Chapter 12
Zinc Finger Nuclease-Mediated Gene Targeting in Plants

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DNA Double-Strand Breaks (DSBs)

The creation and repair of DNA DSBs is of central importance to the recombination between DNA sequences (Xu and Price 2011). Pioneering studies in yeast have highlighted the importance of DSBs in both meiotic (Keeney 2001) and mitotic (Lisby and Rothstein 2007) DNA recombination. The induction of genomic DSBs and their repair via various homologous and nonhomologous processes is well established (Haber 2007). Many of the genes involved in DSB repair have been elucidated and found to be conserved across a broad range of life-forms (Li et al. 2011), although the contributions of each to the DNA repair process have dramatically changed during evolution (Sonoda et al. 2006). These studies have highlighted the dual role of DSB formation and resolution as a means of both promoting genetic diversity by facilitating DNA sequence exchange and conserving genomic integrity via DNA repair.

DSBs can be repaired using homologous sequences, i.e., from a sister chromatid or other related template DNA, via pathways involving a collection of proteins which facilitate strand resection, invasion, annealing, and synthesis reactions resulting in an intact DNA sequence (Rajesh et al. 2011). Alternative pathways of DSB repair involve nonhomologous end joining (NHEJ) of DNA sequences whereby cleaved ends are religated without regard for homology, often resulting in deletions or insertions at the cleavage site (Wu et al. 2012). These complexes of apparently competing processes effectively repair DSBs with varying degrees of fidelity (Shibata et al. 2011).

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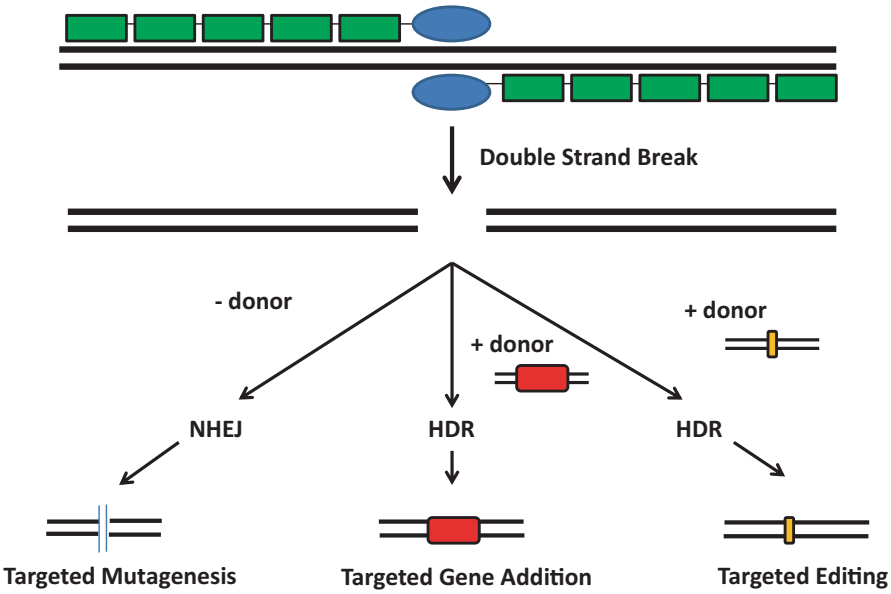

Fig. 12.1 ZFNs facilitate targeted genome modifications. ZFNs can be designed to specific genomic sequences to enable targeted gene addition, gene editing, and targeted mutagenesis. Delivery of ZFNs into cells results in targeted double-strand DNA breaks that are repaired by cellular repair mechanisms such as NHEJ and HDR. Repair of the double-strand DNA break by NHEJ leads to introduction of indels (insertions/deletions) at the cut site and targeted mutagenesis in the genome. Repair in the presence of an exogenous DNA molecule carrying a gene of interest (donor) with homology to the break site leads to targeted gene insertion. Presence of specific mutations in the donor result in edits at desired locations in the genomic sequence. *DSB* double-strand break, *NHEJ* nonhomologous end joining, *HDR* homology-directed repair, *indels* insertions/deletions. Zinc finger DNA-binding domains are represented by *green rectangles* and the *blue circle* represents the Fok1 nuclease domain

In higher plants, it appears as if DSBs are most typically repaired via NHEJ where sequence-independent repair often results in deletions, insertions, and/or rearrangements at the break site (Gorbunova and Levy 1999; Puchta 2005). Although not completely understood, it appears as if several NHEJ pathways in plants operate to repair DSBs (Charbonnel et al. 2011). If homologous sequences are in close proximity to the DSB, high-fidelity, homology-directed repair has been observed to occur in plant cells (Roth et al. 2012; Siebert and Puchta 2002).

