

EMERGING TOOLS FOR SYNTHETIC BIOLOGY IN PLANTS

Synthetic nucleases for genome engineering in plants: prospects for a bright future

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SUMMARY

By inducing double-strand breaks (DSB), it is possible to initiate DNA recombination. For a long time, it was not possible to use DSB induction for efficient genome engineering due to the lack of a means to target DSBs to specific sites. This limitation was overcome by development of modified meganucleases and synthetic DNA-binding domains. Domains derived from zinc-finger transcription factors or transcription activator-like effectors may be designed to recognize almost any DNA sequence. By fusing these domains to the endonuclease domains of a class II restriction enzyme, an active endonuclease dimer may be formed that introduces a site-specific DSB. Recent studies demonstrate that gene knockouts via non-homologous end joining or gene modification via homologous recombination are becoming routine in many plant species. By creating a single genomic DSB, complete knockout of a gene, sequence-specific integration of foreign DNA or subtle modification of individual amino acids in a specific protein domain may be achieved. The induction of two or more DSBs allows complex genomic rearrangements such as deletions, inversions or the exchange of chromosome arms. The potential for controlled genome engineering in plants is tremendous. The recently discovered RNA-based CRISPR/Cas system, a new tool to induce multiple DSBs, and sophisticated technical applications, such as the *in planta* gene targeting system, are further steps in this development. At present, the focus remains on engineering of single genes; in the future, engineering of whole genomes will become an option.

Keywords: gene technology, double-strand break repair, synthetic nucleases, targeted mutagenesis, gene targeting.

INTRODUCTION

More than three decades ago, gene technology was initiated in plants when it became possible to transform cells via *Agrobacterium* (Herrera-Estrella *et al.*, 1983) and later via direct DNA transfer (Paszowski *et al.*, 1984). Since then, large numbers of transgenic plants of various species have been obtained for basic research purposes as well as for agriculture.

Although early attempts were made to target genes (Paszowski *et al.*, 1988), as performed previously in yeast, initial efforts to establish a feasible gene targeting (GT) technique in plants failed (Puchta and Fauser, 2013). The main reason for this failure is that the incoming DNA is integrated randomly into the plant genome, even when sequences that are identical ('homologous') to the genomic DNA are used.

The random integration of incoming DNA is the most common mechanism for most multi-cellular eukaryotes,

including mammals. Nevertheless, a GT technique was developed in mouse embryonic stem cells (Doetschman *et al.*, 1987; Thomas and Capecchi, 1987); this system has been applied efficiently to determine the function of thousands of mammalian genes over the years (Collins *et al.*, 2007). However, similar success was not achieved in other cell types and other animals. This is most likely because embryonic stem cells are more proficient in homologous recombination (HR) than other cell types (Kass *et al.*, 2013), and because transformation vectors and selection markers are highly optimized in these cells.

Over the past two decades, plant biologists have had no means to mutate specific genes in a directed manner. Mutants have been classically produced in a stochastic way using chemical mutagens such as ethyl methyl sulfonate. Large-scale projects were initiated to elucidate gene function first in *Arabidopsis* and later in rice and other

crops; the goals of these project were to isolate randomly obtained insertion mutants produced by T-DNA transformation or by use of transposons or retrotransposons (Ostergaard and Yanofsky, 2004). Although the elucidation of many gene functions was possible using these methods, the situation is by no means comparable to that in yeast or embryonic stem cells. The use of random insertions does not often result in complete knockout of the ORF of interest; aberrant protein products, which may have partial function or even a dominant-negative effect, may be obtained instead. As a rule, several insertion mutants are required to obtain sufficient and reliable information on a knockout phenotype. Moreover subtle changes, such as the exchange of single amino acids, cannot be introduced into genomic DNA using such methods. As an alternative, chemical mutagenesis and 'TILLING' (targeting induced local lesions in genomes) methods have been used. However, mutant plants have been obtained that carry many off-site mutations in addition to the mutation of interest; these off-site mutations are very difficult to eliminate, even after repeated out-crossing (Wang *et al.*, 2012).

RNAi-related techniques (using RNAi, small RNAs and microRNAs) have also been applied in plants to elucidate gene function (Small, 2007). In this case, only knockdown of the specific mRNA may be achieved. As off-site effects cannot be excluded (Auer and Frederick, 2009), RNAi-based approaches are less reliable than DNA-based knockout strategies. Additionally, the silencing is often partial and not stable from one generation to the next.

In 1997, it was demonstrated that *Physcomitrella patens* genes may be targeted efficiently by transforming protoplasts with plasmid DNA carrying homologies to the target locus; this moss therefore became a useful model species for the study of specific aspects of plant metabolism (Schaefer and Zryd, 1997). Nevertheless, this finding did not lead to an understanding of why it is difficult to target genes in higher plants.

A breakthrough in efficient genome engineering in plants was finally achieved by copying the major natural mechanism of recombination initiation: double-strand break (DSB) induction. The presence of DSBs initiates meiotic recombination in all eukaryotes, and initiates mating type switching in yeast as well as immunoglobulin switching in mammals. Studies in yeast demonstrated early on that induction of artificial DSBs at various sites in the yeast genome results in initiation of recombination reactions at these sites (Paques and Haber, 1999).

To better understand the current genome engineering approaches in plants, we first describe the mechanisms by which DSBs are repaired in somatic plant cells. We then discuss the current means available to induce DSBs at specific sites. An overview of the various strategies that may be applied to obtain specific changes in plant genomes follows. Finally, we discuss the further prospects for

DSB-induced genome engineering. For a general review on synthetic nucleases, see Gaj *et al.* (2013); for recent reviews on genome modification in plants, see also Tzfira *et al.* (2012) and Voytas (2013).

HOW PLANTS REPAIR DOUBLE-STRAND BREAKS

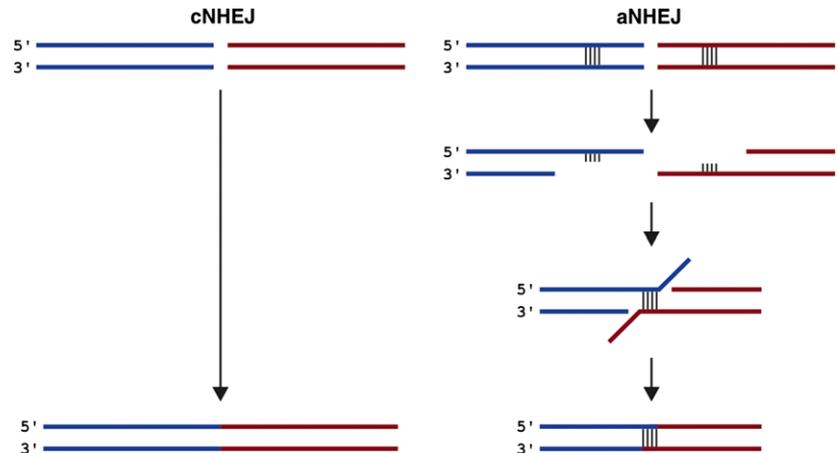
In principle, there are two general ways to repair DSBs. These involve identical ('homologous') or almost identical ('homeologous') sequences or the re-joining of free ends with or without the involvement of a small number of identical bases ('microhomologies'). For recent reviews on DSB repair in plants, see also Lieberman-Lazarovich and Levy (2011) and Waterworth *et al.* (2011). The principle features and the basic actors in the HR and non-homologous end joining (NHEJ) mechanisms are conserved in eukaryotes. However, the efficiency of these pathways differs not only between species but also between cell types. We concentrate here on the DSB repair pathways that are relevant for genome engineering in plants (Puchta, 2005).

