Genome Editing with Engineered Nucleases in Plants

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Numerous examples of successful 'genome editing' now exist. Genome editing uses engineered nucleases as powerful tools to target specific DNA sequences to edit genes precisely in the genomes of both model and crop plants, as well as a variety of other organisms. The DNA-binding domains of zinc finger (ZF) proteins were the first to be used as genome editing tools, in the form of designed ZF nucleases (ZFNs). More recently, transcription activator-like effector nucleases (TALENs), as well as the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) system, which utilizes RNA–DNA interactions, have proved useful. A key step in genome editing is the generation of a double-stranded DNA break that is specific to the target gene. This is achieved by custom-designed endonucleases, which enable site-directed mutagenesis via a non-homologous end-joining (NHEJ) repair pathway and/or gene targeting via homologous recombination (HR) to occur efficiently at specific sites in the genome. This review provides an overview of recent advances in genome editing technologies in plants, and discusses how these can provide insights into current plant molecular biology research and molecular breeding technology.

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-stranded DNA break; GEEN, genome editing using engineered nucleases; gRNA, guide RNA; GT, gene targeting; HR, homologous recombination; MMEJ, microhomology-mediated end-joining; NHEJ, non-homologous end-joining; PAM, protospacer adjacent motif; RVD, repeat-variable di-residue; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease.

Introduction

Genome editing using engineered nucleases ('GEEN') is an effective genetic engineering method that uses 'molecular scissors', or artificially engineered nucleases, to target and digest DNA at specific locations in the genome. The engineered nucleases induce a double-stranded DNA break (DSB) at the target site that is then repaired by the natural processes of homologous recombination (HR) or non-homologous end-joining (NHEJ) (**Figs. 1**, **2**). Sequence modifications then occur at the cleaved sites, which can include deletions or insertions that result in gene disruption in the case of NHEJ, or integration of exogenous sequences by HR. Currently, four types of engineered nucleases are used for genome editing: engineered homing endonucleases/meganucleases (EMNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated)9 (**Fig. 3**). In particular, TALEN and CRISPR/Cas9 are now used widely in various organisms (**Figs. 4B**, **5**).

To understand gene function, genetic analysis has traditionally studied the effects of natural and artificial mutations induced by physical and chemical mutagens such as γ-rays and ethyl methanesulfonate (EMS) (Sikora et al. 2011). These methods require screening to identify important traits as a consequence of mutations that are introduced randomly into the genome. Determining the effects of sequence-specific modifications on the host organism helps to identify gene function. However, performing site-specific mutagenesis in eukaryotes remains challenging. Other methods, such as post-transcriptional silencing of genes of interest using short interfering RNA (siRNA), have been utilized effectively, but gene knockdown by siRNA can be variable and incomplete.

GEEN enables the creation of sequence-specific DSBs and therefore can, in principle, digest any targeted site in the genome to modify endogenous gene sequences (Urnov et al. 2010). In the ZFN and TALEN systems, targeting specificity is provided by binding pairs of ZFNs or TALENs to two closely spaced DNA sequences. One partner in the pair recognizes a DNA sequence on one strand and the other partner recognizes a DNA sequence on the opposite strand. The spacing of the partners is designed to allow the *Fok*I nuclease domains bound to each partner to dimerize and cause a DSB in the DNA 'spacer region' between the two partners (Urnov et al. 2005, Christian et al. 2010). Recently, a new method has been developed that uses the type II prokaryote-specific adaptive immune system nuclease, CRISPR/Cas9. The CRISPR/Cas9 system is based on RNA-guided engineered nucleases (RGNs) that use complementary base pairing to recognize DNA sequences at target sites (Cong et al. 2013, Mali et al. 2013a). For sequence-specific silencing, CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA) participate in target sequence recognition. The CRISPR/Cas9 system has now been utilized widely for editing the genome of various organisms, including bacteria, yeast, animals and plants (Gasiunas et al. 2012, Cong et al. 2013, DiCarlo
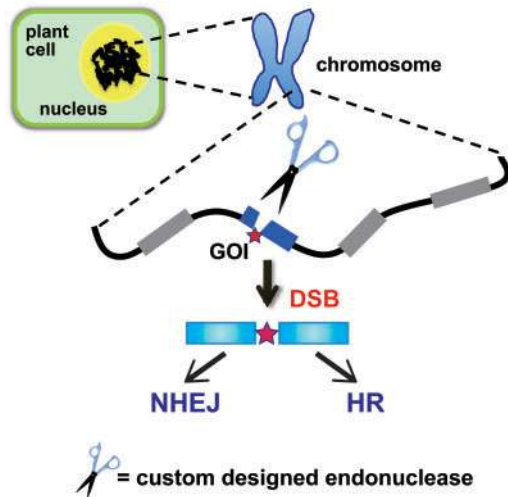
Fig. 1 Targeted genome editing in plants using engineered nucleases ('GEEN'). Engineered nucleases are used to induce a double-stranded DNA break (DSB) at a specified locus of the gene of interest (GOI). DSBs are repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR).

et al. 2013, Jinek et al. 2013, Li et al. 2013, Mali et al. 2013a, Nekrasov et al. 2013, Sugano et al. 2014).

Here, we review recent advances in genome editing technologies. The generation of site-directed sequence modifications by engineered nucleases can be applied not only to model plant species, but also to crop plants. We discuss this and other approaches and suggest applications and future prospects of these technologies in plant molecular breeding and biotechnology.