The ability to generate DSBs, thereby stimulating the cell's DNA repair processes, represents a means of facilitating genetic modification (Fig. 12.1). The error-prone nature of NHEJ repair makes induction of DSBs a method for inducing mutations (Carroll 2011). Intervening sequence elimination following the formation and repair of concurrent DSBs is a means of generating various sorts of gene deletions (Lee et al. 2010). Homology-directed repair of DSBs enables transgene integration (Lombardo et al. 2011) and genome editing (McMahon et al. 2012).

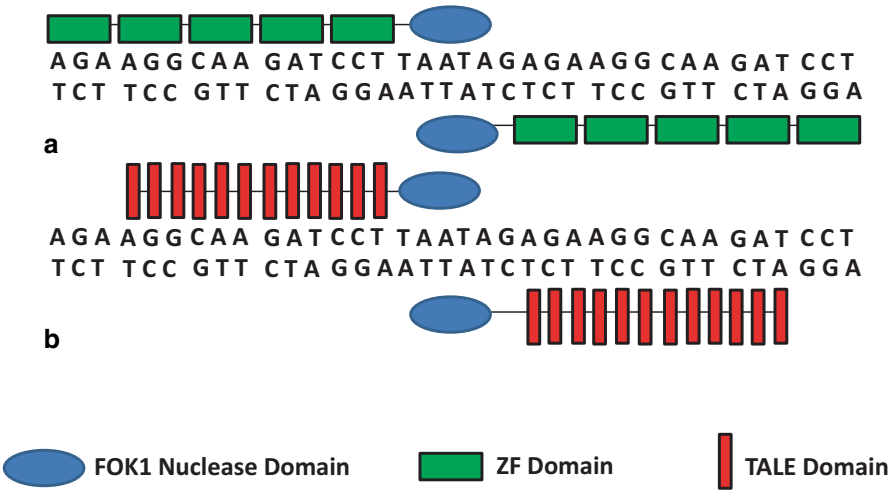

Fig. 12.2 Double-strand DNA binding by site-specific nucleases. Schematic representation of a ZFN (a) ZFN and a TALEN (**b**) bound to DNA. ZFNs recognize and bind DNA through the zinc finger domains (*green*, **a**) and each finger binds a nucleotide triplet. DNA binding by TALENs is mediated by TALE effector (*red*, **b**) domains with single nucleotide specificity. The nonspecific *Fok-1* nuclease domain is shown in *blue*

Designed Zinc Finger Nucleases (ZFNs)

To take advantage of DSB repair for controlled genome modification, a method is required for targeted DNA cleavage (Puchta and Fauser 2013). Previously, targeted DSBs could only be made in plant genomes following pre-integration of restriction enzyme cleavage sites and expression of genes encoding the corresponding restriction enzyme (Salomon and Puchta 1998). Recently, ZFNs have been described that allow for DSB formation at endogenous plant loci (de Pater et al. 2013; Shukla et al. 2009; Townsend et al. 2009; Zhang et al. 2010). ZFNs are engineered restriction enzymes consisting of a nonspecific cleavage domain and sequence-specific DNA-binding domains designed to create site-specific DSBs (Porteus and Carroll 2005). In this way, DSBs can be targeted to investigator-specified sites by engineering and delivering novel sequence-specific restriction enzymes capable of binding and cleaving endogenous genomic DNA (Tzfira et al. 2012).

Zinc finger protein domains consist of ~30 amino acids which, upon chelating a zinc atom, fold into ββαstructures capable of binding specific DNA triplets (Pabo et al. 2001). Key amino acid residues in the α helix dictate sequence-specific binding, while the remaining amino acids maintain a consensus backbone structure with a modular architecture (Durai et al. 2005). Linking such modular structures together allows for the creation of DNA-binding domains capable of recognizing predetermined stretches of sequence (Fig. 12.2a). The development of designed ZFNs that cleave DNA at predetermined sites depends on the reliable creation of zinc finger

