triple mutant. In bread wheat, thousand-kernel weight also exhibited an increase after the three homo-alleles of GASR7, a negative regulator of kernel width and weight, were knocked out using CRISPR/Cas9 [78]. It is nevertheless important to remark that increased grain yield per plant and higher thousand-grain weight does not necessarily translate into improved crop yield, because large-scale field trials are necessary to verify the potential agronomic improvements.

Improved Oil Composition

A high content of polyunsaturated fatty acids, particularly linolenic acid, in oils results in poor oxidative and frying stability which limits their applications. Fatty acid desaturase (FAD) genes have been targeted to change fatty acid composition and improve oil quality. The FAD2 gene family is responsible for the conversion of oleic acid (monounsaturated) into linoleic acid while enzymes encoded by the FAD3 gene family catalyze the production of linolenic acid from linoleic acid. TALENs were used to simultaneously knock out two soybean FAD2 genes, FAD2-1A and FAD2-1B, resulting in vastly improved oil quality: oleic acid increased from 20% to 80% and linoleic acid decreased from 50% to <4% [79]. To further improve oil composition, mutations in FAD3A were introduced into the previously produced fad2-1A/fad2-1B soybean plants by TALEN, resulting in further increased levels of oleic acid and decreased levels of linolenic acid. Recently, two independent groups used CRISPR/Cas9 to simultaneously knock out all three FAD2 homeolog genes in the allohexaploid camelina sativa, producing a significant enhancement in oil composition [77].

Biotic and a Biotic Resistance

Genome editing has been applied to increase disease resistance by editing disease-related gene. In rice, Li, T. et al. (2012) [80] targeted the rice bacterial blight susceptibility gene OsSWEET14 for TALEN-based disruption and observed strong resistance to infection with normal phenotypes. Moreover, Wang et al. (2016) [81] modified OsERF922 and observed significantly enhanced blast resistance with no effect on important agronomic traits. In wheat, by inactivating all three MILDW-RESISTANCE LOCUS (MLO) genes, Wang et al. (2014) [82] showed that the infection rates were significantly reduced combined with a race non-specific resistance. With a similar approach in cucumber, virus resistance could be produced by editing the recessive elf4E (eukaryotic translation initiation factor 4E), without affecting plant development [83]. In terms of abiotic stress tolerance, genome editing can be a valuable weapon in generating novel allelic variation for breeding, as demonstrated in maize. The generation of novel ARGOS8 variants produced elevated expression across multiple tissues and at different developmental stages resulting in increased grain yield under drought stress conditions in the field [84].

In more recent papers, a transgene integration-free targeted mutagenesis has been developed for hexaploid bread wheat and tetraploid durum wheat, as well as for corn. Zhang et al. (2016) [78] used plasmids encoding Cas9 and a conserved target site in the sgRNA for targeting all TaGASR7 homoeologs. After particle bombardment of immature embryos of two bread wheat varieties, out of 2,400 bombarded embryos 101 mutants were obtained out of which eight plants showed simultaneous knock outs in all six alleles. In addition, they targeted four more genes in bread wheat and TdGASR7 in durum wheat and got also mutations in these genes. In a second approach, Zhang et al. (2016) [78] used in vitro synthesized RNA of the coding region of Cas9 and the sgRNA to target all homoeologs of TaGW2, a gene which controls grain weight. They bombarded the RNA into immature embryos of bread wheat and could regenerate plants that showed mutations in all homoeologs in the first generation that were assumed to be free of transgenes, whereas in the DNA-based approach most plants carried the transgene [78]. Finally, a DNA-free genome editing was established in maize and wheat through biolistic delivery of rib nucleoprotein (RNP)
complexes of Cas9 protein and in vitro synthesized sgRNA to immature embryos of bread wheat and corn [85,86]. In maize, target sites for four different genes were created and the RNP complexes were bombarded into immature embryos. From the regenerated plants, 2.4% to 9.7% had mutated alleles [86]. In a similar approach in wheat, all three homoeologs of TaGW2 were targeted with one conserved target region. Regenerated mutants could be obtained for single homoeologs in a frequency of about 4% of the used embryos and, by deep sequencing, it was shown that no off-target effects were present [85].