Genome Editing Technologies: Modern Tools for Mutagenesis

DSB is a key mechanism in genome editing

Traditionally, natural or induced mutagenesis has been used in plant breeding to generate genetic variation that increases crop yields. In recent decades, mutagens, such as EMS and γ-rays, have been used to induce new traits by randomly introducing mutations into the genome (Sikora et al. 2011). To obtain superior mutant organisms using these strategies, mutant populations must be screened to identify individual plants that have
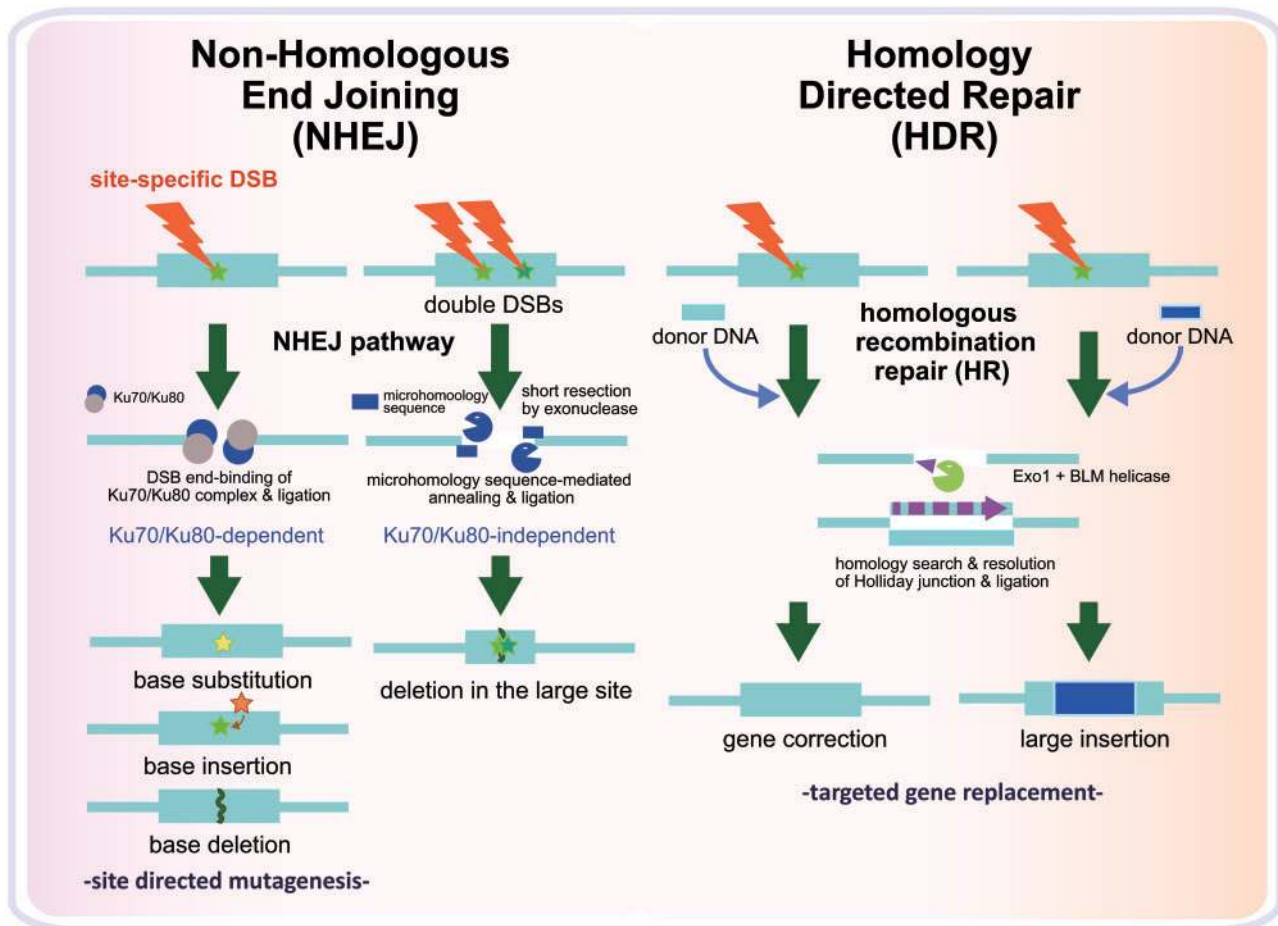
Fig. 2 DNA repair pathways involved in the repair of DSBs generated by engineered nucleases. The NHEJ-mediated pathway leads to the generation of variable insertion or deletion mutations and can be used for site-directed mutagenesis. HR with double-stranded donor DNAs leads to the creation of precise nucleotide substitutions or insertions and is used for targeted gene replacement.
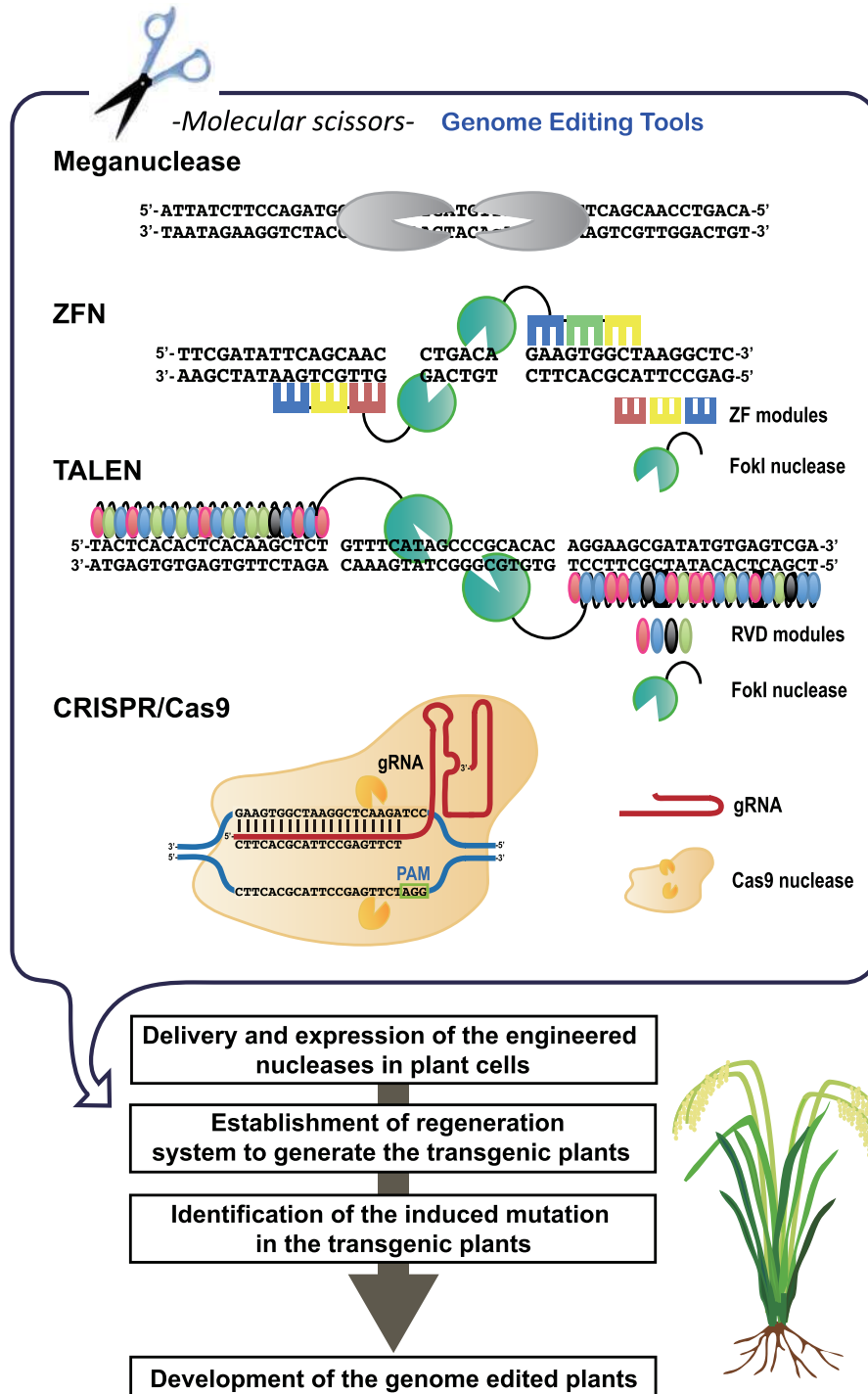
Fig. 3 Current methods in GEEN and an overview of the workflow of plant genome editing. Homing endonucleases/meganucleases (EMNs) recognize long (∼20 bp) DNA sequences. *Fok*I nuclease is used as the DNA cleavage domain in ZFN, which bind the target DNAs via engineered C2H2-zinc finger (ZF) domains and in TALEN that recognize the targets by the engineered TALEs composed of RVD domains derived from the plant pathogen *Xanthomonas*. The CRISPR/Cas9 system utilizes RNA-guided engineered nucleases (RGNs) that use a short guide RNA (gRNA) to recognize DNA sequences at the target sites.