Table 12.1 Use of ZFNs for targeted genome modification in plants

Species	Description of the study	Reference
Targeted mutagenesis		
Arabidopsis thaliana	Stably integrated a ZFN cleavage site along with a ZFN gene. Mutated target sequence	Lloyd et al. (2005)
Arabidopsis thaliana	Stably integrated a *GUS* reporter gene disabled with a stop codon in a ZFN cleavage site. Mutated the stop codon following retransformation with a ZFN gene	Tovkach et al. (2009)
Arabidopsis thaliana	Stably integrated a ZFN cleavage siteRetransformed with ZFN to generate targeted mutations	de Pater et al. (2009)
Arabidopsis thaliana	Transformed with a gene encoding a ZFN designed to cleave, *ADH1* and *TT4*	Zhang et al. (2010)
Arabidopsis thaliana	Transformed with a gene encoding a ZFN designed to cleave *ABI4*	Osakabe et al. (2010)
Glycine max	Transformed with a gene encoding a ZFN designed to cleave *DCLa* and *DCLb*	Curtin et al. (2011)
Nicotiana tabacum	Transformed with a gene encoding a ZFN designed to cleave *SuRA*	Maeder et al. (2008); Townsend et al. (2009)
Zea mays	Transformed with a gene encoding a ZFN designed to cleave *IPK1*	Shukla et al. (2009)
Gene deletion		
Nicotiana tabacum	Stably integrated a ZFN cleavage site-flanked *GUS* reporter gene in one plant and a corresponding ZFN in another. Deleted reporter gene in hybrid and progenies	Petolino et al. (2010)
Site-specific transgene integration		
Arabidopsis thaliana	Stably integrated a ZFN cleavage site Retransformed with ZFN and homologous donor for targeted transgene integration	de Pater et al. (2009)
Nicotiana tabacum	Co-delivered ZFN and homologous donor DNA to repair a nonfunctional *GUS*/*NPTII* fusion gene	Wright et al. (2005)
Nicotiana tabacum	Gene addition into a pre-integrated partial *PAT* gene flanked by ZFN cleavage sites via co-delivery of homologous donor DNA and ZFN gene	Cai et al. (2009)
Nicotiana tabacum	Targeted *PAT* gene integration into *CHN50* using a designed ZFN	Cai et al. (2009)
Nicotiana tabacum	Stably integrated a ZFN cleavage site-flanked *GFP* gene and replaced with an incoming ZFN cleavage site-flanked *HPT* gene co-delivered with a ZFN gene	Weinthal et al. (2013)

Table 12.1 (continued)

Species	Description of the study	Reference
Zea mays	Targeted PAT gene integration into *IPK1* with autonomous and nonautonomous homologous donor DNA and designed ZFNs	Shukla et al. (2009)
Zea mays	Stably integrated an *AAD1* gene flanked by ZFN cleavage site and targeted a *PAT* gene into the locus	Ainley et al. (2013)
Genome editing		
Arabidopsis thaliana	Modified an endogenous *PPO* gene using homologous donor DNA with 2 mutations and a designed ZFN	de Pater et al. (2013)
Nicotiana tabacum	Generated specific mutations of *SuRA* and *SuRB* using homologous donor DNA and designed ZFNs	Townsend et al. (2009)

ZFN zinc finger nuclease

protein domains that can specifically recognize the chosen target site within a genome. The design, assembly, and validation of such DNA-binding proteins based on modular zinc fingers are becoming more routine (Hurt et al. 2003; Isalan et al. 2001; Maeder et al. 2008; Mandell and Barbas 2006). ZFN design services are commercially available (e.g., ComposZr® from Sigma-Aldrich).

DNA cleavage is facilitated by a sequence-independent nuclease domain from the bacterial type IIS restriction endonuclease *Fok*I(Kim et al. 1996). To cut DNA and generate a DSB, the *Fok*I nuclease domain needs to dimerize at the cleavage site (Bitinaite et al. 1998). A ZFN is created by linking the *Fok*I cleavage domain to the C-terminus of a tethered series of zinc finger protein domains designed to bind a specific DNA sequence. Upon binding of two adjacent ZFN pairs to sequences flanking the intended cleavage site in a precise orientation and spacing relative to each other, the *Fok*I domains dimerize thereby facilitating DSB formation (Fig. 12.2a). ZFNs have been used to create targeted DSBs and enable genome modification in a broad spectrum of genomes, including human (Lombardo et al. 2007; Moehle et al. 2007; Perez et al. 2008; Porteus and Baltimore 2003; Provasi et al. 2012; Sebastiano et al. 2011; Urnov et al. 2005; Wilen et al. 2011), hamster (Santiago et al. 2008), mouse (Osiak et al. 2011), pig (Hauschild et al. 2011), frog (Young et al. 2011), zebra fish (Doyon et al. 2008), insect (Beumer et al. 2006; Bibikova et al. 2002), roundworm (Morton et al. 2006), and *Plasmodium* (Straimer et al. 2012). The present chapter reviews the use of designed ZFNs for inducing targeted DSBs and facilitating genome modification in plants (Table 12.1).

Targeted Mutagenesis

The ability to modify specific gene sequences is an indispensable tool for systematic analysis of plant gene function (Perry et al. 2003). Since DSB repair in plants appears to be primarily via NHEJ (Gorbunova and Levy 1999; Puchta 2005) and, since NHEJ in plants tends to be rather error-prone (Britt 1999), targeted DSB formation is a path toward targeted mutagenesis (Lyznik et al. 2012). Designed ZFNs appear to be ideally suited for such an application.