Non-homologous end joining

NHEJ is the main mechanism of DSB repair in somatic plant cells, and is required for random integration of DNA into plant genomes. Our current understanding indicates that there are at least two mechanisms of NHEJ that operate in plant cells; these may be distinguished by the pattern of repair as well as by the factors that are involved (Figure 1). These mechanisms are the canonical or classical NHEJ (cNHEJ) pathway and the more recently discovered 'alternative NHEJ' (aNHEJ) pathway (Mladenov and Iliakis, 2011). After induction of endonuclease-induced genomic DSBs, two classes of repair junctions may be found in plant cells: the first class contains a small number of identical base pairs at the junction site ('microhomologies'), combined with the deletion or insertion of some nucleotides, and the second class lacks these features (Salomon and Puchta, 1998). These two patterns appear to be conserved in eukaryotes. It is generally accepted that, after DSB induction in the canonical repair pathway, the two double-stranded ends are protected from degradation by binding of the Ku heterodimer. With or without minor end processing, a specific ligase (ligase 4) is targeted to the break site, and the two open ends are re-ligated (Figure 1). Mostly, the ligation of ends restores the original sequence. Sometimes genetic information is lost, and microhomologies are rarely found at the newly formed junctions. These are the cases that are interesting from the point of view of genome manipulation: if a DSB occurs within an ORF, deletion of one or two nucleotides results in a frameshift; depending on the position of the break within the ORF, which often leads to a complete knockout of gene function.

In the aNHEJ pathway, which is not as well-characterized as the cNHEJ pathway, a certain amount of 3' resection of the broken ends occurs. A junction is formed via annealing

Figure 1. Two mechanisms of NHEJ. The canonical or classical NHEJ (cNHEJ) pathway and the alternative NHEJ (aNHEJ) pathway differ in the pattern of repair and their respective enzyme machinery. The Ku heterodimer and ligase 4 are responsible for cNHEJ; PARP1 and XRCC1 are characteristic of aNHEJ. In cNHEJ, the degradation of double-stranded DSB ends is blocked by the Ku heterodimer after DSB induction. This is followed by ligase 4-mediated re-ligation. As a result, the change in genetic information is often minor in cNHEJ. In contrast, double-strand ends are resected during aNHEJ. Complementary nucleotides are able to anneal, leading to re-ligation at sites of microhomology. Larger deletions therefore arise.



of the two single strands at sites where a few complementary nucleotides are present. After the ends are trimmed, re-ligation occurs and microhomologies are found at the junction site (Figure 1). It has been demonstrated that the poly (ADP-ribose) polymerase I (PARP1) and X-ray repair cross-complementing protein 1 (XRCC1) proteins involved in this pathway are conserved in mammals and plants (Charbonnel *et al.*, 2011; Jia *et al.*, 2013). Due to occurrence of these deletions, genetic information is lost. Therefore, aNHEJ may also be regarded as a highly mutagenic way to repair DSBs.

Interestingly, both NHEJ pathways appear to compete for DSBs. In a study of the Ku80 mutant in *Arabidopsis*, the error-prone re-joining frequency increased by 2.6-fold compared with wild-type, and increased end degradation was documented (Osakabe *et al.*, 2010). It is unclear whether the two NHEJ pathways work with the same efficiency in different plant species and in different cell types (Kirik *et al.*, 2000; Lloyd *et al.*, 2012). Recent evidence indicates that there may be a third NHEJ pathway that is responsible for the joining of at least some DNA ends when the cNHEJ and aNHEJ pathways are knocked out (Charbonnel *et al.*, 2011).

Interestingly, DSB repair via NHEJ may also be associated with insertions. In an initial study, it was demonstrated that sequences from elsewhere in the genome may be inserted into DSB sites, as may incoming T-DNAs (Salomon and Puchta, 1998). In most of these cases, microhomologies were found at the junctions; either an aNHEJ mechanism or a mode of copying similar to the synthesis-dependent strand annealing model described below for homologous DSB repair may be responsible for this phenomenon. The fact that unique inserted genomic sequences are still found at their original sites after DSB repair strongly suggests that a copying mechanism is involved (Salomon and Puchta, 1998). The suggested mechanism is shown in Figure 2.

In the case of genome engineering, it is notable that DSB-induced NHEJ may be used for induction of muta-

tions as well as sequence-specific DNA integration (Chilton and Que, 2003; Waterworth *et al.*, 2011; Weinthal *et al.*, 2013), and also for various other covalent changes in DNA molecules, such as deletion, inversion and exchange of sequences. For these changes, more than one DSB has to be induced (see below). This is due to the fact that, in a certain number of cases, the original linked DSB ends are not re-joined. Joining instead occurs between ends that were previously unlinked. Therefore, new combinations of genetic elements are obtained.

Homologous recombination

HR is the basis for GT. In plants, HR is efficient during meiosis in a way that is conserved in other eukaryotes (Osman *et al.*, 2011). In somatic cells, HR is a minor DSB repair pathway that occurs mainly during the S and G₂ phases of the cell cycle (Puchta, 2005). The mechanisms of DSB repair during somatic and meiotic HR differ to a certain extent.

We discuss the two most prominent pathways in somatic cells, which are single-strand annealing (SSA) and synthesis-dependent strand annealing (SDSA). These pathways are shown in Figure 3. In both pathways, resection of the double-stranded DNA occurs after DSB induction such that 3' single-stranded overhangs are produced. SSA may only operate if the DSB occurs between two homologous sequences. It is a non-conservative mechanism, as the sequence information between these homologies is lost (Figure 3). SSA is especially important in genomic regions with tandem duplications; in these regions, up to one of every three DSBs is repaired using this mechanism (Siebert and Puchta, 2002). In contrast, SDSA is a conservative mechanism, as the homology of the donor is copied into the break site without loss of sequence (Figure 3). SDSA appears to be approximately five to ten times less efficient than SSA under comparable conditions (Orel *et al.*, 2003). In the case of SSA, overhangs at both ends of the break carry complementary sequences. The two single strands