the desired phenotype. Following natural or induced DSBs, DSB repair systems are responsible for the generation of mutations in the genome, and NHEJ and HR are the main pathways by which this occurs (**Figs. 1**, **2**) (Osakabe et al. 2012). NHEJ uses various repair enzymes to join the ends of the DNA

following a DSB. This repair system has two subpathways: the Ku-dependent NHEJ pathway and the Ku-independent NHEJ pathway (Deriano and Roth 2013). In Ku-dependent NHEJ, the DNA end protection factors—Ku70/80 proteins—bind to the end of the DNA strand at the break site and recruit the repair
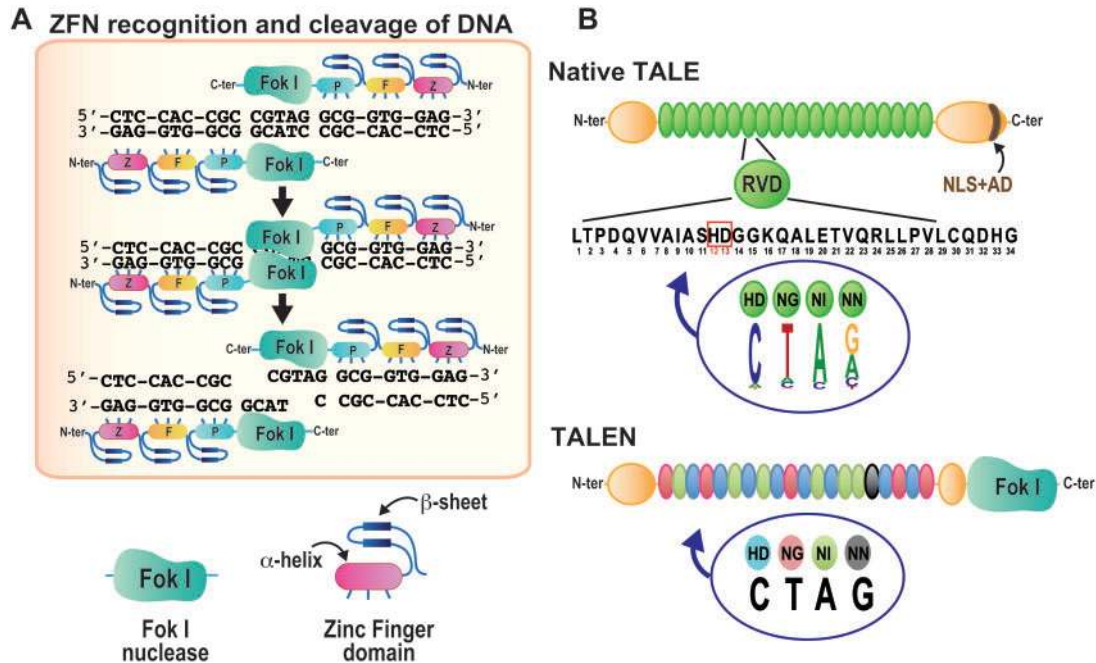
Fig. 4 Mechanisms of target recognition by ZFN and TALEN. (A) An engineered ZF domain that consists of approximately 30 amino acids composed of a ββα fold; each domain contacts 3 or 4 bp in the target DNA. The ZFN target sites consist of two ZF-binding sites. A 5–7 bp spacer site, which is recognized by the *Fok*I domain, is located between two ZF binding sites. (B) Native TALE from *Xanthomonas* is composed of tandem repeat domains of 33–35 amino acids. Each monomer has two repeat variable di-residues (RVDs) that recognize a single base in the target site. The target base frequency for each RVD (Boch et al. 2009) is illustrated. Designed TALENs can recognize the target sites of two TALE-binding sites that are separated by a 12–20 bp spacer region. The spacer is recognized by the *Fok*I domain to generate DSBs. NLS, nuclear localization signal; AD, activation domain of the native TALE.