The first proof-of-concept study demonstrating ZFN-mediated targeted mutagenesis in plants involved the mutation of an introduced construct comprising a ZFN cleavage site and a corresponding ZFN under the control of a heat shock promoter (Lloyd et al. 2005). The experimental system involved an *Eco*R1 restriction sequence within the ZFN cleavage site which could be lost upon mutation, due to NHEJ-induced deletion or insertion, thereby allowing mutations to be identified. TOPO-cloning of polymerase chain reaction (PCR) products amplified from genomic DNA from heat-treated T_1 *Arabidopsis* seedlings with single copy integration of the construct revealed mutation frequencies across multiple independent transgenic events, measured by lost *Eco*R1 restriction sites, to be in the range of 1.7–19.6 % based on a random sampling of clones. Sequencing of the *Eco*R1-minus clones illustrated the types of mutations resulting from DSB repair. Most of the mutations (78 %) were simple deletions of 1–52 bp. Simple insertions (1–4 bp) and combinations of insertions and deletions were also observed at lower frequency. These frequencies should be considered to represent an underestimate of the actual mutation frequency. Based on the design of the targeting construct, larger deletions (>62 bp) which removed the PCR primer binding sites would not have been observed in this study. In fact, NHEJ-mediated deletions of 0.2–2.0 kb have been commonly observed and 50 % of all such deletions were found to be >100 bp (Gorbunova and Levy 1999). Nonetheless, this study clearly demonstrated that ZFN-mediated DSB formation can lead to targeted mutations.

In a similar study, *Arabidopsis* plants, stably transformed with a target construct comprising an *Eco*R1-containing ZFN cleavage site, were retransformed with corresponding ZFN-expressing constructs driven by various promoters (de Pater et al. 2009). Most *Eco*R1-resistant DNA fragments amplified from transgenic plants contained deletions ranging from 1 to 80 bp. Small insertions (1–14 bp) and larger deletions (up to 200 bp) were also observed. Mutation frequency was estimated to be about 2 % based on a random sampling of cloned PCR fragments. Reverse transcription polymerase chain reaction (RT-PCR) was used to estimate relative ZFN expression. Driving the ZFN gene with a stronger promoter appeared to be more effective at generating mutations.

Additional examples of the ability of ZFN expression to mediate targeted genome modification via NHEJ DSB repair involved the mutation of a disabled reporter gene (Cai et al. 2009; Tovkach et al. 2009). In one study (Tovkach et al. 2009), a *GUS* gene, engineered to carry a TGA stop codon within a ZFN cleavage site—and thereby rendered nonfunctional, was stably transformed into tobacco. As expected,

transgenic tissue did not express the *GUS* reporter gene. Cocultivation of transgenic tissue with an *Agrobacterium* strain harboring a construct containing a corresponding ZFN expression cassette resulted in small sectors of positive GUS staining. Similarly, *Arabidopsis* plants stably transformed with the nonfunctional *GUS* gene and a ZFN under the control of a heat shock promoter expressed GUS following high-temperature induction. Sequence analysis of the target site following PCR amplification identified several single nucleotide deletions and substitutions resulting in an open reading frame expected to encode an active *GUS* gene. This mutation was also facilitated using viral delivery of a ZFN (Vainstein et al. 2011). In another study (Cai et al. 2009), a reporter construct carrying a *GFP* gene disabled by the insertion of a 2.8-kb stretch of heterologous DNA containing a ZFN-binding site was stably integrated into tobacco cell cultures. A tandem repeat of 540 bp in the two *GFP* gene fragments served as a substrate for intrachromosomal repair. Upon retransformation with a ZFN gene, fluorescent foci were visible and PCR analysis confirmed homology-directed repair of the targeted DSB.

Mutations at endogenous gene loci have also been demonstrated following expression of designed ZFNs (Maeder et al. 2008; Shukla et al. 2009; Townsend et al. 2009). Tobacco protoplasts were transformed with a ZFN designed to cleave a specific site within the *SuRA* gene. Among 66 transgenic plants regenerated, three displayed single base mutations in the SuRA gene (Maeder et al. 2008). Similarly, ZFNs designed to cleave *SuRA* and *SuRB* genes displayed varying degrees of specificity relative to creating site-specific mutations (Townsend et al. 2009). A ZFN designed to cleave within the maize *IPK1* gene was transiently expressed in cultured maize cells after which multiple deletions and insertions were observed following deep sequencing of PCR amplified products (Shukla et al. 2009).