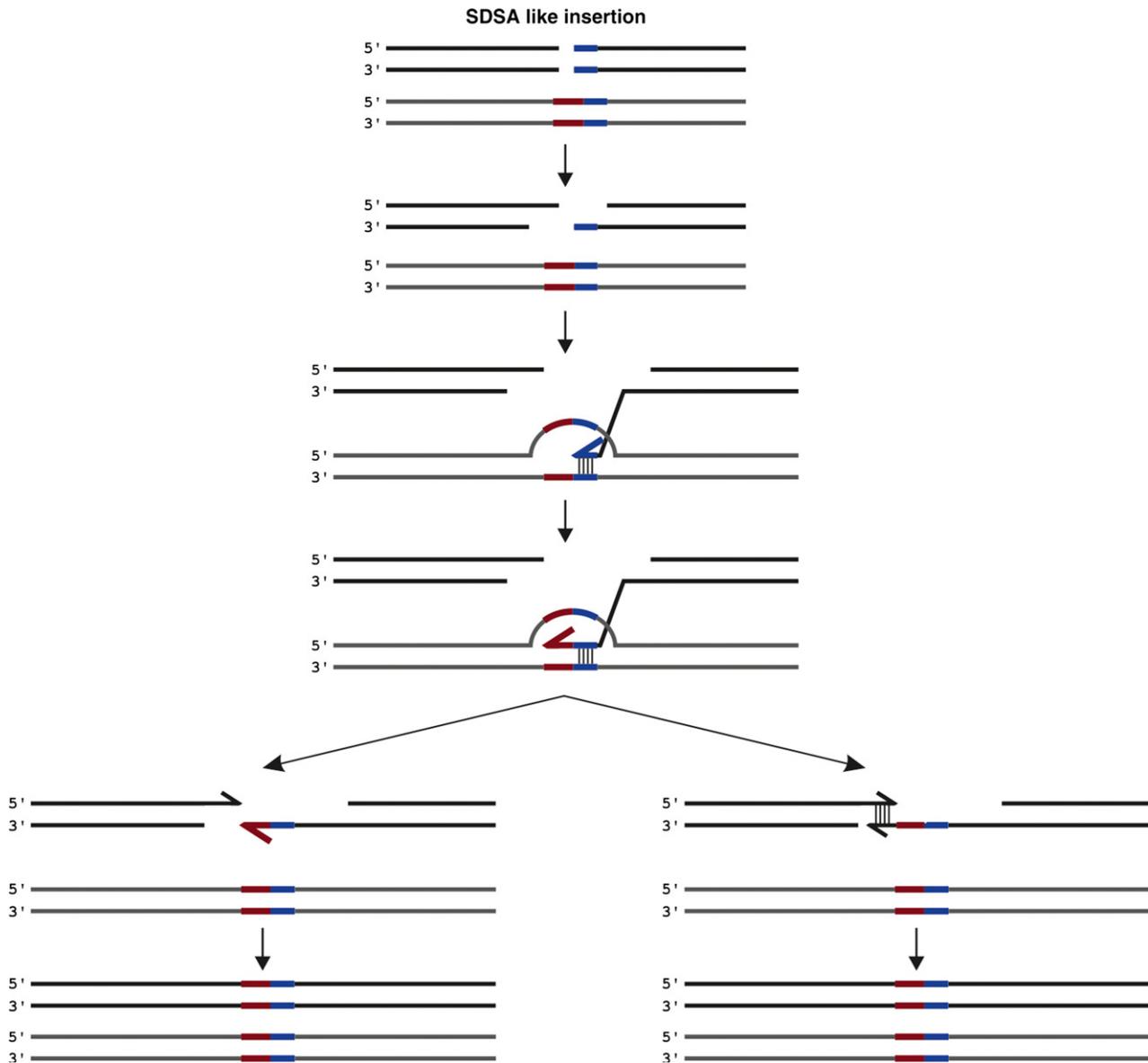


Figure 2. SDSA-like insertions. Microhomology-mediated DSB repair may also result in insertions within the original DSB. Microhomologies are used to initiate the copying process of sequences from elsewhere in the genome or from extra-chromosomal DNA into the DSB. Microhomologies may also be involved for second end capture.

may directly anneal with one another to form a chimeric DNA molecule (Figure 3). If the molecule contains a 3' overhang, it is trimmed; otherwise, the single-stranded regions are filled in by repair synthesis. In the case of SDSA, a single 3' end invades a homologous double-strand, forming a D-loop structure (Figure 3). Repair synthesis starts by using the newly paired strand as a template. After elongation, the strand is displaced from the D-loop structure and anneals with the 3' homologous strand that becomes available due to resection at the second end of the DSB. The final result of this reaction is a gene conversion event. In contrast with the SSA mecha-

nism, no sequence is lost; however, the information content may be changed. Under natural conditions, the repair matrices used are mostly sequences in close proximity on the same chromosome or the sister chromatid. Ectopic or allelic homologies are rarely used in DSB repair (Gisler *et al.*, 2002; Puchta, 1999).

As the two mechanisms differ quite drastically, it is not surprising that the involvement of DNA repair proteins differs considerably between the SSA and SDSA pathways. For SDSA, a strand exchange reaction is required; the RecA homologues AtRAD51 and AtXRCC3, as well as the SNF2/SWI2 ATPase AtRAD54, are essential for SDSA but are not