enzyme ligase IV and its cofactor (Deriano and Roth 2013). This Ku-dependent pathway can sometimes generate several nucleotide insertions or deletions at the break site. When the factors involved in Ku-dependent NHEJ are absent, the alternative end-joining (Ku-independent) pathway can repair DSBs in eukaryotes (Frit et al. 2014). Microhomology-mediated end-joining (MMEJ) is a major Ku-independent NHEJ pathway. A recent study of the MMEJ pathway proposed that the microhomology (MM) regions are resected, annealed at the MM regions, filled in by DNA polymerase and finally re-ligated by DNA ligase (Crespan et al. 2012). Thus, MMEJ produces a longer deletion at the DSB site compared with Ku-dependent NHEJ.

In contrast to error-prone NHEJ pathways, HR is usually an error-free DSB repair pathway, because HR uses a DNA template to replace the DNA sequence at the break point accurately. HR is restricted to late S/G2 phases in the cell cycle, whereas NHEJ functions throughout the entire cell cycle. Therefore, NHEJ is the major DSB repair pathway in eukaryotes (Sonoda et al. 2006) (**Figs. 1**, **2**). Both NHEJ and HR repair pathways can be exploited for nuclease-based genome editing. Based on these DSB repair mechanisms, site-directed mutagenesis mediated via NHEJ- or HR-directed gene knock-in/correction can be performed at specific locations in the genome. In addition, recent studies have shown that targeting cleavage to specific loci can induce chromosomal rearrangements, such as deletions of a few mega base pairs (Lee et al. 2010, Xiao et al.

2013), duplications (Lee et al. 2012), inversions (Lee et al. 2012, Xiao et al. 2013) and translocations (Brunet et al. 2009).

Genome editing by custom-designed site-specific endonucleases

To create site-directed DSBs in the genome, site-specific restriction endonucleases can be used. To date, four types of engineered nucleases have been developed: meganucleases, ZFNs, TALENs and CRISPR/Cas9. Meganucleases, which are commonly identified in microbes, are unique enzymes with high activity and long recognition sequences (>14 bp) resulting in site-specific digestion of target DNA (Epinat et al. 2003, Smith et al. 2006). A further study described the synthesis of hybrid enzymes using two meganucleases, I-Cre I and I-Dmo I, that recognize new target sequences (Epinat et al. 2003). Specialized methods of mutagenesis and high-throughput screening have also been used to create novel meganuclease variants that recognize unique sequences and possess improved nuclease activity (Smith et al. 2006, Arnould et al. 2007, Grizot et al. 2009).

ZFNs are chimeric proteins composed of a synthetic zinc-finger-based DNA-binding domain and a DNA cleavage domain (**Fig. 4**). ZFNs can be designed to cleave almost any long stretch of double-stranded DNA by modification of the zinc finger DNA-binding domain (Durai et al. 2005, Camenisch et al. 2008). ZFNs form dimers from monomers composed of a non-specific DNA cleavage domain of *Fok*I endonuclease
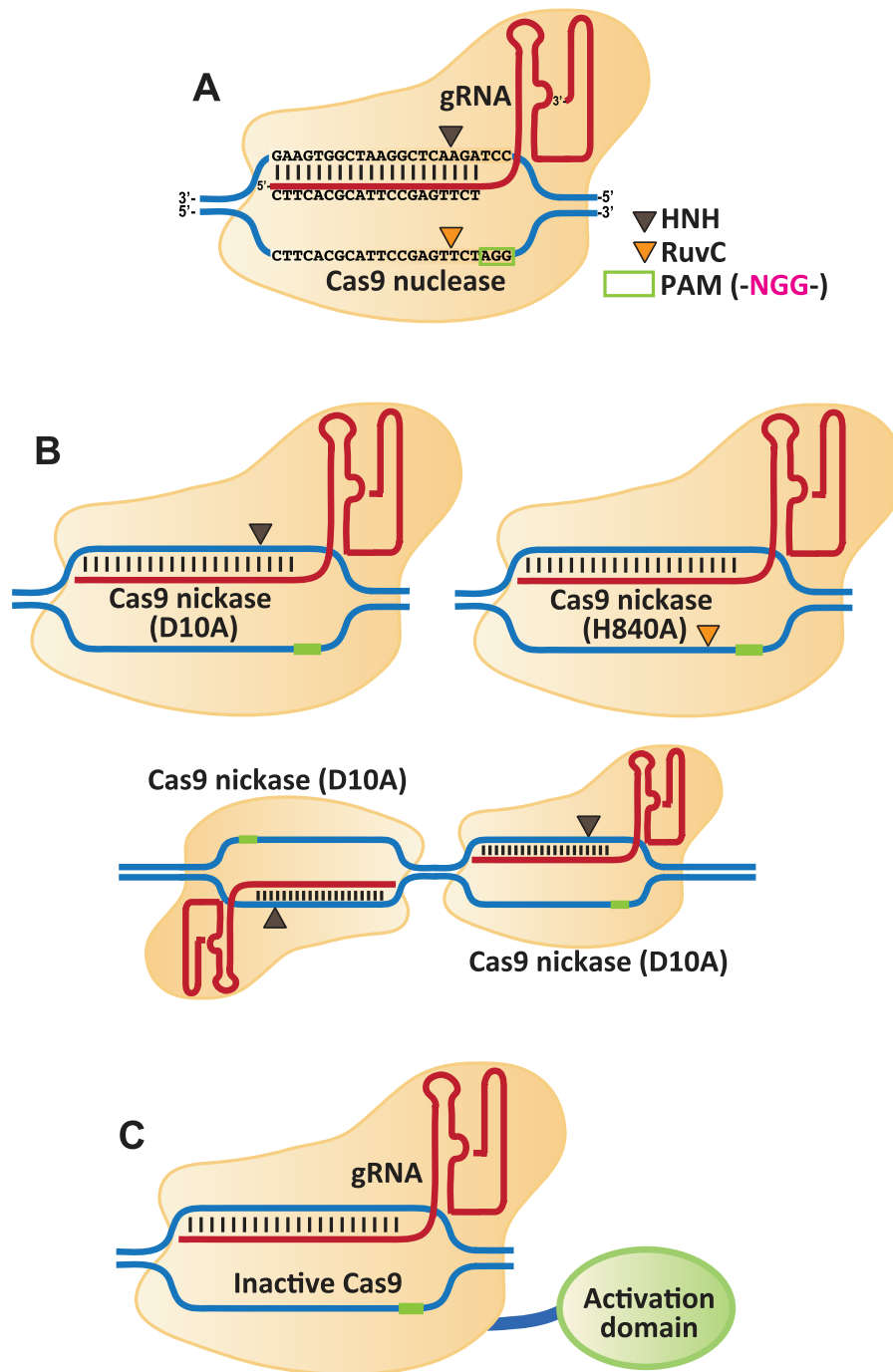
Fig. 5 Overview of various CRISPR/Cas9 mediated-genome modifications. (A) Cas9 has two nuclease domains (RuvC and HNH) to digest different DNA strands to create DSBs at the target sites via direction of the gRNA to the PAM sequence. (B) A mutation of Cas9 nuclease containing a single inactive nuclease domain, RuvC with D10A or HNH with H840A, gives rise to modified versions called 'Cas9 nickases'. The Cas9 nickase D10A cleaves only one DNA strand in the direction of the gRNA to create a single-strand break, whereas H840A cleaves only one strand without the interaction of the gRNA and creates a single-strand break. The two nickases D10A under the direction of a pair of gRNAs can create DSBs. (C) Mutation in both RuvC (D10A) and HNH (H840A) creates an inactive form of Cas9 (called 'dCas9') that can be recruited by the gRNA without cleaving DNA. dCas9 can be applied easily in Cas9/CRISPR techniques, not only in genome editing but also in the modification of gene expression, such as in fusion of the activation domains to up-regulate specific target genes.