CsLOB1 is a host disease-susceptibility gene which erupment pustule formation in citrus [87]. Recently, two groups generated canker-resistant citrus cultivars by CRISPR/ Cas9-targeted modification of the CsLOB1 promoter. Knockout of the ERF transcription factor OsERF922, a negative regulator of rice blast resistance, resulted in enhanced resistance. Editing of the wheat TaMLO gene is another good sample of the use of gene editing to introduce disease resistance into susceptible crop varieties. Loss-of-function mlo alleles in barley, Arabidopsis and tomato produce broad-spectrum and durable resistance to Blumeria graminisf. Sp. tritici (Bgt) which cause powdery mildew [88]. Using the same approach, CRISPR/Cas9-mediated gene disruption of the tomato SIMLO1 gene resulted in rapid generation of tomato fully resistant to powdery mildew [88].

| Crop    | Gene          | Function               | Methods                  | Tools            | References |
|---------|---------------|------------------------|--------------------------|------------------|------------|
| Corn    | ALS1, ALS2    | Herbicide resistance   | Promoter disruption      | CRISPR/Cas9      | [89]       |
| Cotton  | hppd, epsps   | Herbicide resistance   | Promoter disruption      | Meganuclease     | [90]       |
| Flax    | EPSPS         | Herbicide tolerance    | HDR-mediated base change | CRISPR/Cas9      | [63]       |
| Rice    | OsALS         | Herbicide resistance   | Gene knockout            | CRISPR/Cas9      | [91]       |
| Rice    | OsEPSPS       | Herbicide resistance   | Gene knockout            | CRISPR/Cas9      | [50]       |
| Soybean | ALS1          | Herbicide resistance   | HDR-mediated base change | CRISPR/Cas9      | [92]       |
| Tomato  | ANT1          | Anthocyanin accumulation| Viral gene disruption    | CRISPR/Cas9      | [93]       |
| wheat   | TaMLO         | Disease resistance     | Gene knockout            | TALEN            | [82]       |
| Tobacco | Region in viral genome | Virus resistance | Viral gene disruption    | CRISPR/Cas9      | [94]       |

Table 2: Examples of successful genome editing for crop improvement.

**Conclusion**

In the last several years, genome editing has emerged as a technology and revolutionized the field of functional genomics and crop improvement in various plants. Genome editing tools are becoming popular molecular tools of choice for crop improvement, especially engineered nucleases, have had a revolutionary influence on basic research in plants as well as crop improvement. These technologies rely on engineered endonucleases to generate double stranded breaks (DSBs) at target loci. CRISPR/Cas9 has emerged as the most promising approach due to its simplicity, ease of use, versatility, accuracy and tolerable off-target effects. The genome editing system holds great promise in generating crop varieties with enhanced disease resistance, improved oil composition, biotic and abiotic stress resistance, and herbicide tolerance.

