REVIEW

Gene targeting and editing in crop plants: a new era of precision opportunities

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Abstract With increasing global food demands in the face of challenging biotic and abiotic pressures on crop production, there is a vital need for good crop improvement strategies. Gene editing and gene targeting using designer nucleases are relatively new, sophisticated approaches that can be used for crop improvement. Designer nucleases are molecules that can be engineered to cleave virtually any endogenous DNA target sequence, making this technology inherently more powerful over current, essentially random mutation strategies. These molecules can also be used to promote targeted DNA insertions and homologous recombination. Further modifications of these molecules can convert them into designer transcription factors that can activate or suppress a gene of choice. Four designer nuclease platforms are currently available: meganucleases, zinc finger nucleases, TALENs and the more recently developed CRISPR/Cas9 system. All four of these systems have been shown to function in crop plants and have been used for site-specific gene targeting and gene editing. Herein, we describe the basis of each designer nuclease platform, highlighting the advantages and disadvantages of each, and give examples of their application in crop improvement.

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Introduction

In an era where pressure on food production is rapidly growing in the face of challenging global environmental climate changes, the need to produce hardy crops with higher yields but less input is vital (Tester and Langridge 2010). Unfortunately, a number of tools available for crop improvement suffer from a lack of precision and are reliant upon random events for outcomes. For example, mutagenesis, facilitated by mutagenic chemicals, irradiation or DNA insertion sequences, relies upon the random distribution of mutations throughout the plant genome. While screening technologies for mutations in a specific gene have greatly advanced (e.g. TILLING, genome sequencing, flanking sequence tag libraries) (Parry et al. 2009), the actual site of mutation within a gene sequence remains uncontrolled. RNAi technology allows highly specific, targeted post-transcriptional suppression of a gene (Small 2007); however, it results in gene silencing rather than a complete loss of gene activity and does not allow precise, targeted modification of an endogenous gene sequence.

As another example, a major advance in germplasm improvement of crop species has been the development of transgenic plants which has enabled the introduction

of DNA sequences from any source, either biological or synthetic, for agronomic benefit (Gasser and Fraley 1989). The introduction of this additional genetic material, however, has been overwhelmingly achieved by random insertion into the plant genome by nonhomologous recombination. This nontargeted gene insertion precludes the subsequent insertion of additional transgenes at the same locus which would greatly simplify future breeding efforts if it was possible. The ability to subtract specific genes present at a multitransgene locus would also be of commercial benefit, enabling precise gene combinations to be developed depending upon the intellectual property demands of specific commercial relationships.

A further highly desirable technology is the ability to facilitate *in planta* homologous recombination. This process enables alteration of endogenous gene sequences to create new alleles with beneficial agronomic traits. Some success in altering endogenous gene sequences has been achieved via the introduction of short oligonucleotide sequences into plant cells which can cause sequence change in target alleles by mismatch repair (Beetham et al. 1999; Zhu et al. 1999; Kochevenko and Willmitzer 2003; Okuzaki and Toriyama 2004; Iida and Terada 2005; Dong et al. 2006). However, published reports of this process in tobacco, maize, rice and wheat have shown very low efficiencies. Nonetheless, companies such as Cibus (San Diego, USA) offer this service as a "Rapid Trait Development System", and the resultant products are claimed to be considered nontransgenic.

Major technological advances have been achieved in overcoming the current limitations described above by the advent of designer nuclease technologies which include meganucleases, Zn finger nucleases, TALENs and more recently CRISPR\Cas9. This review aims to summarise these technologies and provide examples of their current and potential future application to agricultural crop improvement.

Custom-designed nucleases and nickases

Custom-designed nucleases are all similar in that they each can be engineered to specifically recognise any DNA target sequence usually around 20 nucleotides in length and cleave this target sequence to create a double strand (DS) DNA break. DS DNA breaks within the plant genome are primarily repaired either by nonhomologous end joining (NHEJ) or homologous recombination (Puchta 2005). NHEJ occurs far more commonly and is error prone resulting in insertions or deletions at the break site. These nucleases can therefore create small insertions/deletions (indels) at very precise locations within an endogenous DNA sequence allowing highly targeted mutagenesis to be undertaken. DS DNA breaks can also promote the insertion of foreign DNA sequences at these sites by NHEJ. For example, Tzfira reported that 2.5 % of T-DNA insertions occurred preferentially in an enforced break in the tobacco genome amongst 620 transgenic plants produced (Tzfira et al. 2003).

DNA breaks can also promote homologous recombination. When DNA is introduced into plants by either Agrobacterium or biolistic transformation, homologous recombination has been reported to take place once for every 10^4–10^7 illegitimate recombination events (Puchta et al. 1996; Hannin et al. 2001; Puchta 2002; Wright et al. 2005 and reference therein; Tzfira et al. 2012). In spite of its rarity, homologous recombination can be detected in plants by either extensive screening or by homologous recombination-dependent selection strategies such as reconstitution of a selectable marker gene (Tzfira et al. 2012). However, the creation of a targeted DS DNA break combined with the introduction of a sequence flanked with homologous ends to this target can dramatically increase the frequency (i.e. up to 10^{-2}) of homologous recombination at this site (Puchta et al. 1996; Puchta 2002; Wright et al. 2005). Modified versions of nucleases termed "nickases", described below, have also been engineered that cleave only a single strand of DNA at a target site, which further increases the likelihood of homologous recombination occurring, rather than NHEJ (van Nierop et al. 2009; Chan et al. 2011; Fauser et al. 2014).