fused to a zinc finger array engineered to bind a target DNA sequence. The DNA-binding domain of a ZFN is typically composed of 3–4 zinc finger arrays (**Fig. 4**). The amino acids at positions −1, +2, +3 and +6 relative to the start of the zinc finger α-helix, which contribute to site-specific binding to the target DNA, can be changed and customized to fit specific target sequences. The other amino acids form the consensus backbone to generate ZFNs with different sequence specificities. Several studies have described the rules for selecting target sequences for ZFNs (e.g. Mandell and Barbas 2006, Sander et al.

2007). **Fig. 4** shows an example of the modular assembly method for a ZF array binding to the sequence 5′-GCG-GTG-GAG-3′. 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 ZFN monomer can cut the target site if the two ZF-binding sites are palindromic.

To generate ZF arrays that recognize specific DNA sequences, the modular assembly method described above is initially established, and individual zinc fingers with known specificities for triplet sequences are combined to cover the required sequence (Bae et al. 2003, Segal et al. 2003). Sangamo BioSciences Inc. has developed ZF module archives, and has exploited these to synthesize highly specific ZFNs (Urnov et al. 2005, Shukla et al. 2009). Currently, custom ZFNs designed by this method are available from Sigma-Aldrich (http://www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology/custom-zfn.html). Another method, oligomerized pool engineering (OPEN), has been developed for use in plant genome modification (Townsend et al. 2009, Zhang et al. 2010). OPEN is a combinatorial selection-based method used to engineer zinc finger arrays by genetic selection. Libraries of three-finger protein variants, and the identification of combinations that bind to target DNA with high specificity in vivo, are applied in this system (Maeder et al. 2008). A simpler platform to generate ZFN has been developed, known as context-dependent assembly (CoDA). CoDA uses pre-selected two-finger units, and arrays can be assembled rapidly using simple techniques (Sander et al. 2011b, Curtin et al. 2012). Bacterial one-hybrid screening of zinc finger libraries has also been used to generate ZFNs (Joung et al. 2000, Durai et al. 2005). Improvements to the methods used to generate ZFNs seek to simplify this highly technical procedure; however, target sequences remain a limitation of all ZFN-based methods.

TALEN is a recently developed platform for GEEN. TALE proteins are DNA-binding domains derived from various plant bacterial pathogens of the genus *Xanthomonas*. The *Xanthomonas* pathogens secrete TALEs into the host plant cell during infection. The TALE moves to the nucleus, where it recognizes and binds to a specific DNA sequence in the promoter region of a specific gene in the host genome (Boch and Bonas 2010). TALE has a central DNA-binding domain composed of 13–28 repeat monomers of 34 amino acids (**Fig. 4**). The amino acids of each monomer are highly conserved, except for hypervariable amino acid residues at positions 12 and 13. These two variable amino acids are called repeat-variable diresidues (RVDs). The crystal structure of TALE revealed that the first RVD residue forms a stabilizing contact with the backbone of the RVD loop, while the second makes a base-specific contact with DNA (Deng et al. 2012, Mak et al. 2012). The amino acid pairs NI, NG, HD and NN of RVDs preferentially recognize adenine, thymine, cytosine and guanine/adenine, respectively (**Fig. 4**), and modulation of RVDs can recognize consecutive DNA bases. TALEN is composed of a DNA-binding domain and an endonuclease *Fok*I domain. Like ZFN, TALEN dimerizes when two monomers recognize individual DNA target sites (**Figs. 3**, **4**). Since it has several advantages, especially the

large range of engineered TALEs for target binding, TALEN has been used as an effective genome editing tool in various organisms and cells (human cells, Miller et al. 2011; rat, Tesson et al. 2011, Mashimo et al. 2013; livestock, Carlson et al. 2012; pig, Lillico et al. 2013; zebrafish, Huang et al. 2011, Sander et al. 2011a; *Drosophila*, Liu et al. 2012; *Caenorhabditis elegans*, Wood et al. 2011; *Xenopus*, Ishibashi et al. 2012, Lei et al. 2012) including plants (Arabidopsis, Christian et al. 2013; tobacco, Zhang et al. 2013; rice, T. Li et al. 2012).