Genes encoding ZFNs designed to recognize *Arabidopsis ADH1* and *TT4* driven by an estrogen-inducible promoter resulted in somatic mutation frequencies of 7 and 16 %, respectively (Zhang et al. 2010). The mutations were typically 1–142 bp insertions or deletions localized at the ZFN cleavage site and were often found to be biallelic, i.e., homozygous. A ZFN gene, designed to recognize the *Arabidopsis ABI4* gene sequence, driven by a heat shock promoter, upon induction, resulted in up to 3 % mutagenesis of the binding site and the appearance of expected phenotypes, i.e., abscisic acid (ABA) and glucose insensitivity, in homozygous progeny (Osakabe et al. 2010). In a similar study, independent mutations in the paralogous *DCLa* and *DCLb* soybean genes involved in RNA silencing were generated using designed ZFNs (Curtin et al. 2011). Taken together, these results suggest the general utility for basic and applied studies of making site-specific mutations by expressing ZFNs designed to create targeted DSBs and induce NHEJ repair.

Mutation breeding in plants has resulted in numerous commercially relevant varieties in a broad spectrum of crop species (Maluszynski 2001). Conventional methods of mutagenesis used to generate genetically-modified crops typically involve random perturbations in the DNA sequence, using treatment with chemicals such as ethyl methanesulfonate (Watanabe et al. 2007), physical methods such as fast neutron radiation (Li et al. 2001) or naturally occurring genetic mechanisms such as transposable elements (Mathieu et al. 2009) combined most recently with

sequence-specific screening (McCallum et al. 2000). Such approaches have serious limitations, such as the lack of observable phenotypes, in highly duplicated genomes such as those found in modern domesticated crop species (Pham et al. 2010). More targeted transgenic approaches, such as RNAi-based gene silencing, have been fraught with unanticipated phenotypic consequences presumably due to lack of specificity and potential off-target effects (Duxbury and Whang 2004). The ability to modify single or multiple gene copies in duplicated genomes of crop species would represent a powerful means of generating new genetic variants. Targeted mutagenesis via sequence-specific DSB formation and repair using designed ZFNs enables such a capability.

Gene Deletion

As complete plant genomic sequences become elucidated, the need to assign functions to unknown genes becomes increasingly important. This is most effectively approached via reverse genetics and the analysis of gene disruptions, including silencing (Baulcombe 1999), insertional mutants (Feldmann 1991), and deletions (Koornneeff et al. 1982). Conventional methods of creating plant gene deletions, such as exposure to fast neutron emission, combined with molecular analysis of pooled arrays of mutant DNA, have resulted in the assembly of large deletion libraries covering most known genes in *Arabidopsis* and rice (Li et al. 2001). The ability to generate investigator-specified deletions by creating targeted DSBs, followed by subsequent intervening sequence removal via DNA repair, represents an increasingly powerful refinement for genome modification. In human cell cultures, predetermined genomic DNA segments up to 15 mega-bp were deleted following expression of ZFNs designed to cleave at specific loci (Lee et al. 2010). Targeted deletions of promoter or exon sequences by generating DSBs in intergenic regions or introns could result in targeted gene knockouts, including multigene disruption. By virtue of the polyploid nature of most crop species, agronomically relevant genes exist as multiple copies such that single gene disruptions may not result in discernable phenotypes (Pham et al. 2010). The ability to knockout multiple homologous genes simultaneously with carefully designed ZFNs might be particularly useful for crop improvement.

Proof of concept for ZFN-mediated gene deletion was obtained in a recent study involving the removal of a ZFN cleavage site-flanked reporter gene from a stably transformed plant by crossing it with a second plant expressing a corresponding ZFN gene (Petolino et al. 2010). A target construct, containing a *GUS* reporter gene flanked by ZFN cleavage sites, was used to generate transgenic tobacco target events. A second construct, containing a ZFN gene driven by a strong constitutive promoter, was used to generate separate transgenic ZFN events. Homozygous T_1 target plants, which expressed the *GUS* reporter gene, were crossed with homozygous T_1 ZFN plants, which expressed the ZFN gene. Numerous *GUS*-negative hybrid plants were observed (up to 35 % in one cross). Evidence for complete deletion of a 4.3-kb sequence between the ZFN cleavage sites was obtained and sequence

verified in hybrid plants and progenies. Since ZFNs can be designed to cleave a wide range of DNA sequences, the results from this study constitute a general strategy for creating targeted deletions.

Site-Specific Transgene Integration

The ability to introduce exogenous DNA into a predetermined location within the plant genome would greatly enhance the precision and predictability of transgenic technology. The potential mutagenic effects of random DNA integration and the unpredictable consequences of position effect on transgene behavior could be circumvented by targeting transgenes to specific genomic locations.