**Herbicide Tolerance**

Herbicide tolerance is a very important trait in agriculture worldwide. So far, only four glyphosate tolerant crops are grown on a large scale: corn, soybean, rapeseed and sugar beet. They are genetically modified plants, as their tolerance results from the transformation with a bacterial EPSPS gene. The EPSPS gene encodes a 5-enolpyruvylshikimate-3-phosphate synthase, which is necessary for the biosynthesis of aromatic amino acids essential for plant survival. In plants, EPSPS is a target for glyphosate, a widely used herbicide which binds to EPSPS functional sites to prevent its activity. The usual method to introduce glyphosate tolerance in plants is to modify the EPSPS protein structure in order to disrupt herbicide binding while maintaining its catalytic activity. CRISPR/Cas9 has been used in Linum usitatissimum (Flax) to substitute two nucleotides in the EPSPS glyphosate binding site through HDR-based genome editing [63]. A similar approach has been used to introduce base substitutions in the rice EPSPS gene resulting in glyphosate-resistant rice [50]. ALS encodes the acetolactate synthase enzyme that participates in the biosynthesis of branched-chain amino acids like valine, leucine, and isoleucine. Inhibitors of ALS eventually lead to inhibition of DNA synthesis, but specific point mutations within the conserved region of ALS can confer resistance to these herbicides. ALS is the target of numerous herbicides including sulfonylurea, imidazolinones, triazolopyrimidines, pyrimidinylloxycarboxylic acids, and sulfonyl amino carbonyl triazolinones [75].
improved yield and quality and novel agronomic traits which will be beneficial for farmers and consumers. The technology has been successfully used for targeted mutagenesis in various crops.

Acknowledgement

I am indebted to many people in writing this Review. My sincerest gratitude and appreciation go to my advisor and my friends for their constructive advice and guidance.

References

1. Pray L (2008) Discovery of DNA structure and function Watson and Crick. Nature Education 1(1): 100.
2. Pratt J, Venkatraman N, Brinker A, Xiao Y, Blasberg J, et al. (2012) Use of zinc finger nuclease technology to knock out efflux transporters in C2BBe1 cells. Curr Protoc Toxicol 52(1).
3. Podevin N, Davies HV, Hartung F, Nogue F, Casacuberta JM (2013) Site directed nucleases a paradigm shift in predictable knowledge based plant breeding. Trends Biotechnol 31(6): 375-383.
4. Voytas DF, Gao C (2014) Precision genome engineering and agriculture: opportunities and regulatory challenges. PLoS biology 12(6): 1001877.
5. Voytas DF (2013) Plant genome engineering with sequence-specific nucleases. Annual review of plant biology 64: 327-350.
6. Jinek M, East A, Cheng A, Lin S, Doudna J (2013) RNA-programmed genome editing in human cells. elife 2: 00471.
7. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, et al. (2012) A programmable dual-RNA- guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096): 816-821.
8. Paszkowski J, Baur M, Bogucki A, Potrykus I (1988) Gene targeting in plants. The EMBO Journal 7(13): 4021-4026.
9. JE, Stuart K, Shaw JMS, Feagin L (1988) Editing of kinetoplastid mitochondrial mRNAs by uridine addition and deletion generates conserved amino acid sequences and AUG initiation codons. Cell 53(3): 401-411.
10. Park SY, Vagghchipawala Z, Vasudevan B, Lee LY, Shen Y, et al. (2015) Agrobacterium T-DNA integration into the plant genome can occur without the activity of key non-homologous end-joining proteins. Plant Journal 81(6): 934-946.
11. Henikoff S, Till BJ, Comai L (2004) TILLING Traditional Mutagenesis Meets Functional Genomics. Plant Physiol 135(2): 630-636.
12. Wang N, Shi L (2015) Screening of mutations by TILLING in plants. In Plant Genotyping 193-203.
13. Hannon GJ (2002) RNA interference. Nature 418: 244-251.
14. Puchta H (2002) Gene replacement by homologous recombination in plants. Plant Molecular Biology 48(1-2): 173-182.
15. Puchta H, Dujon B, Hohn B (1993) Homologous recombination in plant cells is enhanced by in vivo induction of double strand break into DNA by a sitespecific endonuclease. Nucleic Acids Res 21(22): 5034-5040.
16. Puchta H, Fauser F (2013) Gene targeting in plants: 25 years later. Int J Dev Biol 57(6-8): 629-637.
17. Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, et al. (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326(5959): 1509-1512.
18. Sorek R, Lawrence CM, Wiedenheft B (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu Rev Biochem 82: 237-266.
19. Smith J, Bibikova M, Whitby FG, Reddy AR, Chandra segaran S, et al. (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. Nucleic Acids Res 28(17): 3361-3369.
20. Carroll D (2014) Genome engineering with targetable nucleases. Annu Rev Biochem 83: 409-439.
21. Hansen K, Coussens MJ, Sago J, Subramanian S, Gjoka M, et al. (2012) Genome editing with CompoZr custom zinc finger nucleases (ZFNs). J Vis Exp 14(64): 3304.
22. Osborn MJ, DeFeo AP, Blazar BR, Tolar J (2011) Synthetic zinc finger nuclease design and rapid assembly. Hum Gene Ther 22(9): 1155-1165.
23. Lloyd A, Plaisier C, Carroll D, Drews GN (2005) Targeted mutagenesis using zinc-finger nucleases in Arabidopsis. Proc Natl Acad Sci 102(6): 2232-2237.