The application of designer nucleases therefore exploits endogenous DNA repair mechanisms to create site-specific indels or to promote precise DNA insertions or homologous recombination. Similar exploitation of DNA repair systems has been undertaken using site-specific recombination systems such as Cre/loxP, R/RS and FLP/FRT (Wang et al. 2011). However, the major difference between these site-specific recombination systems and designer nucleases is that the former systems are generally limited to a single, specific target sequence or closely related derivative sequences. In contrast, designer nucleases can be engineered to target any short DNA sequence of choice, making them inherently more flexible.

While all designer nucleases are similar in that they generate targeted DNA cleavage, they differ in their origin and the mechanism by which target sequence specificity is achieved. The designer nuclease platforms that are currently available are summarised as follows.
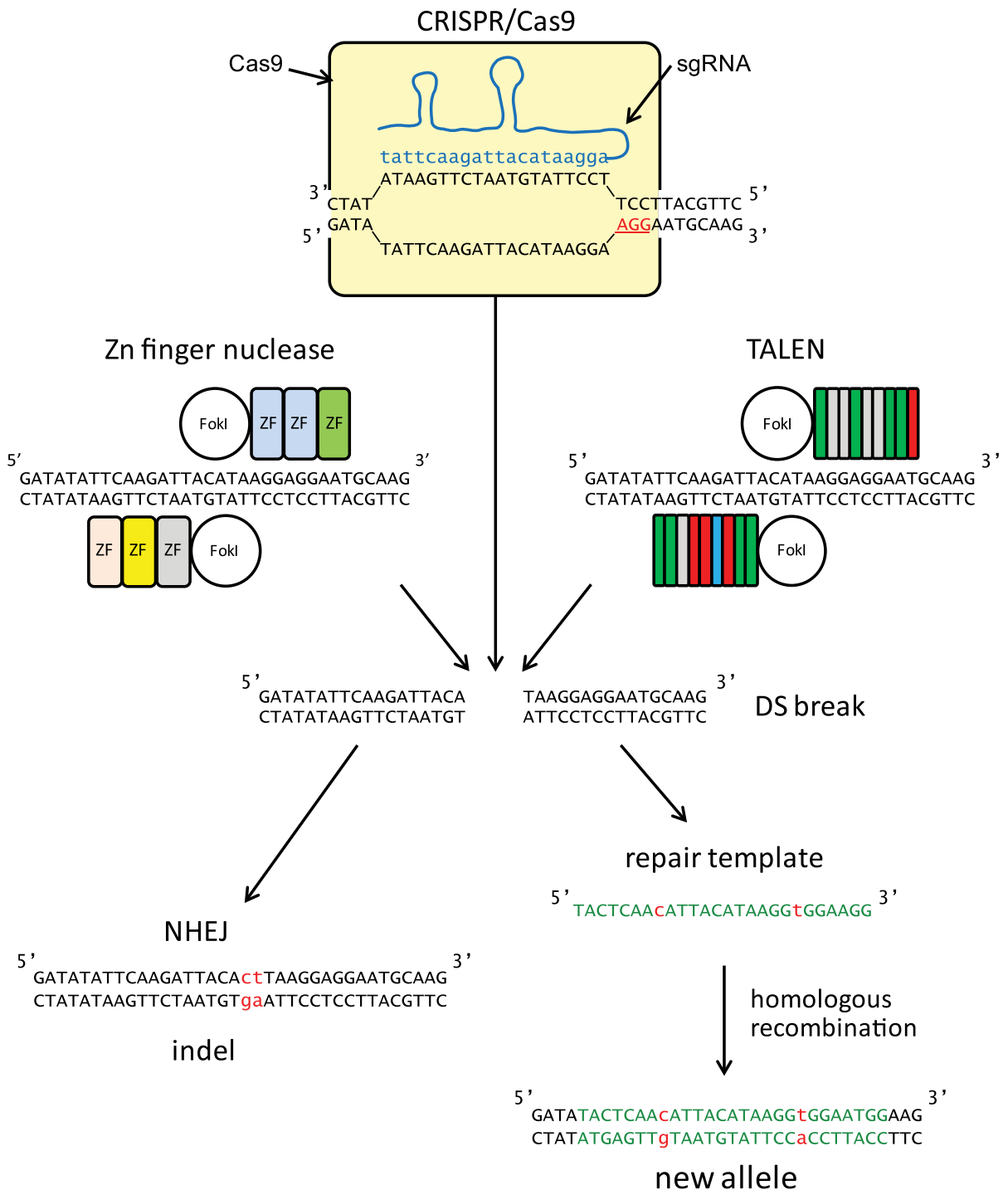
Meganucleases

Meganucleases, or homing nucleases, are natural restriction endonucleases that are components of mobile genetic elements. These enzymes recognise specific DNA sequences that range from >12 to 40 bp in size, whereupon they produce a DS DNA break (Paques and Duchateau 2007). Several hundred members have been identified that are found in eukaryotes, bacteria and archea and are often encoded on mobile class I introns and inteins (Paques and Duchateau 2007). Given the size of meganuclease recognition sites, an entire plant genome may contain no, or just a few, recognition sites for a given nuclease. These rare cutting nucleases have been successfully used to target DNA insertions in a number of plant species including Arabidopsis, tobacco and maize (D'Halluin et al. 2008; Yang et al. 2009). However, obvious limitations exist in that endogenous target sites are uncommon and are fixed a priori, or alternatively, the target site has to be introduced encoded on a transgene. Re-engineering of meganucleases to recognise new DNA sequences has been achieved but has proven complex (Gao et al. 2010; Tzfira et al. 2012) although it continues to improve (Arnould et al. 2011). Meganucleases with nickase activity have also been developed (McConnell-Smith et al. 2009).