Early attempts at targeted transgene integration used a combination of integrated, nonfunctional selectable marker genes and exogenous DNA homologous and complementary to the integrated target (Offringa et al. 1990; Paszkowski et al. 1988). Transgene integration into the target site was achieved under selective conditions following correction of the nonfunctional selectable marker gene at very low frequency, i.e., estimated to be in the range of 10^{-4}–10^{-5}. In similar approaches, nonfunctional *ALS* gene fragments, carrying mutations that specified resistance to various herbicides, were used to target the endogenous gene loci in tobacco (Lee et al. 1990) and rice (Endo et al. 2007). Using herbicide selection, transgenic events were obtained that suggested that homologous recombination between the exogenous DNA and the endogenous gene had occurred at estimated frequencies in the range of 10^{-4}–10^{-5}. "Brute force" attempts at generating transgenic events via homologous recombination without direct selection corroborated the extremely low frequency of targeted transgene integration (Miao and Lam 1995). Some success was reported using a combination of positive and negative selection to enrich for targeted events, whereby a targeting construct containing an antibiotic resistance gene within and a cytosine deaminase gene outside sequences homologous to an endogenous locus allow for selection against random integration in the presence of fluorocytocine (Xiaohui Wang et al. 2001). Subsequently, rice *Waxy* and *adh2* genes were successfully targeted using a similar approach whereby a diphtheria toxin gene was used as a negative selectable marker (Terada et al. 2007; Terada et al. 2002). Attempts to enhance targeted transgene integration by modifying DNA repair pathways, such as co-expressing recombinase genes (Reiss et al. 2000; Shaked et al. 2005; Shalev et al. 1999), or knocking out genes associated with NHEJ (Jia et al. 2012), have met with limited success. Clearly, homology-directed repair does occur in plants and can facilitate targeted transgene integration; however, the frequency of targeted versus random integration appears to be too low for practical use with conventional transformation technology.

The yeast mitochondrial endonuclease, *I-sceI,* which has an 18-bp recognition sequence, has been used to demonstrate the importance of homology-directed repair of DSBs for targeted transgene integration (Puchta et al. 1996). A target construct containing an *I-sceI* restriction site flanking a partially deleted antibiotic resistance

gene was transformed stably into tobacco. Retransformation with a repair construct containing sequences homologous to the target construct and complementary to the deleted antibiotic resistance gene together with an *I-sceI* expression construct resulted in targeted transgene integration at the *I-sceI* cleavage site. Using different ratios of *Agrobacterium* strains harboring the repair versus the *I-secI* construct, it appeared as if the induction of DSBs by the *I-sceI* was rate limiting, i.e., the best targeting frequency (18.8×10^{-3}) was achieved using a 1:9 ratio of repair: *I-sceI* strain. Thus, the induction of DSB formation and its repair via homology-directed processes are a key to targeted transgene integration.

Using analogous strategies, targeted transgene integration into transgenic reporter loci via homology-directed repair has also been demonstrated after ZFN-mediated DSB formation in tobacco (Cai et al. 2009; Wright et al. 2005). Following stable integration of a defective *GUS/NPT*II reporter gene containing a 600-bp deletion and a ZFN cleavage site, transgenic protoplasts were electroporated with DNA encoding the corresponding ZFN and donor DNA homologous to the target and capable of correcting the deletion. Homology-directed repair of the reporter gene occurred in more than 10% of the protoplasts across multiple transgenic events, i.e., target chromosomal positions (Wright et al. 2005). In a similar study, a pre-integrated reporter construct containing a 3′ partial herbicide resistance gene fragment flanked by ZFN binding sites allowed for in vitro selection following targeted integration of a complementary 5′ sequence from an incoming donor DNA co-transformed with a ZFN-expressing construct (Cai et al. 2009). Approximately 6 kb of target sequence between two ZFN cleavage sites was excised and replaced by 1.9 kb of donor DNA sequence using 1.2 and 1.7 kb of homology directly flanking each of two induced DSBs. These studies clearly illustrate the efficacy of ZFN-mediated DSB induction and the ability to effectively target exogenous DNA using homology-directed repair. NHEJ-mediated repair of DSBs has also been used to integrate DNA sequences in a targeted manner (Weinthal et al. 2013). ZFN-mediated cassette exchange was facilitated between an incoming promoter-less *hpt* gene and a pre-integrated GFP reporter gene both flanked with the same ZFN cleavage sites.