24. Wright DA, Townsend JA, Winfrey RJ, Irwin PA, Rajagopal J, et al. (2005) High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J 44(4): 693-705.

25. Baltes NJ, Gil-Humanes, Cermak T, Atkins PA, Voytas DF (2014) DNA replications for plant genome engineering. Plant Cell 26(1): 151-163.

26. Weeks DP, Spalding MH, Yang B (2016) Use of designer nucleases for targeted gene and genome editing in plants. Plant Biotechnol J 14(2): 483-495.

27. Faitelson LE, Samach A, Bessudo CM, Ragolsky NA, Levy AA (2011) Localized egg-cell expression of effector proteins for targeted modification of the Arabidopsis genome. Plant J 68(5): 929-937.

28. Petolino JP, Worden A, Curlee K, Connell J, Moynahan TLS (2010) Zinc finger nuclease-mediated transgene deletion. Plant Moleculer Biology 73(6): 617-628.

29. Qi Y, Zhang Y, Zhang F, Baller JA, Cleland SC, et al. (2013) Increasing frequencies of site-specific mutagenesis and gene targeting in Arabidopsis by manipulating DNA repair pathways. Genome Res 23(3): 547-554.

30. Chen W, Qian Y, Wu X, Sun Y, Wu X, et al. (2014) Inhibiting replication of begomo viruses using artificial zinc finger nucleases that target viral-conserved nucleotide motif. Virus Genes 48(3): 494-501.

31. Takasu Y, Kobayashi I, Beumer K, Uchino K, Sezutsu H, et al. (2010) Targeted mutagenesis in the silkworm Bombyx mori using zinc finger nuclease mRNA injection. Insect Biochem Mol Biol 40(10): 759-765.

32. Ochiai H, Fujita K, Suzuki K, Nishikawa M, Shibata T, et al. (2010) Targeted mutagenesis in the sea urchin embryo using zinc-finger nucleases. Genes Cells 15(8): 875-885.

33. Young JJ, Cherone JM, Doyon Y, Ankoudinova I, Faraji FM, et al. (2011) Efficient targeted gene disruption in the soma and germ line of the frog Xenopus tropicalis using engineered zinc-finger nucleases. Proc Natl Acad Sci 108(17): 7052-7057.

34. Mashimo T, Takizawa A, Voigt B, Yoshimi K, Hiai H, et al. (2010) Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. PloS one 5(1): 8870.

35. Carbery ID, Ji D, Harrington A, Brown V, Weinstein EJ (2010) Targeted genome modification in mice using zinc-finger nucleases. Genetics 186(2): 451-459.

36. Osakabe K, Osakabe Y, Toki S (2010) Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proceedings of the National Academy of Sciences 107(26): 12034-12039.

37. Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, et al. (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature 459(7245): 437-441.

38. Marton I, Zuker A, Shklarman E, Zeevi V, Tovkach A (2010) Non-transgenic genome modification in plant cells. Plant Physiology 154(3): 1079-1087.