Zn finger nucleases

Zinc finger nucleases (ZFNs) are composed of two functional domain types: zinc finger (ZF) DNA recognition domains, common to some transcription factor families, and a non-sequence-specific nuclease domain (Porteus and Carroll 2005). The DNA recognition domain consists of an array of Cys_2–His_2 ZF domains with each finger binding a specific nucleotide triplet. ZFs have been identified that recognise all GNN and ANN nucleotide triplets and most CNN and TNN triplets. Combining ZFs that have different recognition specificities enables the resultant multimeric protein to bind a specific DNA sequence. The nuclease domain of the ZFN is responsible for DNA cleavage immediately adjacent to the ZFN-binding site. This is usually catalysed by the 196 amino acid C-terminal domain from the nuclease FokI (Kim et al. 1996). This FokI domain functions as a dimer; hence, two ZFNs are required to bind in close proximity to enable dimerisation and production of a DS DNA break at the target site, with each ZFN recognising a different DNA sequence on either side of this site (Fig. 1), (Mani et al. 2005). Typically, each ZFN consists of 3–4 ZF domains with each finger recognising a nucleotide triplet (Klug 2005). A functional pair of ZFNs, each containing 3 ZF domains, would therefore recognise two specific 9 bp sequences that flank an internal 5–7 bp DNA cleavage site (Fig. 1). ZFNs, like TALENs and the CRISPR\Cas9 systems described below, are therefore true designer nucleases in that many DNA sequences can be selectively targeted in the plant genome, making these systems remarkably powerful. In addition, fusion of zinc fingers to transcriptional activation domains can generate synthetic transcription factors that can be potentially designed to target many regulatory sequences of choice (Stege et al. 2002; Sanchez et al. 2006). Some examples of these synthetic transcription factors are described later in this review. ZFN pairs have also been modified by inactivating the FokI cleavage domain in one of the ZFNs to produce a nickase activity (i.e. cleavage of a single DNA strand only) to promote homologous recombination (Gaj et al. 2013).

The requirement for ZFN to act as dimers for DNA cleavage increases targeting specificity as the likelihood of off-target site binding by both ZFNs at the same site is low. However, toxicity of ZFNs has been reported presumably due to some off-target cleavage (Paques and Duchateau 2007; Tzfira et al. 2012). Attempts to ameliorate this problem have been by either engineering ZFNs with increased specificity or by including additional modifications such as a FokI nuclease heterodimerisation requirement or engineering additional cofactor requirements (Szczepek et al. 2007; Miller et al. 2007; Pruett-Miller et al. 2009; Townsend et al. 2009; Ramalingam et al. 2011). Individual ZF domains do not always behave as predicted in a multimeric context; therefore, selective synthesis cycles are required to produce ZFNs with desired specificity outcomes (Joung and Sanders 2013; Straub and LaHaye 2013).

CRISPR/Cas9

Cas9   sgRNA

tattcaagattacataagga
ATAAGTTCTAATGTATTCCT
3' CTAT   TCCTTACGTTC 5'
5' GATA   AGGAATGCAAG 3'
TATTCAAGATTACATAAGGA

Zn finger nuclease

FokI ZF ZF ZF

5' GATATATTCAAGATTACATAAGGAGGAATGCAAG 3'
CTATATAAGTTCTAATGTATTCCTCCTTACGTTC

ZF ZF ZF FokI

TALEN

FokI

5' GATATATTCAAGATTACATAAGGAGGAATGCAAG 3'
CTATATAAGTTCTAATGTATTCCTCCTTACGTTC

FokI

5' GATATATTCAAGATTACA   TAAGGAGGAATGCAAG 3' DS break
CTATATAAGTTCTAATGT   ATTCCTCCTTACGTTC

NHEJ

5' GATATATTCAAGATTACActTAAGGAGGAATGCAAG 3'
CTATATAAGTTCTAATGTgaATTCCTCCTTACGTTC

indel

repair template

5' TACTCAAcATTACATAAGGtGGAAGG 3'

homologous
recombination

5' GATATACTCAAcATTACATAAGGtGGAATGGAAG 3'
CTATATGAGTTgTAATGTATTCCaCCTTACCTTC

new allele

In plants, ZFNs have been successfully used in Arabidopsis (Lloyd et al. 2005; de Pater et al. 2009; Tovkach and Zeevi 2009; Osakabe et al. 2010; Zhang et al. 2010; Qi et al. 2013a; de Pater et al. 2013), tobacco (Bibikova et al. 2003; Wright et al. 2005; Cai et al. 2009; Townsend et al. 2009; Petolino et al. 2010),