The ability to design ZFNs to cleave virtually any DNA sequence and thereby create investigator-modified, site-specific DSBs has allowed for targeted transgene integration into endogenous gene loci. Using a yeast-based system for screening ZFN efficacy (Doyon et al. 2008), ZFNs were designed against native gene sequences including, tobacco endochitinase (Cai et al. 2009) and maize *IPK1* (Shukla et al. 2009). An herbicide resistance gene driven by a constitutive promoter flanked on each side by 750 bp of endochitinase, *CHN50*, gene sequence was co-delivered with a ZFN expression cassette via *Agrobacterium* (Cai et al. 2009). Although the majority of the resulting transgenic events were the result of random integration, 5–10% of the events appeared to be targeted to the *CHN50* locus. Four different ZFN pairs targeting exon 2 of the maize *IPK1* gene were independently co-delivered with donor constructs containing a herbicide resistance gene cassette flanked by 815 bp of sequence homologous to IPK1 (Shukla et al. 2009). Two different donor constructs were used for targeted integration into the maize *IPK1* gene locus. One carried an autonomous herbicide resistance gene with its own promoter, whereas a second comprised a nonautonomous, i.e., promoter-less, gene that relied on precise trapping of

the endogenous *IPK1* promoter for expression and herbicide resistance. All four ZFN pairs drove targeted gene addition into their respective target sites, albeit with different efficiencies. In addition, site-specific transgene integration was successful using either donor construct with frequencies ranging from 3.4–22.3 % and 16.7–100 % for autonomous and nonautonomous constructs, respectively. Moreover, both monoallelic and biallelic insertions into the *IPK1* locus were observed. These exciting results with designed ZFNs not only extend transgenic technology to targeted transgene integration into endogenous genomic loci but also to include important crop species.

Genome Editing

The ability to make specific modifications to plant genome sequences in order to truly edit genes in a precise and predicable fashion would not only enhance basic understanding of plant biology but also ultimately result in genetically enhanced crops with new traits and improved performance. A recent study suggests that this capability might not be too far from reality (Townsend et al. 2009). Specific mutations in *SuR* genes in tobacco result in resistance to different imidazolinone herbicides. ZFNs were designed to cleave a specific sequences within the tobacco *SuRA* and *SuRB* genes. Electroporation of protoplasts with DNA encoding these engineered ZFNs along with donor DNA templates containing specific mutations resulted in herbicide resistance resulting from homology-directed processes. A surprising outcome was that mutation frequencies in the range of 2 % were observed with up to 1.3 kb removed from the DSB. Although this study relied on herbicide resistance for identifying edited events, the frequencies observed were high enough for screening via high-throughput DNA analysis. A ZFN designed to recognize the *Arabidopsis PPO* gene was co-delivered with a truncated *PPO* gene containing two mutations resulting in tolerance to the herbicide butafenacil using *Agrobacterium* floral dip transformation (de Pater et al. 2013). Targeted PPO modification was observed at a frequency of 3.1×10^{-3}. The combination of sequence-specific DNA cleavage by designed ZFNs and homology-directed DSB repair at investigator-specified break sites makes precise genome modification a reality. This capability, in combination with rapid advances in genome sequencing and bioinformatics, bodes well for the future of plant functional genomics and crop improvement.

Alternative Nuclease Technologies

Although ZFNs have become the most well-established tools for precise genome engineering, alternative nucleases are also available, such as those based on DNA binding domains from transcription activator-like effector (TALE) proteins (Boch and Bonas 2010) or "meganucleases" encoded by mobile introns (Arnould et al. 2011). TALEs are a family of proteins, first discovered in the plant pathogen *Xanthomonas* sp., that contain variable N- and C-terminal domains and a conserved central domain

for DNA binding (Boch et al. 2009). The DNA-binding domain consists of a variable number of tandem 34 amino acid repeats (Fig. 12.2b), whereby binding specificity is determined by the repeat-variant di-residues (RVDs) at positions 12 and 13, which specifically recognize a single nucleotide (Bogdanove and Voytas 2011; Deng et al. 2012; Moscou and Bogdanove 2009). A one-to-one correspondence of the RVDs to a single nucleotide enables TALE designs for any target DNA sequence of interest with a high degree of specificity, though the RVD binding is not completely independent of its neighbor in TALE derivatives (Streubel et al. 2012). TALE-*Fok1* nuclease (TALENs) fusions have been shown to facilitate genome modifications in several species, including human (Hockemeyer et al. 2011), rat (Tesson et al. 2011), zebra fish (Sander et al. 2011), worms (Wood et al. 2011), and plants (Cermak et al. 2011).