39. Lombardo A, Genovese P, Beausjour CM, Colleoni S, Lee Y, et al. (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25(11): 1298-1306.

40. Connelly JP, Barker JC, Miller SP, Porteus MH (2010) Gene correction by homologous recombination with zinc finger nucleases in primary cells from a mouse model of a generic recessive genetic disease. Molecular therapy 18(6): 1103-1110.

41. Cost GJ, Freyvert Y, Vafiadis A, Santiago Y, Miller JC, et al. (2010) BAK and BAX deletion using zinc-finger nucleases yields apoptosis-resistant CHO cells. Biotechnol Bioeng 105(2): 330-340.

42. Watanabe M, Umeyama K, Matsunari H, Takayanagi S, Haruyama E, et al. (2010) Knockout of exogenous EGFP gene in porcine somatic cells using zinc-finger nucleases. Biochem Biophys Res Commun 402(1): 14-18.

43. Boch J (2011) TALEs of genome targeting. Nat Biotechnol 29(2): 135-136.

44. Joung JK, Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 14(1): 49-55.

45. Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, et al. (2011) Genetic engineering of human pluripotent...
cells using TALE nucleases. Nat Biotechnol 29(8): 731-734.

46. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli and identification of the gene product. J Bacteriol 169(12): 5429-5433.

47. Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327(5962): 167-170.

48. Bhaya D, Davison M, Barrangou R (2011) CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. Annual Rev Genet 45: 273-297.

49. Cong L, Ran FA, Cox D, Lin S, Barretto R, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121): 819-823.

50. Li M, Li X, Zhou Z, Wu P, Fang M, et al. (2016) Reassessment of the four yield-related genes Gn1a, DEP1, GS3 and IPA1 in rice using a CRISPR/Cas9 system. Frontiers in plant science 7: 377.

51. Li Q, Zhang D, Chen M, Liang W, Wei J (2016) Development of japonica photo- sensitive Genic Male Sterile Rice Lines by Editing Carbon Starved Anther Using CRISPR/Cas9. J Genet Genomics 43(6): 415-419.

52. Pyott DE, Sheehan E, Molnar A (2016) Engineering of CRISPR/Cas9-mediated potyvirus resistance in transgene-free Arabidopsis plants. Mol Plant Pathol 17(8): 1276-1288.

53. Li JF, Norville JE, Aach J, McCormack M, Zhang D, et al. (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol 31(8): 688-691.

54. Feng Z, Zhang B, Ding W, Liu X, Yang DL, et al. (2013) Efficient genome editing in plants using a CRISPR/Cas system. Cell Res 23(10): 1229-1232.

55. Feng Z, Mao Y, Xu N, Zhang B, Wei P, et al. (2014) Multi generation analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc Natl Acad Sci 111(12): 4632-4637.

56. Jiang W, Zhou H, Bi H, Fromm M, Yang B, et al. (2013) Demonstration of CRISPR/Cas9/ sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res 41(20): 188.

57. Lawrenson T, Shorinola O, Stacey N, Li C, Ostergaard L, et al. (2015) Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. Genome Biol 16: 258.

58. Chen X, Lu X, Shu N, Wang S, Wang J, et al. (2017) Targeted mutagenesis in cotton (Gossypium hirsutum L.) using the CRISPR/Cas9 system. Sci Rep 7: 44304.

59. Baek K, Kim DH, Jeong J, Sim SJ, Melis A, et al. (2016) DNA-free two-gene knockout in Chlamydomonas reinhardtii via CRISPR-Cas9 ribonucleoproteins. Sci Rep 6: 30620.

60. Janga MR, Campbell LM, Rathore KS (2017) CRISPR/Cas9-mediated targeted mutagenesis in upland cotton (Gossypium hirsutum L). Plant Mol Biol 94(4-5): 349-360.

61. Li C, Unver T, Zhang B (2017) A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in cotton (Gossypium hirsutum L.). Sci Rep 7(1): 43902.