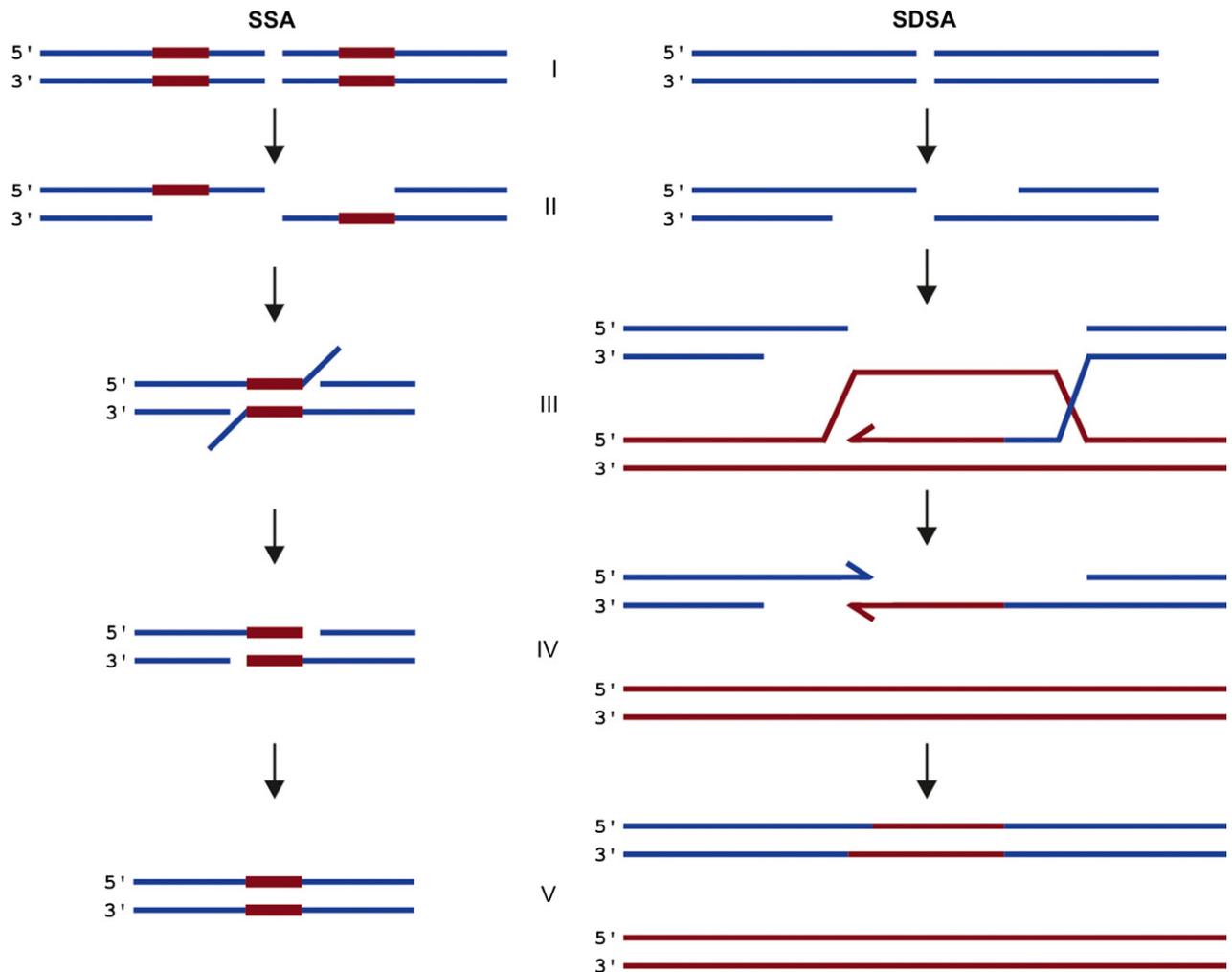


Figure 3. SSA and SDSA: two mechanisms for homologous DSB repair. In somatic plant cells, DSBs may be repaired via SSA or SDSA. SDSA is considered to be a conservative DSB repair pathway; SSA is a non-conservative pathway. Both pathways are initiated by a DSB (I), followed by the resection of the 5' ends to produce 3' overhangs (II). In the non-conservative SSA pathway, homologies within the single-stranded 3' overhangs support immediate annealing of both strands (III); this leads to deletion of sequences flanked by the homology sites (IV and V). The SDSA pathway is initiated via a free 3' strand that invades a homologous double-stranded DNA molecule (III). The invade 3' end is elongated using the double-stranded DNA molecule as a donor of genetic information. Once the 3' end is set free, it re-anneals with the original strand. Single-stranded gaps are repaired via fill-in synthesis. In comparison with SSA, SDSA results in a restored double-stranded DNA molecule without loss of genetic information. The SDSA pathway was initially described in plants by Gorbunova and Levy (1997) and Ruben and Levy (1997).

required for SSA (Roth *et al.*, 2012). The DNA helicases AtRECQ4A and AtFANCM, as well as nucleases such as AtMUS81, play some roles in SDSA and minor roles in SSA (Mannuss *et al.*, 2010). There are strong indications that the RAD1/RAD10 heterodimer, a structure-specific flap-like endonuclease, is involved in trimming the complementary strand before ligation in SSA (Dubest *et al.*, 2002). No other factors that are essential for SSA have yet been characterized.

In genome engineering, the SSA mechanism may be used for DSB-induced deletion of sequences between genomic repeats. DSB-induced GT most likely occurs via an SDSA-like mechanism. A unique feature of SDSA is that

both ends of the DSB interact with their homologous counterparts independently of one another. If GT occurs via an SDSA-like mechanism, DSB-induced GT should be possible using a targeting vector that contains homology to only one end of the break. Indeed, this has been demonstrated for T-DNA-mediated GT in tobacco (*Nicotiana tabacum*). GT was approximately half as frequent as when a vector containing homology on both ends of the targeted DSB was used, some of the GT events included vector sequences at the target locus; this suggests that one site of the break was repaired via HR and the other was repaired via NHEJ (Puchta *et al.*, 1996; Wright *et al.*, 2005).

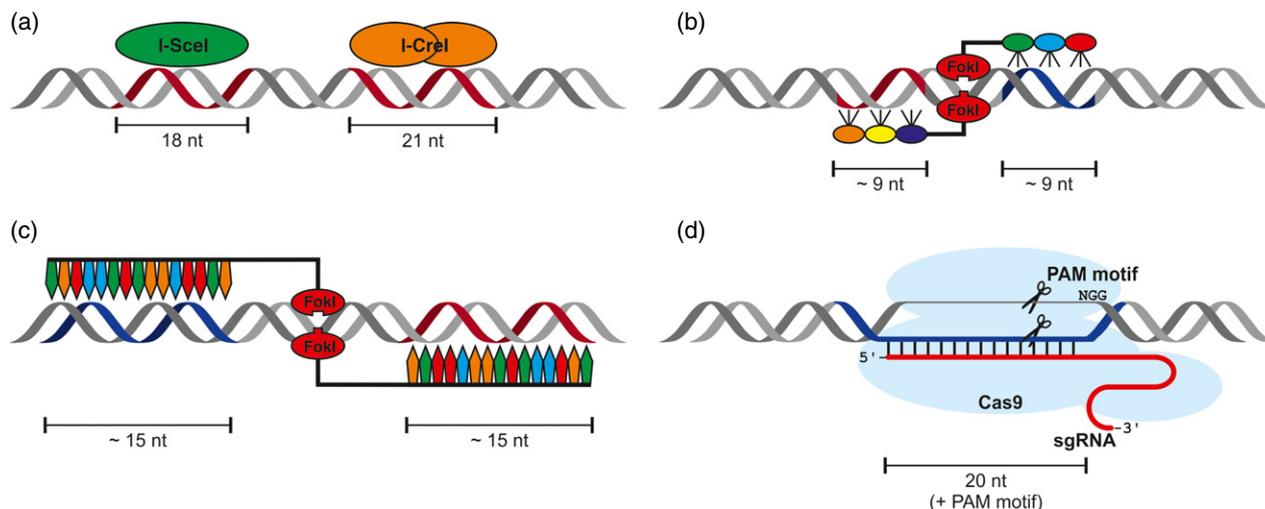


Figure 4. Various tools to induce DSBs.

(a) The naturally occurring homing endonucleases I-SceI and I-CreI are shown. I-SceI is a monomeric meganuclease that binds and cuts an 18 nt recognition site. The dimeric meganuclease I-CreI recognizes a 21 nt binding site. The DNA-binding domain of I-CreI was successfully modified to recognize artificial targets.

(b) Zinc-finger nucleases (ZFNs) act as dimers. These enzymes consist of two independently constructed subunits. Every subunit is divided into a DNA-binding domain and a nuclease domain. Typically, a heterodimeric version of FokI is used as a nuclease, and arrays of three to four zinc fingers are fused to a DNA-binding domain. Each zinc finger recognizes 3 nt, resulting in 9–12 nt DNA recognition sites.