The flanking region of the DNA-binding domain in the TALEN protein affects TALEN activity in higher eukaryotes including plant species (Voytas 2013). Constructs containing all of the N- and C-terminal regions flanking the DNA-binding domain in TALEN have successfully targeted sequences for mutagenesis in Arabidopsis and rice (Cermak et al. 2011, T. Li et al. 2012); however, several reports showed that TALEN activity was enhanced when the N- and C-terminal regions were truncated (Miller et al. 2011, Mussolino et al. 2011, Sun et al. 2012, Zhang et al. 2013). Deletion of both N- and C-terminal amino acids in TALEN may stabilize the protein and/or facilitate protein folding (Voytas 2013). To create a custom TALEN, a ligation-based method, the so-called 'Golden Gate system', using multiple plasmids encoding different TALE repeats, has been developed to assemble similar repeats in TALE (Cermak et al. 2011, L. Li et al. 2012, Sanjana et al. 2012). The Golden Gate system is one of the most powerful tools available to generate custom TALENs. Recently, Sakuma et al. (2013) described an improved system with high TALEN activity and TALE repeat assembly, namely the 'Platinum TALEN and Platinum Gate system'. Platinum TALEN has been used effectively to introduce mutations in the frog and rat genomes. These platforms for TALEN assembly are available from a non-profit plasmid repository, Addgene (http://www.addgene.org).

Recently, a novel method for site-directed mutagenesis using an adaptive bacterial and archeal immune systems has emerged. The CRISPR/Cas9 system is an alternative to the commonly used *Fok*I-based methods ZFN and TALEN. The CRISPR system is an acquired immune system that protects against invading phages and plasmid DNA. The CRISPR locus is composed of an operon encoding the Cas9 protein and a repeated array of repeat spacer sequences (Wiedenheft et al. 2012). The repeat spacers are transcribed and processed to generate mature crRNAs, which are complementary to the exogenous DNA, and then crRNA is annealed to the tracrRNA; a hybrid crRNA–tracrRNA (a single guide RNA; gRNA) then guides the Cas9 nuclease to cleave the DNA. This system has the advantage that the researcher need make only a single construct for expression or RNA synthesis of gRNA (Jinek et al. 2013, Jinek et al. 2014) (**Fig. 5**). Therefore, the CRISPR/Cas9 system is able to target and create DSBs in any genomic DNA sequence using a specifically designed gRNA together with a PAM sequence (protospacer adjacent motif—a short nucleotide sequence at the 3′ end of the target sequence in the genome; **Fig. 5**). The Cas9 from *Streptococcus pyogenes* (SpCas9) recognizes 5′-NGG-3′ as the PAM sequence. PAM plays an important role in target binding and cleavage by the Cas9–gRNA complex (Sternberg et al. 2014). SpCas9 is directed to the desired

genomic target by a 20-nucleotide guide sequence in the gRNA, thus the CRISPR/Cas9 system is simple, easy to design and highly effective (Deveau et al. 2010, Deltcheva et al. 2011, Gasiunas et al. 2012, Jinek et al. 2012, Cong et al. 2013, Mali et al. 2013a). The CRISPR/Cas9 system has been used successfully to cleave target DNA and introduce mutations in eukaryotic cells, including human cells (Cong et al. 2013, Mali et al. 2013a, Mussolino and Cathomen 2013) and plants (Li et al. 2013, Nekrasov et al. 2013, Shan et al. 2013b).

In genome editing technologies, specific target recognition is critical, since any off-target effects generated by custom nucleases are generally toxic, and unexpected mutations throughout the genome cannot be monitored easily. The high specificity of target recognition in the CRISPR/Cas9 system, where specificity is determined by a 20 nucleotide guide RNA, has recently been reported (Cong et al. 2013, Fu et al. 2013, Hsu et al. 2013). Although target specificity is dependent on the number and position of mismatches between the gRNA and the target DNA sequence, recent studies suggest that the 3′ end of the guide sequences that flank PAM confer target specificity, while mismatches at the 5′ end are tolerated (Cong et al. 2013, Fu et al. 2013, Hsu et al. 2013, Jiang et al. 2013a, Mali et al. 2013b, Pattanayak et al. 2013). To avoid potential off-target effects, and to improve the specificity of CRISPR/Cas9, several approaches to genome editing have been used (Ran et al. 2013, Fu et al. 2014). Ran et al. (2013) developed a strategy that combines a mutated version of Cas9 (D10A, Cas9 nickase; Cas9n) (**Fig. 5**), which can induce nicks (single-stranded DNA breaks; SSBs) to improve the specificity of CRISPR/Cas9 (Gasiunas et al. 2012, Jinek et al. 2012, Cong et al. 2013, Ran et al. 2013). Double nicking using two sets of Cas9n with distinct gRNAs can generate two SSBs on complementary strands of the target sequence; thus a DSB is created specifically at the target site, followed by NHEJ. This strategy of using paired nickases effectively minimizes Cas9-induced off-target effects of gRNA in human cells (Ran et al. 2013). Several studies have suggested that single monomeric Cas9n can induce indel mutations at several loci in the genome (Mali et al. 2013a, Mali et al. 2013b, Ran et al. 2013, Fu et al. 2014). Therefore, further optimization of paired nickases is needed to enhance the co-dependent activity of nickase monomers (Sander and Joung 2014) (**Fig. 5**). Paired nickases have also been utilized in ZFN-mediated genome editing, in which the ZFN nickases reduced off-target mutagenesis in the same way as Cas9n (Kim et al. 2012, Ramirez et al. 2012, Wang et al. 2012), and HR induced by ZFN nickases has also been identified in this system (McConnell et al. 2009).