62. Iaffaldano B, Zhang Y, Cornish K (2016) CRISPR/Cas9 genome editing of rubber producing dandelion Taraxacum kok-saghyz using Agrobacterium rhizogenes without selection. Industrial Crops and Products 89: 356-362.

63. Sauer NJ, Vasquez JN, Mozoruk J, Miller RB, Warburg ZJ, et al. (2016) Oligonucleotide-mediated genome editing provides precision and function to engineered nucleases and antibiotics in plants. Plant Physiol 170(4): 1917-1928.

64. Ren C, Liu X, Zhang Z, Wang Y, Duan W, et al. (2016) CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (Vitis vinifera L.). Sci Rep 31(6): 322-389.

65. Woo JW, Kim J, Kwon SI, Corvalan C, Cho S W, et al. (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33(11): 1162-1164.

66. Sugano SS, Shirakawa M, Takagi J, Matsuda Y, Shimada T, et al. (2014) CRISPR/Cas9 mediated targeted mutagenesis in the liverwort Marchantia polymorphaL. Plant Cell Physiol 55(3): 475-481.
67. Sugano SS, Ueta R, Osakabe Y, Watanabe T, Ishihara R, et al. (2016) Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. Sci Rep 26(6): 266-285.

68. Gaj T, Gersbach CA, Carlos F, Barbas (2013) ZFN, TALEN and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31(7): 397-405.

69. Cho SW, Kim S, Kim JM, Kim JS (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nature biotechnology 31: 232.

70. Noman A, Aqeel M, He S (2016) CRISPR-Cas9: tool for qualitative and quantitative plant genome editing. Front Plant Sci 21(7): 1740.

71. Mao Y, Zhang H, Xu N, Zhang B, Gou F, et al. (2013) Application of the CRISPR-Cas system for efficient genome engineering in plants. Mol Plant 6(6): 2008-2011.

72. Puchta H (2005) The repair of double-strand breaks in plants: Mechanisms and consequences for genome evolution. J Exp Bot 56(409): 1-14.

73. Xu R, Yang Y, Qin R, Li H, Qiu C, et al. (2016) Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. J Genet Genomics 43(8): 529-532.

74. Ordon J, Gantner J, Kemna J, Schwalgun L, Reschke M, et al. (2017) Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome editing toolkit. Plant J 89(1): 155-168.

75. Zhou H, He M, Li J, Chen L, Huang Z, et al. (2016) Development of commercial thermo-sensitive genic male sterile rice accelerates hybrid rice breeding using the CRISPR/Cas9-mediated TMS5 editing system. Scient Rep 6: 373-395.

76. Terada R, Uraw H, Inagaki Y, Tsugane K, Iida S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20(10): 1030-1034.

77. Zhang H, Zhang J, Lang Z, Botella JR, Zhu JK (2017) Genome Editing- Principles and Applications for Functional Genomics Research and Crop Improvement. Critical Reviews in Plant Sciences 36(4): 291-309.
89. Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, et al. (2015) Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant Physiol 169(2): 931-945.

90. Halluin KD, Vanderstraeten C, Hulle J, Rosolowska J, Brande IVD, et al. (2013) Targeted molecular trait stacking in cotton through targeted double strand break induction. Plant Biotechnol J 11(8): 933-941.

91. Endo M, Mikami M, Toki S (2016) Biallelic gene targeting in rice. Plant Physiol 170(2): 667-677.

92. Sun X, Hu Z, Chen R, Jiang Q, Song G, et al. (2015) Targeted mutagenesis in soybean using the CRISPR-Cas9 system. Scientific Reports 5: 10342.

93. Zhang H, Zhang J, Wei P, Zhang B, Gou F, et al. (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12(6): 797-807.

94. Ali Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, et al. (2015) CRISPR/Cas9 mediated viral interference in plants. Genome biology 16: 238.