(c) Transcription activator-like effector nucleases (TALENs): TALENs are dimeric enzymes with an architecture related to ZFNs. Again, FokI is used as a nuclease domain. The DNA-binding array is more flexible because it is based on a ‘one module per nucleotide’ code. The DNA-binding domain consists of modules repeats that are specific for particular nucleotides.

(d) The CRISPR/Cas system: In nature, the Cas9 nuclease complexes with a crRNA (CRISPR RNA) that is base-paired with the tracrRNA (trans-activating crRNA). For biotechnological applications, these RNAs are fused, resulting in a chimeric sgRNA (single-guide RNA) that is responsible for the specificity of the Cas9 nuclease after the functional site-specific Cas9 is built. Within this system, a 20 nt spanning protospacer defines the recognition site, which may be modified with ease if a protospacer-adjacent motif (PAM) is present (sequence NGG).

INDUCTION OF DSBs BY SYNTHETIC NUCLEASES

Homing endonucleases/meganucleases

The basic mechanisms of DSB repair in the plant genomes were elucidated by use of rare cutting endonucleases; the homing endonuclease I-SceI (Figure 4), discovered in yeast mitochondria, has been used most frequently (Jacquier and Dujon, 1985). Initially, the applicability of these enzymes to induce DSBs *in vivo* was demonstrated using plasmid molecules in *Nicotiana* protoplasts (Puchta *et al.*, 1993). To utilize I-SceI in genomic DSB repair, transgenic plant lines were produced that harboured an artificial I-SceI site in a marker construct such that they may be used to monitor specific types of DSB repair mechanisms. By 1996, the principle of DSB-induced GT was demonstrated by use of I-SceI (Puchta *et al.*, 1996). Many basic features of DSB repair were elucidated using I-SceI (Puchta, 2005). I-SceI is still widely used in the field to study the mechanisms of DSB repair; it has been especially important in defining the roles of specific factors involved in these pathways (Mannuss *et al.*, 2010; Roth *et al.*, 2012; Wei *et al.*, 2012) and demonstrating the applicability of new genome engineering techniques (Ayar *et al.*, 2013; Fauser *et al.*, 2012).

Homing endonucleases, also known as meganucleases, were the first tools used for DSB-induced genome manipulations. The DNA-binding sites of these enzymes have

been manipulated to target DSBs to natural sites of interest in plant genomes. Because homing endonucleases are small proteins, the domains responsible for DNA binding and endonuclease activity overlap. The dimeric I-CreI endonuclease was used to change the recognition site specificity rather than the monomeric I-SceI endonuclease (Grizot *et al.*, 2011). I-CreI-based enzymes (Figure 4a) have been successfully used in NHEJ-mediated targeted mutagenesis in maize (*Zea mays*) (Gao *et al.*, 2010), the excision of transgene sequences in *Arabidopsis* (Antunes *et al.*, 2012), and gene stacking in cotton (*Gossypium hirsutum*) (D’Halluin *et al.*, 2013). However, we do not expect that these enzymes will play an important role in genome engineering in the future. In comparison to the synthetic nucleases that are now available (see below), production of modified homing endonucleases is too time-consuming and not sufficiently flexible.

Zinc-finger nucleases

The development of zinc-finger nucleases (ZFNs) as efficient tools for genome manipulation was a long and laborious process. Many important proof-of-concept experiments were eventually performed using ZFNs; these experiments have demonstrated the tremendous potential of artificial nucleases in general. ZFNs were originally developed by the group of Srinivasan Chandrasegaran

(Kim *et al.*, 1996) and also Dana Carroll and colleagues pioneered the set-up of ZFNs (Smith *et al.*, 2000). These enzymes consist of two independent regions: the endonuclease domain of the restriction enzyme FokI and the zinc-finger binding arrays found in transcription factors (Kim *et al.*, 1996; Smith *et al.*, 2000). The DNA-binding domain of the ZFN enzyme normally consists of three to four binding arrays, which typically recognize three consecutive nucleotides each. In total, 9–12 nt of the genomic sequence may be recognized per protein monomer. To become active, two FokI endonuclease domains must dimerize; this results in a staggered cut in the double-stranded DNA. This dimerization may be achieved by designing the binding domain to recognize sequences in close proximity on opposite strands. As a result, the recognition site of a functional ZFN dimer is 18–24 nt, excluding the spacer region (Figure 4b).

The research group under Dan Voytas pioneered use of ZFNs for gene editing in plant cells. They were able to achieve a decisive breakthrough by demonstrating that ZFNs may be used for GT in plants. Using restoration of a defective selection marker gene in tobacco protoplasts, they demonstrated that GT frequencies may be enhanced by up to 10^{-1} in comparison with random integration (Wright *et al.*, 2005). At the same time, Lloyd *et al.* (2005) demonstrated that ZFNs may be used to mutate an artificially introduced restriction site in the *Arabidopsis* genome via NHEJ.

More time and effort was required before the first reports on use of ZFNs for the engineering of non-transgenic sequences in plant genomes appeared. In 2009, Dan Voytas' group, as well as scientists from Dow Chemicals, demonstrated independently that it was possible to modify endogenous genes in tobacco and maize by DSB-induced HR using ZFNs (Townsend *et al.*, 2009 and Shukla *et al.*, 2009). In tobacco, the *SuRA* and *SuRB* loci were modified at the single-nucleotide level via ZFN-mediated GT to establish resistance to various herbicides. An impressively high GT frequency, up to several per cent, was reported (Townsend *et al.*, 2009). In maize, ZFN-mediated GT of the maize *IPK1* gene was achieved in such a way that expression of the recombinant *IPK1* resulted in an herbicide-resistant phenotype. The reported GT frequencies were extremely high, reaching more than 10% in most experiments (Shukla *et al.*, 2009). ZFN-mediated GT was also recently described in *Arabidopsis*, although the frequencies were not as high as in maize and tobacco (de Pater *et al.*, 2013). In 2010, it was shown that ZFNs are efficient tools for the knockout of natural genes in *Arabidopsis* via NHEJ (Osakabe *et al.*, 2010; Zhang *et al.*, 2010). The list of species with genomes that have been engineered by ZFNs is growing; nine endogenous soybean (*Glycine max*) genes have now been mutated using ZFNs (Curtin *et al.*, 2011).

ZFNs have therefore become an efficient tool for various types of genome editing in plants. Nevertheless, the domi-

nance of ZFNs may already be over because of the recent development of newer and even more attractive tools for site-specific DSB induction (see below). A general problem with artificial nucleases is that they may cut additional sites in the genome that are similar but not identical to the target site. Such 'off-target effects' are a source of concern (Voytas, 2013) as unpredicted mutations may be induced. The respective mutations may lead to unwanted secondary effects. Indeed, it has been observed that some ZFNs have negative effects on cell proliferation. This suggests that these nucleases do cause unwanted DSBs at secondary sites. Further uncertainty stems from the fact that the various binding modules in the zinc-finger binding arrays influence each other. Therefore, construction of domains for new genomic sites is not as predictable and efficient as expected. Although kits for building ZFNs are available, the construction of ZFNs still appears to be more time-consuming than that for TALENs or the CRISPR/Cas system (see below) and less versatile, as ZFN design is often limited by the lack of suitability of the target sequence.