Another approach to improving the specificity of CRISPR/Cas9 systems is the use of specialized gRNAs, i.e. truncated gRNAs with a target sequence shorter than 20 nucleotides (Fu et al. 2014). A recent study suggested that gRNAs truncated at the 5′ end of their complementary sequences (17–18 nucleotides) can also decrease off-target effects (Fu et al. 2014). Furthermore, the careful design of gRNAs has been effective in avoiding off-target effects (Mali et al. 2013b, Pattanayak et al. 2013), and optimization of gRNA and Cas9 expression levels are related to the specificity of these effects (Fu et al.

2013, Pattanayak et al. 2013, Ran et al. 2013). The crystal structure of Cas9 was determined recently by Jinek et al. (2014) and Nishimasu et al. (2014), and their findings suggest a bi-lobed enzyme architecture that undergoes conformational changes upon binding to gRNA. Another study by Sternberg et al. (2014) using single-molecule imaging, showed that the Cas9–gRNA complex forms a long-lived interaction with target sites that contain a PAM, whereas binding at non-target sequences and at complementary sequences without PAMs is only transient. Furthermore, this study also showed formation of a gRNA–DNA heteroduplex, and that subsequent DNA cleavage was initiated near, and was dependent on, the PAM. This suggests that PAM has an important function that stimulates Cas9 activity (Sternberg et al. 2014). Together with the findings of evolutionary variability in the Cas9 structure (Jinek et al. 2014), these studies may lead to improved specificity and activity of the Cas9–gRNA complex.

Genome Editing in Plants

Site-directed mutagenesis of plant species using genome editing tools

As alternatives to traditional methods in plant breeding, the genome editing techniques described above using ZFN, TALEN and CRISPR/Cas9 have led to genome engineering targeting important genes. In this section, we highlight the genome editing studies that have led to the modification of various plant genomes. ZFN has been reported as a useful genome editing technique to create target-specific mutations in Arabidopsis using a heat shock promoter (HSP) (Lloyd et al. 2005). Highly effective site-directed mutagenesis in Arabidopsis using ZFNs driven by an estrogen-inducible system has also been reported, leading to mutations in alcohol dehydrogenase and chalcone synthase genes (Zhang et al. 2010). We have also reported ZFN mutagenesis of the gene for *ABSISCIC ACID INSENSITIVE 4* in Arabidopsis using a HSP. The resulting novel *abi4* mutant showed a nearly identical response to the plant hormone ABA as the ABA-insensitive mutant studied earlier (Osakabe et al. 2010). Together, these, and most other ZFN studies (Tovkach et al. 2009, Tovkach et al. 2010), have utilized the controlled expression of ZF proteins to create DSBs and induce repair by NHEJ. ZFNs have also been applied for agricultural purposes in studies by groups from Dow AgriSciences, Inc. and Sangamo BioScience, Inc. Ainley et al. (2013) reported site-specific trait stacking using ZFNs. These authors produced a maize line containing a herbicide-resistant marker and a synthetic ZFN target site. By using the ZFN target site and the corresponding ZFNs, they site-specifically integrated an additional herbicide-resistant marker gene (second trait) flanked with a new ZFN target site. Therefore, this system allows multiple trait stacking at a specific locus.

Using extensions of the TALEN methodology, several studies have recently succeeded in creating target-specific mutations (Cermak et al. 2011, Mahfouz et al. 2011, T. Li et al. 2012, Christian et al. 2013, Gurushidze et al. 2014, Shan et al. 2013a, Zhang et al. 2013, Wendt et al. 2013). Cermak et al. (2011)

developed the assembly of custom TALE arrays known as the Golden Gate system referred to above, and used it to perform site-directed mutation in the *ADH1* gene in Arabidopsis protoplasts (Cermak et al. 2011). A de novo engineered TALEN has been created using Hax3 from the Brassicaceae pathogen *Xanthomonas campestris* pv. *armoraciae* strain 5 (Mahfouz et al. 2011). Hax3, which recognizes a 12 bp DNA, was used to construct an engineered nuclease for targeted mutagenesis in a transient assay using *Nicotiana benthamiana* (Mahfouz et al. 2011). Targeted mutagenesis with TALENs in the Arabidopsis genome using a stable transgenic approach was subsequently reported (Christian et al. 2013). Because of the relatively low toxicity of TALEN in comparison with ZFN, TALEN can be driven by strong constitutive promoters as well as the conditional inducible promoters used for ZFN expression (Zhang et al. 2010). Indeed, Christian et al. (2013) found that the use of a strong constitutive promoter was more efficient than using an inducible promoter for TALEN-mediated target mutagenesis.

TALEN technology has also been used in crops such as rice, barley and maize (T. Li et al. 2012, Shan et al. 2013a, Wendt et al. 2013, Gurushidze et al. 2014, Liang et al. 2014). The rice disease susceptibility gene and the sucrose efflux transporter gene *OsSWEET14* were mutated by TALEN, resulting in the generation of disease-resistant rice with normal phenotypes (T. Li et al. 2012). Furthermore, via genetic segregation, these latter authors successively obtained disease-resistant rice lacking the selection marker and TALEN genes. Although further analyses will be needed to demonstrate whether all transgenes have been removed from the TALEN-mediated mutant lines, these mutant plants can be discussed as candidates for non-genetically modified organisms without foreign DNA.

Large-scale targeted mutagenesis by TALEN in rice and *Brachypodium* has been reported by Shan et al. (2013a). This group generated rice plants with eight *Brachypodium* genes knocked out with high efficiency, as well as large genomic deletions (e.g. 1.3 kb) achieved by simultaneous expression of two sets of TALEN pairs (Shan et al. 2013a). Recently, Gurushidze et al. (2014) developed gene knockout systems using TALENs to disrupt genes in barley embryonic pollen cultures consisting primarily of haploid cells. These findings have high applicability for the detailed study of gene function and molecular breeding in various crop species.