◄ **Fig. 1** Three designer nuclease platforms. Schematic diagram of zinc finger nucleases, TALENs and CRISPR/Cas9 nuclease systems. A single Zn finger nuclease (ZFN) consists of three zinc finger (ZF) domains that each recognises a specific nucleotide triplet, coupled to a FokI nuclease domain. A pair of ZFNs is required for activity due to a homo-dimerisation requirement of the FokI nuclease domain. TALENs also function in pairs with a single TALEN molecule consisting of nine individual TALE repeats (*rectangles*) fused to the FokI nuclease domain. Each TALE repeat recognises a specific nucleotide. The CRISPR/Cas9 system differs in that target sequence recognition is via a small guide RNA (sgRNA, in *blue*) containing a 20 base sequence (lower case) that recognises a genomic target sequence via complementary base pairing. The target sequence must have a two invariable guanine bases at the 3′ end which form a protospacer-adjacent motif sequence (PAM, *underlined* in *red*). Associated with the sgRNA is a Cas9 nuclease protein that subsequently cleaves the target site. All three nuclease systems produce a double-stranded (DS) DNA break unless additional nuclease domain modifications are made. DS DNA breaks are preferentially repaired by nonhomologous end joining (NHEJ) which usually results in insertions (two bases shown in *red lower case*), deletions or substitutions of a few nucleotides at the target site. Addition of a homologous repair template (*green*) in the presence of a DS DNA break can facilitate homologous recombination which enables designer alleles to be produced by incorporating sequence modifications (*red bases* shown in *lower case*) into the repair template

soybean (Curtin et al. 2011), petunia (Marton et al. 2010) and maize (Shukla et al. 2009; Ainley et al. 2013). Expression of ZFNs in Arabidopsis and tobacco has produced heritable, targeted mutations in transgenes and endogenous genes at frequencies as high as 3–7 %, depending upon the ZFN and target sequence (Townsend et al. 2009; Lloyd et al. 2005; Zhang et al. 2010; Osakabe et al. 2010). In tobacco, targeted transgene integration was as high as 10 % (Cai et al. 2009) and homologous recombination with an endogenous gene to generate herbicide resistance up to 4 % (Townsend et al. 2009). Commercially produced ZFN expression plasmids can be purchased from Sigma-Aldrich as part of a propriety platform (CompaZr) with Sangamo Biosciences (Richmond, CA, USA) which hold ZFN patent rights (Thomas Scott 2005; DeFrancesco 2011). This commercial production alleviates the extensive confirmation of ZFN specificity and activity by the end user (Gaj et al. 2013; Tzfira et al. 2012; Johnson et al. 2013).

TALENs

Transcription activator-like effector nucleases (TALENs) are similar to Zn finger nucleases in that they allow true designer targeting of most DNA sequences. Transcription activator-like effectors (TALEs) are a group of proteins first identified in the bacterial plant pathogen *Xanthamonas oryzae* (Bogdanove et al. 2010; Schornack et al. 2013). These proteins are directly introduced into plant cells by the bacterium to promote bacterial colonisation. Each TALE binds to a specific DNA sequence in the vicinity of an endogenous plant gene and then transcriptionally activates this host gene to promote bacterial pathogenesis (Bogdanove et al. 2010). Within the TALE protein are 33–35 amino acid repeats that each recognises a specific DNA base, with a hypervariable region at amino acid positions 12 and 13 determining base specificity (Boch et al. 2009; Moscou and Bogdanove 2009). Most engineered TALE repeat arrays published to date use multimers of four domains that contain at hypervariable residues amino acids NN, NI, HD or NG for the recognition of guanine, adenine, cytosine and thymine nucleotides, respectively (Joung and Sander 2013). Having deciphered the DNA-binding code of these proteins, it is now possible to produce synthetic TALEs that transcriptionally activate or repress a gene of interest by targeting a specific sequence in the 5' region of the chosen gene. This ability is potentially a very powerful tool for altering plant gene expression for desirable traits (Morbitzer et al. 2010; Mahfouz et al. 2012).

Further engineering of TALEs has enabled the development of TALENs by fusion of a FokI nuclease domain to the TALE protein, as described above for ZFNs (Christian et al. 2010; Miller et al. 2011; Li et al. 2011; Mahfouz et al. 2011; Joung and Sanders 2013; Schornack et al. 2013). As for ZFNs, TALENS also function in pairs, again due to the homodimeric requirement for DS DNA cleavage by the FokI nuclease domain, with each TALEN targeting a specific sequence either side of the cleavage site (Fig. 1). TALENS can also be used for nickase activity rather than DS nuclease activity by inactivating one of the FokI domains. TALENs have been suggested to show less target sequence restrictions than ZFNs and equal or better efficiencies at mediating target site cleavage (Cermak et al. 2011). The assembly of tandemly repeated TALE DNA-binding domains, however, is challenging using conventional cloning techniques although improved cloning strategies have been developed (Joung and Sanders 2013; Straub and LaHaye 2013). TALENs have been used in a variety of eukaryotic organisms including Arabidopsis (Cermak

et al. 2011), tobacco (Mahfouz et al. 2011; Zhang et al. 2013), rice (Li et al. 2012), wheat (Shan et al. 2013b; Wang et al. 2014); soybean (Haun et al. 2014), maize (Liang et al. 2014) and barley (Wendt et al. 2013; Gurushidze et al. 2014). They are commercially available from companies including Cellectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA) and Life Technologies (Grand Island, NY, USA) (Gaj et al. 2013). Two patent positions cover TALEN technology with one being exclusively licensed to the Two Blades Foundation, a USA-based charitable organisation, for commercial use in plants who in turn have licensed these rights to LifeTechnologies while the latter patent has been licensed to Cellectis Research (DeFrancesco 2011).