Transcription activator-like effector nucleases

In ground-breaking studies, Ulla Bonas and her group discovered that the transcription activator-like effector protein is delivered into its host by the bacterial pathogen *Xanthomonas* (Bonas *et al.*, 1989; Van den Ackerveken *et al.*, 1996). This protein carries a DNA-binding domain that binds to various plant promoters (Boch *et al.*, 2009; Kay *et al.*, 2007; Romer *et al.*, 2007). In an accompanying review, Thomas Lahaye gives a detailed overview of transcription activator-like effector proteins and their potential for various applications in synthetic biology and biotechnology (Lahaye, this issue). Here we concentrate on TALE-based nucleases that are constructed as dimers, similar to ZFNs (see Figure 4c). The DNA-binding domains of transcription activator-like effector nucleases (TALENs) consist of up to 30 copies of highly conserved repeats that span 34 amino acids each. There are several obvious advantages of the use of TALENs in comparison to ZFNs. As each repeat is able to recognize a single base, new binding sites may be easily assembled for virtually any DNA sequence; the management of binding specificity is easy compared with ZFNs (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). TALENs may be assembled in the laboratory within 1 week using publically available kits, e.g. by Golden Gate cloning (Cermak *et al.*, 2011). Due to the longer recognition sites of TALENs, these nucleases may cause fewer unwanted off-target effects than ZFNs.

It is therefore not surprising that TALENs are becoming widely used in the plant community. Recent studies have demonstrated the huge potential use of TALENs for genetic engineering in plants (Christian *et al.*, 2010; Mahfouz *et al.*, 2011). By TALEN-mediated introduction of a mutation into the promoter of the *OsSWEET14* gene in rice, it was demon-

strated that the binding motif for a pathogen-based transcription factor was destroyed; this led to enhanced disease resistance in rice (Li *et al.*, 2012b). Again, important contributions were made by Dan Voytas and his group. They introduced targeted mutations within the *ALS* gene in up to 30% of transformed tobacco protoplasts using TALENs. They also performed a TALEN-mediated GT experiment in tobacco using a donor template that created an in-frame gene fusion between *ALS* and a YFP marker gene. This made it possible to quantify GT efficiency by YFP fluorescence using flow cytometry. The GT frequencies were as high as an astonishing 14%. In a second set of experiments, this group used a TALEN and a 322 bp homologous donor DNA differing by 6 bp from the *ALS* coding sequence. Even without selection, 4% of the regenerated calli showed evidence of targeted gene replacement (Zhang *et al.*, 2013). Recently, TALENs have also been used for targeted mutagenesis in *Brachypodium* (Shan *et al.*, 2013a) and barley (*Hordeum vulgare*) (Wendt *et al.*, 2013).

The CRISPR/Cas system

As with TALENs, host–microbe interaction studies resulted in the discovery of another exciting means of site-specific DSB induction. The type II ‘clustered, regularly interspaced, short palindromic repeats’ (CRISPR) interference system is part of the adaptive immunity system in bacteria. The CRISPR genomic locus encodes the Cas9 (CRISPR-associated) endonuclease, which forms a complex with two short RNA molecules known as CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA). These two RNAs allow the Cas9 enzyme to recognize and cleave a site in foreign DNA (e.g. phage DNA or plasmid DNA) which has invaded the bacterial cell afore. In an elegant study, it was shown that these two short RNAs may be fused; this results in a chimeric single-guide RNA (sgRNA) comprising functional portions of both progenitors. Together with the Cas9 protein, the sgRNA is able to form a targeted RNA-guided endonuclease (Jinek *et al.*, 2012). The specificity of this nuclease is defined by 20 consecutive nucleotides embedded within the sgRNA (Figure 4d). This makes the design process of new synthetic enzymes extremely easy and attractive. It is only necessary to insert the desired sequence as a DNA oligonucleotide into a vector construct for target site selection. The Cas9 protein does not require any re-engineering and has worked well for all target sites that have been studied so far. The expression of multiple guide RNAs allows for multiplexing (Cong *et al.*, 2013; Mali *et al.*, 2013; Wang *et al.*, 2013), which reduces costs and the time needed to generate plants with multiple targeted mutations.

Over the last year, a number of papers were published that demonstrated the applicability of the CRISPR/Cas system for gene editing via NHEJ and via HR in human cells, mice and zebrafish (Cong *et al.*, 2013; Hwang *et al.*, 2013; Mali *et al.*, 2013; Wang *et al.*, 2013). The knockout and GT

frequencies reported for these experiments were similar to those obtained previously with ZFNs or TALENs. There is no question that the CRISPR/Cas system is an interesting alternative tool to induce DSBs in plant genomes. As the 20 nt recognition sequence is quite short and evolutionarily adapted to relatively small bacterial genomes, it is not yet clear whether the specificity is high enough to avoid off-site effects in eukaryotic organisms, especially within the much larger plant genomes. Recent results in human cells indicate that a high frequency of off-target mutagenesis is induced by various CRISPR/Cas nucleases (Fu *et al.*, 2013).

Very recently, a number of studies were published demonstrating that the CRISPR/Cas system is indeed a suitable means for genome engineering in model as well as crop plants (Mao *et al.*, 2013; Feng *et al.*, 2013; Xie and Yang, 2013; Jiang *et al.*, 2013; Miao *et al.*, 2013; Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013b). Most of these experiments used Cas9 nucleases for endogenous target sites. In *Arabidopsis* protoplasts, NHEJ-based targeted mutagenesis frequencies up to 5.6% were obtained, while in *Nicotiana benthamiana* cells, the frequencies were up to 38.5% (Li *et al.*, 2013). CRISPR/Cas nucleases were also expressed *in planta* via *Agrobacterium*-mediated leaf infiltration (agroinfiltration). The reported frequencies of targeted mutagenesis in *N. benthamiana* after agroinfiltration are in a comparable range of a few per cent (Li *et al.*, 2013; Nekrasov *et al.*, 2013). A similar frequency was reported for *Arabidopsis* (Li *et al.*, 2013). Cas9 activity was also proven in *Arabidopsis* and *N. benthamiana* using a NHEJ-based GFP reporter system and co-transformation via agroinfiltration (Jiang *et al.*, 2013). Similar approaches were used in the same study using a NHEJ-based DsRED2 reporter system in Sorghum via *Agrobacterium*-mediated transformation of immature embryos (Jiang *et al.*, 2013). Additionally, *Arabidopsis* was stably transformed using *Agrobacterium*-mediated floral dipping, and mutation frequencies in T_1 plants of up to 84% were documented (Feng *et al.*, 2013). These results are comparable with other observations made using a multiplex approach in *Arabidopsis* (Mao *et al.*, 2013). Here, most of the transgenic T_1 seedlings showed mutations not only in one or the other but also in both target sites within the *TT4* gene (Mao *et al.*, 2013). It was also shown that plants may be regenerated from *N. benthamiana* cells that have been modified using the CRISPR/Cas system. Two of 30 regenerated plants were found to have a mutated target site (Nekrasov *et al.*, 2013).