Since the first publication reporting plant genome editing using the CRISPR/Cas9 system in 2013, this system has been applied to several plant species, including Arabidopsis (Feng et al. 2013, Jiang et al. 2013b, Li et al. 2013, Mao et al. 2013), tobacco (Jiang et al. 2013b, Li et al. 2013, Nekrasov et al. 2013), sorghum (Jiang et al. 2013b), rice (Feng et al. 2013, Jiang et al. 2013b, Miao et al. 2013, Shan et al. 2013b), wheat (Upadhyay et al. 2013), maize (Liang et al. 2014), sweet orange (Jia and Wang 2014) and liverwort (Sugano et al. 2014), suggesting its broad applicability. The first reference to the CRISPR/Cas9 system in plant genome editing appeared in August 2013, reporting the transient expression of CRISPR/Cas9 in Arabidopsis protoplasts (Li et al. 2013), tobacco cells

(Li et al. 2013, Nekrasov et al. 2013) and rice plants (Shan et al. 2013b) to create DSBs. Li et al. (2013) showed that the transient expression of a CRISPR/Cas9 vector in Arabidopsis protoplasts induced a mutation in homologous members of the multiplex genome as designed (Li et al. 2013). Using a haploid generation of *Marchantia polymorpha* L., Sugano et al. (2014) performed a simple and rapid genome editing procedure using CRISPR/Cas9 in the model species liverwort to study land plant evolution. Recently, Feng et al. (2014) and Jiang et al. (2014) reported the multigeneration analysis of CRISPR/Cas9-induced genome editing in Arabidopsis, showing the heritable mutation of a 1 bp insertion and short deletion with high efficiency. Recently, Zhang et al. (2014) reported efficient gene modification using CRISPR/Cas9 in rice, suggesting that the CRISPR/Cas9 system could become a powerful tool in crop genome engineering. Because the structure of the plant genome and gene families are highly redundant and overlapping, off-target effects are an important issue when considering genome editing. Recently, Fauser et al. (2014) reported that application of nickase resulted in efficient genome engineering in Arabidopsis. Concentrated efforts to extend the aforementioned findings, together with the precise and detailed evaluation of genome-edited plants to detect off-target effects will be extended to allow the further application of genome editing for crop breeding.

Gene targeting and targeted gene addition in plants

Genome editing tools provide new strategies for genetic manipulation in plants and are likely to assist in engineering desired plant traits by modifying endogenous genes. For instance, site-specific gene addition in major crop species can be used for 'trait stacking', whereby several desired traits are linked physically to ensure their co-segregation during the breeding process. Gene targeting (GT) is a genome engineering method used to introduce modifications into endogenous genomic sequences via HR. In GT, a transferred foreign gene with a homologous sequence is used to target the gene as a HR repair template (Osakabe et al. 2006, Osakabe et al. 2012). HR is induced by creating DSBs at the target site. For example, the expression of I-Sce I, a rare-cutting restriction enzyme, has been shown to lead to a significant increase in HR-mediated GT in tobacco cells by using the artifital target site (Puchta et al. 1996); however, rare-cutting enzymes such as I-SceI cannot be used for general purposes to create DSBs for HR in any gene of interest.

To overcome the low efficiency of GT events in the gene of interest, custom-engineered nucleases that induce DSB and HR at target sites have been developed. In the fruit fly, an engineered ZFN that recognizes the yellow gene was expressed in flies in the presence of donor DNA, resulting in GT induction at the target gene with remarkable efficiency (Bibikova et al. 2003). Urnov et al. (2005) reported that engineered ZFN and induced HR can be used to correct human genetic defects, and that the ZFN can also be used to increase GT frequency in plants (Shukla et al. 2009). A ZFN designed for maize genes and a

heterologous donor molecule were introduced into maize cells, and the results suggested that ZFN effectively controlled the addition of the target gene at a specific site in the genome. Up to 20% of selected lines showed GT events. Furthermore, GT events were inheritable and transmitted to the next generation (Shukla et al. 2009). Another study by Townsend et al. (2009) has shown that ZFN-mediated GT can be used in a transient expression system in tobacco. These authors revealed that the efficiency of GT reached 4%. These works demonstrate that cleavage of a chromosomal target by ZFNs dramatically stimulates HR-mediated gene targeting in plants, and provides a basis for future experiments with ZFNs directed to endogenous genomic locations.

Recently, targeted gene insertion using TALEN in tobacco protoplasts has been reported with high efficiency (Zhang et al. 2013). The CRISPR/Cas9 system is also useful in HR-mediated targeted gene insertion in tobacco (Li et al. 2014) and rice (Shan et al. 2013a). Because low HR efficiency was found in several plant species, appropriate optimization and improvements are needed for extended plant genome engineering using custom-engineered nuclease-mediated GT.

Future Prospects

Current molecular biology allows targeted gene mutations to be achieved effectively by GEEN. In gene modification, these targetable nucleases have the potential to become alternatives to standard breeding methods to identify novel traits in economically important plants and animals. However, to extend genome editing methods in order to make them more applicable and useful for various plant species, further improvements are required to overcome their limitations. Currently, the promising nuclease system CRISPR/Cas9 is becoming more widely used because it is easy to set up, and it is easy to design targets and constructs. One major problem of the CRISPR/Cas9 system is the generation of off-target mutations; however, this problem has been overcome by using the double-nicking CRISPR/Cas9 system described above (Ran et al. 2013), as well as new gRNA design systems (Fu et al. 2014). These systems should now be applied to genome editing in plants.

An important issue in plant genome editing is how to deliver and express the engineered nucleases in plant cells (**Fig. 3**), because not all useful plant species have established regeneration and transgenic methods. The selection of appropriate plant tissues, and the optimization of methods for transformation and culture, are major issues that remain to be resolved to provide opportunities to generate novel, useful crops. Thus, efficient systems to deliver genome editing tools into plant cells must be developed. A recent study (Baltes et al. 2014) used geminivirus-based replicons to deliver DNA for genome editing, enabling efficient genome engineering to be applied to various plant species. As new plant breeding techniques develop, these efforts, together with a deeper understanding of the structure and function of whole genomes, will enable the development of future technologies in breeding new and important traits in plants.

Disclosures

The authors have no conflicts of interest to declare.

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