CRISPR/Cas9 system

The CRISPR/Cas system is a prokaryote defence mechanism found in most archeal (90 %) and bacterial species (40 %) and protects these microbes against invading nucleic acids such as viral genomes and plasmids (Horvath and Barrangou 2010). Clustered regular interspaced short palindromic repeats (CRISPR) are short direct repeats (21–47 bp) separated by spacer sequences (21–72 bp) that are usually segments of captured viral or plasmid DNA. CRISPR repeats are often adjacent to CRISPR-associated (Cas) genes which encode a heterogeneous family of proteins that include nucleases, helicases and polymerases, in addition to noncoding RNAs. CRISPR segments are transcribed and these transcripts are processed to form small RNAs. These small RNAs act as guides by binding to complementary foreign nucleic acid sequences by homologous pairing which targets components of the Cas complex, including an endonuclease called Cas9, to these invading sequences resulting in their degradation (Horvath and Barrangou 2010). Obvious parallels exist between the CRISPR/Cas system and eukaryotic RNAi-mediated gene silencing systems in that target sequence recognition is based upon complimentary nucleic acid pairing; however, apart from this similarity, these two systems are mechanistically distinct.

To aid the utility of this natural system in genome editing applications, the complexity of prokaryotic CRISPR/Cas systems has been substantially reduced by engineering it to consist of just two genes, one encoding the Cas9 nuclease protein and the second to encode a synthetic small guide RNA (sgRNA). This latter molecule is approximately 85 bp in length and negates the RNA processing requirements of the endogenous bacterial system (Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013; Qi et al. 2013b). Located at the 5′ end of the sgRNA are 19–22 bases that recognise the DNA target sequence by complementary nucleotide pairing (Fig. 1). This target sequence requires two invariable guanine bases at the 3′ end of the target site which form a protospacer adjacent motif sequence (PAM) of NGG (Straub and LaHaye 2013). Upon target sequence recognition, the Cas9 nuclease cleaves the complementary and noncomplementary DNA strands three and three to eight nucleotides, respectively, from the PAM site in the region of target sequence and sgRNA complementarity (Lozano-Juste and Cutler 2014).

Similar to Zn finger domain proteins and TAL effector proteins, modification of the Cas9 nuclease can also produce nickase activity rather than DS DNA cleavage (Jinek et al. 2012) to facilitate homologous recombination. Combining a nuclease-deficient Cas9 protein with sgRNAs can also produce a transcriptional repressor when appropriately targeted to regulatory sequences of a gene of interest (Qi et al. 2013b). Similarly, fusing a transcriptional activation domain to an inactive Cas9 protein can generate transcriptional activation of a target gene (Perez-Pinera et al. 2013; Maeder et al. 2013). The CRISPR/Cas9 system is therefore as versatile as Zn finger and TAL technologies in that it can function as a designer nuclease or designer transcription factor. Furthermore, this system is suggested to be significantly simpler in application than ZFN and TALENS as the simple sgRNA defines the cleavage site rather than complex engineered proteins containing multimeric ZF or TALE domains (Straub and LaHaye 2013; Belhaj et al. 2013).

One drawback, however, is that the relatively small number of "programmable" target nucleotides is further constrained by the requirement of the PAM sequence. In spite of these target sequence limitations, over 1.4 million potential target sites have been identified in the Arabidopsis genome with more than 99 % of protein-encoding nuclear genes containing at least one target site (Li et al. 2013) and over 90 % of rice genes predicted to also contain suitable target sites (Xie and Yang 2013). In a similar bioinformatic analysis, suitable sgRNA target sites were identified in at least one exon of 83–98 % of genes present in

Arabidopsis, *Medicago*, tomato, soybean, *Brachypodium*, sorghum and rice; however, only 30 % of maize genes contained a target site (Xie et al. 2014). Another caveat is that, similar to the other designer nuclease platforms, off-target modifications by CRISPR/Cas9 can occur (Fu et al. 2013; Hsu et al. 2013; Xie and Yang 2013).

The relatively simple CRISPR/Cas9 system has recently been shown to function effectively in Arabidopsis, *Nicotiana benthamiana*, tobacco, wheat, rice, sweet orange, sorghum and maize cells to generate target site indels and nucleotide substitutions or promote homologous recombination (Shan et al. 2013b; Li et al. 2013; Nekrasov et al. 2013, Upadhyay et al. 2013, Jiang et al. 2013; Feng et al. 2013; Mao et al. 2013; Xie and Yang 2013; Jia and Wang 2014; Liang et al. 2014; Xu et al. 2014; Zhang et al. 2014; Jiang et al. 2014; Fauser et al. 2014). An extensive and insightful summary of many of these experiments is provided by Belhaj et al. (2013). These studies demonstrate the robustness of this technology by its successful application to numerous plants species in such a short span of time. The intellectual property ownership of the CRISPR/Cas9 system remains to be determined; however, the BROAD Institute was recently granted the first patent which covers the components and methodology of this system (Zhang 2014).

Applications

The following examples highlight some of the potential applications for designer nuclease technology in crop plants. These events can be broadly classified as precision gene mutation, in situ engineering of endogenous genes, gene removal, transcriptional reprogramming of endogenous genes and production of large *cis* transgene stacks.

Precision gene mutation

Unlike conventional mutagens and DNA insertion sequences, designer nucleases offer an unparalleled opportunity to target specific regions in a gene of interest. In two examples, the I-CreI homing endonuclease (meganuclease) from *Chlamydomonas reinhardti* was engineered to recognise a 21 bp sequence in 5′ juxtaposition to the maize *liguless1* gene (Gao et al. 2010) and a 22 bp sequence present in MS26, a maize cytochrome P450 gene required for male fertility (Djukanovic et al. 2013). In the former study, 3 % of T0 plants contained mutations at the target site (Gao et al. 2010), while in the latter study, 6 % of T0 plants contained an indel within this gene, and homozygous progeny produced a male sterile phenotype (Djukanovic et al. 2013).