Efficient NHEJ-mediated targeted mutagenesis was also detected in rice protoplasts at frequencies of 14.5–38.0%, as well as in wheat protoplasts at a frequency of 28.5% (Shan *et al.*, 2013b). Other researchers reported efficiencies of 3–8% in rice protoplasts (Xie and Yang, 2013). Rice callus was also bombarded to obtain NHEJ-mediated gene

knockouts in plants, and mutations were detected in nine of 96 (9.4%) independent transgenic plants (Shan *et al.*, 2013b). In another study, almost all independent transgenic T_1 rice plants showed mutations at the target site, and approximately half harboured bi-allelic mutations (Miao *et al.*, 2013). Rice callus was also transformed using *Agrobacterium*, and the targeted mutagenesis frequencies in T_1 plants were up to 75% (Feng *et al.*, 2013).

Activity of the Cas9 nuclease was also shown by co-transforming a GUS reporter construct for SSA-mediated DSB repair (Orel *et al.*, 2003) and the CRISPR/Cas system via bombardment into rice callus (Miao *et al.*, 2013). In a similar approach, Cas9 activity was detected using a split YFP reporter system in *Arabidopsis* protoplasts. For this experimental set-up, HR-mediated DSB repair occurred at a frequency of 18.8% (Feng *et al.*, 2013). An analogous YFP reporter construct was also used to compare Cas9 efficiency with other nucleases such as TALENs and I-SceI in *Arabidopsis* protoplasts (Mao *et al.*, 2013). For this purpose, a YFP reporter was used that harbours a multiple recognition site within the spacer region that separates the partial overlapping YFP fragments. The CRISPR/Cas system used in this approach led to fluorescence in 11.0% of co-transfected protoplasts, while a slightly higher frequency of 18.8% was obtained with TALENs and a comparable frequency of 12.5% was obtained using I-SceI (Mao *et al.*, 2013). The same group also used a GUS reporter construct to detect Cas9 activity in transgenic *Arabidopsis* plants after *Agrobacterium*-mediated transformation (Mao *et al.*, 2013). These three studies demonstrate that Cas9-mediated DSB induction is not only useful for targeted mutagenesis but also for HR-related techniques of genome engineering.

Homology-directed repair-mediated gene replacement was achieved in *N. benthamiana* protoplasts at a frequency of 9.0% (Li *et al.*, 2013), and positive homology-directed repair events were also documented for rice protoplasts (Shan *et al.*, 2013b). In both cases, a donor template offering homologies to the target site was simultaneously supplied with the nuclease to obtain the pre-defined change within the target site. In rice, the repair template was a short (72 nt) single-stranded oligo (Shan *et al.*, 2013b); in *Nicotiana*, a linear double-stranded DNA molecule was used, offering 533 nt homology upstream and 114 nt homology downstream of the DSB (Li *et al.*, 2013).

Thus, the CRISPR/Cas system shows high efficiencies in a number of model and crop plants. In comparison to ZFNs and TALENs, Cas9-mediated cleavage is unaffected by DNA methylation (Hsu *et al.*, 2013). Especially important is the fact that the CRISPR/Cas system allows multiplexing (Li *et al.*, 2013; Mao *et al.*, 2013; Miao *et al.*, 2013). The induction of several DSBs is prerequisite for larger genomic modifications such as deletions (Mao *et al.*, 2013). Thus,

use of the CRISPR/Cas system seems to be extremely promising at first sight. However, use of genome-wide approaches to measure possible off-target effects of the CRISPR/Cas system in plants is required before a final assessment may be made.

STRATEGIES TO MODIFY GENES

At present, the three main applications of synthetic nucleases in plants are single gene knockouts, subtle modification of gene functions (e.g. via GT or homology-directed repair), and sequence-specific integration of foreign genes for gene stacking. As only expression of the enzyme is required for NHEJ-mediated single gene knockout, this appears to be by far the simplest way to eliminate gene function. If programmed changes are to be inserted within the gene, this may only be achieved by co-transformation of a repair template copied into the genomic DSB. This is also true when stacked foreign genes are integrated in an agronomically attractive region of an elite cultivar (Cai *et al.*, 2009). Experiments using DSB-induced genetic engineering are currently being developed for a number of plant species. We expect that, in due course, this strategy will become routine in plant laboratories around the globe. Recently, efficient high-throughput techniques for the assembly of TALENs (Lahaye, 2014) and for the testing of activities *in planta* have been developed (Johnson *et al.*, 2013; Li *et al.*, 2012a). We briefly discuss some strategies for gene knockouts and GT using synthetic nucleases.

Gene knockouts via NHEJ

The best method for knocking out a gene via NHEJ is dependent on the protocols that are available to deliver the synthetic nucleases into the plant species of interest. Various strategies for plants are shown in Figure 5. In *Arabidopsis* and soybean, the nuclease is often stably integrated via *Agrobacterium*-mediated transformation and driven by an inducible, cell type-specific or constitutive promoter (Curtin *et al.*, 2011; de Pater *et al.*, 2009; Even-Faitelson *et al.*, 2011; Gao *et al.*, 2010; Lloyd *et al.*, 2005; Osakabe *et al.*, 2010; Tovkach *et al.*, 2009; Zhang *et al.*, 2010). After selecting for primary transformants (T_1 generation), these plants are further propagated to select for heritable targeted mutagenesis events in the T_2 generation. During plant development in the T_1 generation, the synthetic nucleases may induce DSBs in meristematic or somatic tissue; this leads to mutations via erroneous NHEJ-mediated DSB repair in single cells. These mutations may become clonal and enter the germline. Depending on the activity of the nuclease chosen, the frequencies of heritable targeted mutagenesis events vary drastically. Therefore, test systems have been established for early screening of nuclease activity, e.g. in yeast (Cermak *et al.*, 2011) or protoplasts (Zhang *et al.*, 2010). Nevertheless, nuclease activity assays in plants cannot be totally replaced by such test systems.