In contrast, designing ZFNs is more complex as each finger can only recognize a nucleotide triplet and there are multiple zinc finger designs for a given triplet of base pairs, with complex contextual interactions. Detailed knowledge of DNA binding of individual zinc fingers as well as the influence of various combinations of zinc fingers on binding specificity and affinity is required. Ease of design, high degree of specificity, minimal documented off-target effect, and low cost make TALENs an attractive alternative to ZFNs. Indeed, several recent reports of successful targeted mutagenesis following expression of designed TALENs suggest that this type of nuclease may represent a powerful addition to the arsenal of tools for plant genome modification (Li et al. 2012; Zhang et al. 2013). However, the larger size of TALENs (~3×) might limit their activity in plant cells primarily by effecting their expression negatively. Also, due to their pathogenic origins, TALENs might have a higher regulatory hurdle to cross for product development. Well controlled, comparative studies of ZFNs, and TALENs in plants will be critical for understanding their relative merits for precision genome engineering.

"Meganucleases" are naturally occurring gene-targeting proteins that function as homodimers comprising two identical subunits each 160–200 amino acid residues in size, but also active as a single peptide of two tandem repeat monomers joined together by a linker sequence (Stoddard 2011). Meganucleases typically bind to 20–30 bp DNA target sites which provide remarkable specificity, a primary reason for pursuing these proteins as for genome modification. In contrast to ZFNs and TALENs, the cleavage and DNA-binding domains of meganucleases are not clearly separated. Attempts to reengineer DNA contact points of the endonuclease can be challenging and often compromise nuclease activity (Taylor et al. 2012). Because of these engineering challenges, only a handful of academic groups and companies routinely engineer meganucleases that target novel DNA sites.

Most recently, RNA-guided nucleases from bacteria and archaea, referred to as "clustered regulatory interspaced short palindromic repeats" or CRISPRs have been adapted for genome modification whereby short segments of DNA are transcribed into RNAs which direct sequence-specific cleavage by Cas proteins (Wiedenheft et al. 2012). Using this system, targeted mutations were made in *Arabidopsis* BRI1, JAZ1 and GAI, and in rice ROC5 (Feng et al. 2013).

One of the main challenges associated with the routine deployment of designed nuclease technology for crop improvement is the relative inefficiencies of transgen-

ic event production in all but a few plant species. Recently, *in planta* gene targeting was demonstrated using the meganuclease *I-SceI* (Fauser et al. 2012). In this study, three constructs were transformed independently into *Arabidopsis*: (i) a target with a broken reporter gene and nuclease cleavage sites, (ii) a donor with sequences complementary to the broken reporter, nuclease cleavage sites, and sequences homologous to the target, and (iii) the meganuclease which cuts in both the target and donor. Single copy, homozygous plants for each construct were generated and intermated in the following manner, [(target × donor) × nuclease]. The target contained a 3′ partial *GUS* reporter gene sequence and two *I-SceI* nuclease cleavage sites. The donor contained a 5′ partial *GUS* reporter gene, two *I-SceI* nuclease cleavage sites, sequences homologous to the target and two flanking identical sequences for single strand annealing repair following excision. Nuclease cleavage at the donor locus released the 5′ *GUS* gene fragment and the homologous sequences which provided a template for repair of the target. Observed targeting frequencies were as high as ~1 % on a progeny seed basis. This approach was corroborated in maize whereby inducible expression of I-SceI, combined with in vitro selection on kanamycin, allowed for the detection of the somatic repair of an *NPT*II gene (Ayar et al. 2013).

Future Prospects

The availability of custom targeting reagents such as designed ZFNs, together with the development of high-resolution molecular methods and bioinformatics for trait characterization, is likely to rapidly advance precision genome engineering in plants to enable product development in the near future. It is anticipated that targeted mutagenesis, gene excision, and genome editing will be routinely deployed for functional genomics and trait discovery. Some of these applications of precision genome engineering are likely to be regulated differently, i.e., as non-transgenic (Waltz 2011) and, as such, resulting changes in regulatory policies may have positive economic and social consequences. Similarly, current transgenic product development methods involve the random integration of transgenes into the plant genome, such that generating events and screening them for a trait of interest is time and cost intensive. The ability to target transgene integration into a predetermined genomic site should result in events whereby undesired side effects would be minimized and cycle times associated with product development reduced as event-specific analysis and characterization is simplified. Moreover, additional routes to product development are also likely through retargeting of transgenic loci leading to transgene stacking (Ainley et al. 2013; D'Halluin et al. 2013). In addition, from a trait discovery standpoint, targeting experimental constructs to specific genomic loci effectively removes variability associated with position effect thereby providing a uniform background against which genes and gene constructs can be screened to find lead candidates for new traits. Clearly, the enhanced precision relative to DNA manipulation, made possible by designed ZFNs, opens up some intriguing possibilities for both basic and applied research.

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