Targeted gene mutation using CRISPR/Cas9 were also undertaken in rice protoplasts where four rice genes were successfully mutated (Shan et al. 2013b). Mutation frequencies were estimated by PCR amplification of the target site from total protoplast DNA, and a proportion of PCR products were shown to have lost a restriction enzyme site present in the target sequence. Using this method, approximately 25 % of alleles were estimated to have been effectively mutated in each case. Stable rice transgenics were also produced in which the *OsPDS* and *OsBAD* genes were targeted and mutations detected in 9 and 7 % of T0 plants, respectively, including biallelic mutations in one-third of *OsPDS* mutant plants (Shan et al. 2013b). In another study, 11 genes were independently targeted in the rice genome using CRISPR/Cas, and 44 % of T0 plants on average had a mutation at the targeted locus with 4 % of plants containing homozygous mutations (Zhang et al. 2014). These mutations were stably inherited in progeny, and deep sequencing revealed that off-target genome modifications were rare (Zhang et al. 2014).

Wheat protoplasts have also been mutated using the CRIPSR/Cas system at 28 % efficiency (Shan et al. 2013b). In this case, the target gene was the wheat homologue of the barley *Mlo* gene which is of particular interest given that inactivation of this gene in barley provides broad spectrum resistance to *Blumeria graminis* (powdery mildew) (Buschges et al. 1997). Subsequently, the simultaneous editing of all three wheat *Mlo* homoealleles using TALENs was reported resulting in broad spectrum resistance to powdery mildew disease (Wang et al. 2014). Twenty-seven mutant T0 plants were detected amongst 450 transgenics of which 20 were heterozygous for mutations at a single *Mlo* locus, two plants contained multiple mutations at single loci, four plants had mutations present at two *Mlo* loci and one line was heterozygous for mutations at all three homologous loci. Progeny from this latter line that was homozygous for mutations at all three homologous *Mlo* loci were resistant to powdery mildew disease (Wang et al. 2014).

CRISPR/Cas-targeted mutations have also been produced in the wheat *inox* and *PDS* genes at around 20 % efficiency in suspension-cultured cells (Upadhyay et al. 2013). A remarkable extrapolation of targeted gene knockout using CRIPSR/Cas9 was recently demonstrated in a human cell line where 64, 751 unique sgRNAs were used to screen 18,080 genes for increased drug resistance upon gene knockout (Shalem et al. 2014). Such high throughput, targeted mutagenesis has yet to be applied to plants, but it is an exciting proposition.

TALENs have also been demonstrated to function effectively in rice and produce highly targeted gene knockouts. In one study, four loci were targeted in the rice genome, and PCR assays confirmed TALEN editing in 3–60 % of callus lines depending upon the TALEN pair used (Shan et al. 2013a). Transgenic plants were regenerated after transformation with two TALEN pairs and mutations detected in 19 and 36 % of T0 plants, respectively. In the same study, similar TALEN efficiencies were observed for *Brachypodium distachyon* callus transformed with TALEN pairs (Shan et al. 2013a).

TALENS were also used in soybean to produce simultaneous mutations in two fatty acid desaturase genes (*FAD2-1A* and *FAD2-1B*) for improved oil quality (Haun et al. 2014). Four out of nineteen transgenic plants contained mutations in both *FAD2* genes; however, both mutations were subsequently inherited in T1 progeny from a single plant only. Progeny from this plant were identified that were homozygous for mutations at both genes and that no longer contained TALEN transgenes by segregation. Seed from these plants showed improved oil quality with a dramatic increase in oleic acid and concomitant reduction in linoleic acid (Haun et al. 2014).

All four designer nuclease platforms have therefore been successfully used in crop plants to produce targeted mutations in genes of interest with comparable efficiencies. Unlike conventional mutagenesis, these mutations were targeted to a precise DNA sequence. However, a point to consider is that while designer nucleases may be able to precisely target a short DNA sequence and cause cleavage, there is no control over the subsequent NHEJ process that takes place. Hence, although the site of mutation is highly specific, the resultant structure of the mutated locus is largely random and consists of indels of unspecified size and sequence. Truly precise sequence engineering

of an endogenous locus is restricted to homologous recombination.

In situ engineering of endogenous genes

An efficient homologous recombination system is highly desirable in plant improvement as endogenous gene sequences can be altered to encode allelic variants with improved agronomic traits. Unlike NHEJ, this process can provide absolute designer sequence specificity by providing a recombination template of exact sequence choice (Fig. 1). Homologous recombination in plants remains a challenging process; however, several studies have successfully employed designer nucleases to promote sequence replacement and targeted sequence insertion. In rice, homology-directed repair following TALEN cleavage of the *PDS* locus was achieved by concomitantly providing a 72 bp donor sequence, although the efficiency of this process was undetermined (Shan et al. 2013a). In maize, ZFN-mediated cleavage of the *IPK1* gene, which catalyses the last step in phytate production, was coupled with precise insertion of an herbicide-selectable marker gene at this site using homology-dependent repair mechanisms (Shukla et al. 2009). The resultant plants were both herbicide resistant and had reduced levels of phytate, an anti-nutritional component of feed grain that contributes to environmental pollution via animal waste (Shukla et al. 2009). In this study, selection using an herbicide resistance gene with no promoter, but which acquired adjacent regulatory sequences upon correct integration, resulted in a twofold increase in targeted gene insertion when compared with the same gene containing an autonomous promoter.

Gene removal

Removal of specific transgene sequences after the production of transgenic plants, often selectable marker genes, has been undertaken in numerous species including tobacco, Arabidopsis, rice, maize, barley, sorghum, tomato, soybean and wheat using site-specific recombinase systems (reviewed by Ow 2007). Examples include the development of a selectable marker-free corn line, LY038, developed by Monsanto using the Cre-lox system (Ow 2007; Wang et al. 2011). This system was also used to reduce the complexity of a biolistic transgene locus in wheat (Srivastava et al. 1999). However, as pointed out

earlier, these site-specific recombination platforms are restricted in that each system is confined to a specific recognition sequence not present in the endogenous plant genome.

The following examples demonstrate the utility of designer nucleases by their ability to produce precise deletions in endogenous sequences at sites of choice. In rice protoplasts and callus tissue, two TALEN pairs were introduced that targeted two endogenous sites separated by 1,322 bp in the genome. Deletion alleles could be identified in both tissues with 5 % of calli containing deletions, and in one callus, an inversion of the intervening sequence was detected (Shan et al. 2013a). In wheat, a duplex sgRNA that recognised two separate regions of the endogenous *inox* gene resulted in deletion of the intervening 50 bp sequence between each target site in 3 % of sequences amplified (Upadhyay et al. 2013). In Arabidopsis, three different tandemly arrayed gene families were targeted with ZFNs that recognised multiple members within each cluster (Qi et al. 2013a). The resulting double-stranded DNA breaks and NHEJ produced deletions at these loci up to 55 kb in size. A large chromosomal deletion of 9 Mb was also generated using two ZFN pairs that each recognised a locus at either end of the intervening 9 Mb of sequence. The proportion of somatic cells containing deletions was inversely related to the size of the deletion and varied from 1 to less than 0.1 % (Qi et al. 2013a). However, it is noteworthy that no plants in this study were able to be recovered with germline-transmitted deletions, although the diploid nature of Arabidopsis presumably makes it less amenable for transmission of deletions.

Targeted deletions have also been produced in animal cells. Using zebrafish embryos and mRNA injection, TALENs or TALENS in conjunction with ZFNs were used to generate targeted deletions of endogenous sequences (Gupta et al. 2014). Targeted deletion sizes included 39, 69 kb and 5.5 Mb which were achieved at efficiencies of 3.2, 4.9 and 0.7 %, respectively (Gupta et al. 2014). Similarly in human cell lines, ZFN pairs were used to create precise, large deletions that ranged in size from several hundred base pairs to 15 megabases (Lee et al. 2010). The potential application of this approach for plant improvement is obvious. Deleterious genes linked to traits of interest could be removed, introgressed DNA segments from wild relatives could be reduced in size, and groups of candidate genes could be deleted *en mass* in positional cloning experiments for gene identification, all of which can be carried out with precision.

Transcriptional reprogramming of endogenous genes

A further ingenious application of designer nucleases is the exploitation of their sequence-targeting abilities to reprogram transcriptional regulation of endogenous genes through targeting transcription factor-binding sites or by generating synthetic transcription factors. The following report by Li et al. (2012) is a wonderful example of exploiting a bacterial pathogen's virulence armoury to create disease-resistant rice using TALEN technology. In rice, an endogenous sucrose transporter gene, *Os Sweet14*, is targeted by TALE effectors produced by the bacterial pathogen *Xanthomomas oryzae* pv. *oryzicola* resulting in upregulation of this gene during bacterial infection. Upregulation of *OsSweet14* is essential for successful infection by this pathogen. The *Xoo* effector-binding sites present within the promoter region of *OsSweet14* were identified and then mutagenised using a sequence-specific TALEN. The resultant altered endogenous rice gene was no longer successfully targeted by *Xoo* effector proteins which resulted in significantly enhanced resistance to this bacterial pathogen.

In an alternative approach, ZF proteins were modified to generate a synthetic transcription factor in *Brassica napus* to improve oil quality by reducing the level of saturated fat (Gupta et al. 2012). A ZF protein was engineered to recognise a common region located 50 bp 3′ of the transcriptional start site of two *B*-ketoacyl-ACP synthase II (*KASII*) genes involved in fatty acid elongation. A transcriptional activation domain (V16) from the herpes simplex virus was fused to the ZF protein domain to generate a synthetic transcription factor which when introduced into canola resulted in a concomitant increase in *KASII* gene expression. Transgenic lines showed reduced total saturated fatty acid content in seeds due to a reduction in palmitic acid content resulting in improved oil quality.

Producing *cis* transgene stacks

Transgenic crop plants (cotton, canola, maize) are now being released that contain multiple transgenes (Que et al. 2010) an example being the Dow Agroscience/Monsanto maize line "SmartStax" which contains

eight GM traits (Marra et al. 2010). As more useful transgenic traits are developed, the ability to effectively combine and manipulate large numbers of transgenes becomes more imperative. The most advantageous arrangement of multiple transgenes is at a single locus enabling subsequent simple coinheritance of these traits. A true designer multigene locus would offer the flexibility of addition or subtraction of genes at will to tailor the locus to accommodate various regulatory or commercial requirements.

In numerous studies, recombination-mediated integration using Cre/lox, R/RS and FLP/FRT has been used to target a sequence into a pre-existing recombination site and produce single copy insertions. This has been successfully undertaken in Arabidopsis (Vergunst and Hooykaas 1998; Vergunst et al. 1998; Louwerse et al. 2007), tobacco (Albert et al. 1995; Choi et al. 2000; Day et al. 2000; Nanto et al. 2005; Nanto and Ebinuma 2008; Nanto et al. 2009), maize (Baszczynski et al. 2003; Kerbach et al. 2005), rice (Srivastava and Ow 2002; Srivastava et al. 2004; Chawla et al. 2006; Akbudak et al. 2010; Nandy and Srivastava, 2011; Srivastava 2013), soybean (Li et al. 2009) and aspen (Fladung and Becker 2010). However, again a constraint of these approaches is that a target site must be pre-introduced into the genome through transgenesis and that a limited number of target sites are available for each platform. Nonetheless, sequential rounds of targeted gene insertion theoretically make it plausible to generate large multigene stacks using these technologies.

Site-specific integration has also been achieved using ZFNs in corn whereby a 4.5 kb sequence that encoded a selectable maker gene (*aad1*) and flanking sequences with homology to the target site was precisely integrated in juxtaposition to a pre-existing transgene (pat) in 3 % of transgenic events (Ainley et al. 2013). In this study, a ZFN pair was used to cleave a sequence immediately adjacent to the *pat* transgene. The homologous sequences flanking the incoming *aad1* gene enabled potential homologous recombination between the target site and donor sequence.

Perhaps the most advanced demonstration of sequential *cis* stacking of transgenes in crop plants has been demonstrated in cotton (Dhalluin et al. 2013). A meganuclease was re-engineered to recognise an endogenous target in juxtaposition to a pre-existing transgene sequence that encoded the *cry2Ae* insecticidal protein and BASTA herbicide tolerance gene (*bar*). Using meganuclease cleavage to promote homologous recombination, a second 9 kb sequence encoding two herbicide tolerance transgenes, *epsp* and *hppd* (5.5 kb in total), and flanked by sequence (3.5 kb) with target locus homology was introduced adjacent to the first transgene locus in 2 % of transformed calli. Analysis of T1 progeny from regenerated plants showed simple inheritance of these four *cis* stacked transgenic traits. Interestingly, both this study and the maize studies of Shukla et al. (2009) and Ainley et al. (2013) used homology-dependent repair mechanisms to promote precise transgene insertions.

Targeted transgene insertion does not necessarily require homologous recombination-based processes. In tobacco, 2.5 % of T-DNA insertions occurred in an enforced DS DNA break catalysed by the *I-SceI* meganuclease, and the incoming T-DNA sequence did not contain significant homology to the target integration site (Tzfira et al. 2003). However, in general these insertions lacked the precision of homologous recombination and were frequently associated with small indels at the target site. In a similar set of experiments using ZFNs, a *GFP* ORF was excised and replaced with a promoterless antibiotic selectable marker gene (*hpt*) in both Arabidopsis and tobacco in 5 % of regenerated plants (Weinthal et al. 2013). In this latter experiment, both the *GFP* target and incoming *hpt* gene were flanked by the same ZFN recognition site. Likewise, a promoterless GFP reporter gene flanked by TALEN sites was inserted in juxtaposition to an endogenous gene promoter (*TaMlo*) in wheat protoplasts, albeit with small indels again arising from the NHEJ process (Wang et al. 2014).

When considering the current molecular tools available when producing *cis* transgene stacks, designer nucleases potentially have the advantage in that the number of sequential target sites is not limited. In addition, the initial choice of insertion site within the plant genome can be theoretically predetermined rather than beginning with a random insertion event. This could enable the first transgene insertion to be located next to a desirable endogenous trait, or, as in the cotton example above, a pre-existing transgene that will contribute to the utility of the final transgene stack. Sensible construct design would enable sequential removal of the previous selectable marker during insertion of the next transgene by flanking this selectable marker with appropriate nuclease target sites.

Regulatory considerations: Are they transgenic?

A caveat for the use of designer nucleases is that firstly the species of choice must have a functional transformation system available, and secondly the resulting plants will be considered as transgenic. Or will they? A designer nuclease can be used to precisely cleave a DNA target site which is then repaired by endogenous DNA repair systems. The nuclease transgene can then be segregated away by selecting progeny plants that contain only the targeted mutation and not the transgene. These plants may potentially be considered as nontransgenic. Logically, these plants differ very little to plants with a mutation in the same gene that have arisen by EMS or radioisotope mutagenesis. The only differences being that the designer nuclease-produced plants will contain a mutation in a precisely defined region of choice in the target gene and will also have far less, if any, unknown background mutations when compared with mutagen-derived plants. The regulation and classification of these precision-engineered crops in terms of their GM or nonGM status is yet to be determined (Kuzma and Kokotovich 2011; Waltz 2012; Lusser and Davies 2013; Hartung and Schiemann 2014).

In summary, a number of designer nuclease platforms are available for crop plant improvement. Their applications range from targeted mutations, deletions, homologous recombination, production of *cis* transgene stacks and transcriptional reprogramming of endogenous genes. These technologies have been demonstrated to function effectively in a number of important crop species, and it is likely that new cultivars will contain improved germplasm derived from these technologies in the very near future. This adoption would be greatly facilitated by a sensible ruling regarding the nonGM status of these plants in simple targeted mutation applications.

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