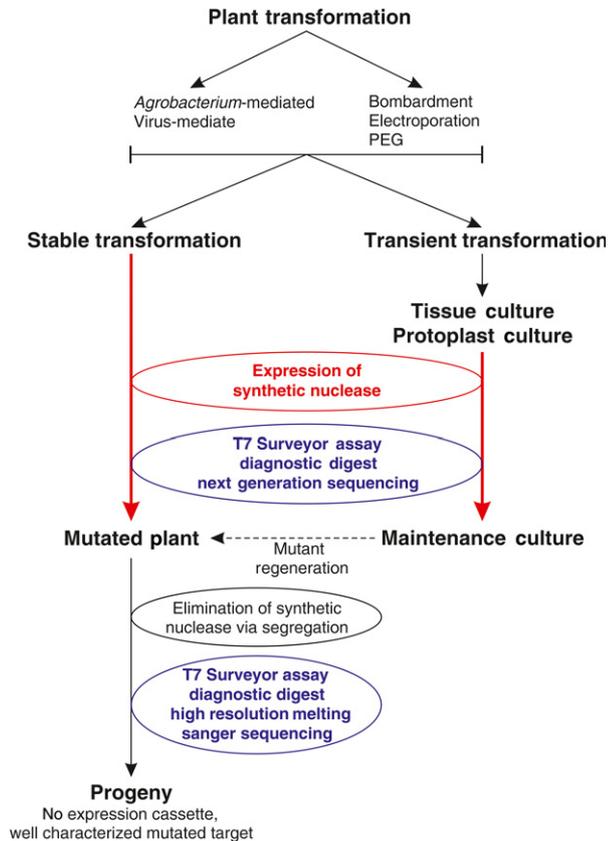


Figure 5. Gene knockouts via DSB-induced NHEJ. The method for NHEJ-mediated knockout of genes is dependent on the transformation protocols available for the respective plant species. Whereas *Arabidopsis* is typically transformed via the *Agrobacterium*-mediated flower-dip protocol, other species such as tobacco and tomato rely on transformation of explants or protoplasts in a stable or transient manner. In addition, transient expression of synthetic nucleases may also be used to assay the cutting efficiency of the respective enzyme. In the case of a stable transformation event, a T_1 plant is obtained that carries the expression cassette for a synthetic nuclease. Depending on the promoter used, the nuclease may be expressed in an induced, constitutive or tissue-specific manner. Typically, mutations are induced in the meristematic or somatic tissue in the T_1 generation. During T_1 development, nuclease activity may be characterized in a qualitative manner using a T7 surveyor assay or a diagnostic digest. Quantitative analysis via next-generation sequencing may also be undertaken. Mutations that enter the germline of a T_1 plant will be clonal in the T_2 generation, resulting in a mutated plant. Mutated plants may then be screened for individuals that carry the mutation in a homozygous state. It is possible to genotype these mutations via T7 surveyor assays, diagnostic digests or high-resolution melting analysis. The nature of the events may be determined by Sanger sequencing. To eliminate the nuclease expression cassette, plants are further propagated. Plants without an expression cassette that are homozygous for the individual mutation may then be identified in the next generation.

Nuclease activity may be tested in T_1 plants using qualitative and quantitative methods. PCR amplification of the respective target locus results in a heterogeneous PCR product if the nuclease has been active. It is possible to screen for such events if 1% or more of the extracted DNA serves as a mutated template. A diagnostic digest may be used for detection of an eliminated restriction enzyme rec-

ognition site. A surveyor assay based on enzymes such as the T7 endonuclease that recognize mismatches may also be used. The sub-cloning of respective PCR products followed by Sanger sequencing is a further option. Next-generation sequencing may be used to quantify nuclease activity; the Roche (<http://www.roche.com>) 454 platform allows detection of larger deletions and insertions due to longer read lengths. When a targeted mutagenesis event enters into the germline of T_1 plants, these events may be either homozygous or heterozygous in the T_2 offspring. When a homozygously mutated target occurs in the T_2 generation, such an event may be explained either by an early event in T_1 leading to segregation of the T_1 plant and a homo-allelic T_2 generation or two independent events occurring later on during development resulting in a hetero-allelic T_2 plant. Nevertheless, plants that harbour a mutated target may be easily genotyped via the qualitative assays mentioned above or by PCR-based high-resolution melting analysis. High-resolution melting analysis allows the detection of single base pair substitutions, insertions and deletions by heteroduplex formation using a saturating dye that measures the exact melting temperature of the amplified product.

Some nucleases may be less specific than others, which leads to unwanted side-effects. This varying specificity is due to different nuclease architectures and complex target sequences. Obligate heterodimeric FokI nuclease domains (Doyon *et al.*, 2011; Miller *et al.*, 2007; Szczypek *et al.*, 2007) and inducible promoter systems may reduce cytotoxic effects caused by non-specific DNA binding that leads to introduction of DSBs at off-target sites (Voytas, 2013). The avoidance of the off-site effects inherent in use of artificial nucleases is of the utmost importance. If expression of a nuclease is accompanied by slow growth or abnormal phenotypes, off-target effects are most likely the cause. It is not advisable to continue experiments with such nucleases, as unrecognized secondary mutations may accumulate in mutant plants. Therefore, we strongly recommend the design of more than just one nuclease for the target site of interest.

Transient expression of synthetic nucleases is preferentially used for the targeted mutagenesis of transformed protoplasts (Mahfouz *et al.*, 2011; Qi *et al.*, 2013; Townsend *et al.*, 2009; Zhang *et al.*, 2013). In addition to *Agrobacterium*-mediated transformation via floral dipping, tissue culture of transformed explants (Salomon and Puchta, 1998; Shukla *et al.*, 2009; Sun *et al.*, 2006) and protoplast transformation, several other methods have been shown to be functional for the delivery of transient nucleases. A virus-based expression system has been used in tobacco and *Petunia hybrida* for delivery of ZFNs (Marton *et al.*, 2010). In *Drosophila* (Beumer *et al.*, 2008) and zebrafish (Doyon *et al.*, 2008), mRNA encoding a nuclease was successfully injected into the embryo, and direct protein transfer was

achieved for various mammalian cell types (Gaj *et al.*, 2012).

Plants resulting from protocols for transient expression do not have foreign DNA integrated into their genome. It is therefore questionable whether they may actually be considered as transgenic organisms. The fast evolving development of synthetic nucleases raises the following question: is our current understanding of transgenic organisms still valid (Hartung and Schiemann, this issue)? Transgenic organisms are defined as organisms that carry foreign DNA from another species. Using synthetic nucleases, we are now able to mutate or modify any natural occurring gene in a defined manner. We are able to exclude transgenes created by transient expression or out-crossing. We are also able to select against randomly occurring off-site effects such as off-target mutations or integration of foreign DNA by chance via re-sequencing of entire genomes using 2nd and 3rd generation sequencing platforms. Should consumers be more concerned about plants carrying only single base pair substitutions introduced by a synthetic nuclease than plants mutated heavily by 'classical' breeding programs using genotoxins, which results in undefined genotypes? We hope that future public discussions concerning this issue will take place at a rational level.

Gene targeting

DSB-induced GT presents the following challenge: a synthetic endonuclease and a template for HR-mediated DSB repair must be supplied simultaneously. In the first experiments in tobacco, the ORF of the nuclease as well as the repair template were co-transformed either by *Agrobacterium* transformation (Puchta *et al.*, 1996) or by direct gene transfer (Wright *et al.*, 2005). To achieve GT, a reasonable transformation frequency must be achieved. Even when GT frequencies reach the per cent range, it is necessary to

produce hundreds of transgenic lines. Moreover, the co-transformation of two different DNAs must also work efficiently. While DSB-induced GT was achieved in maize (Shukla *et al.*, 2009) and at low frequency in *Arabidopsis* (de Pater *et al.*, 2013; Qi *et al.*, 2013), many crop plants are barely transformable. The regeneration of transgenic material into fertile plants presents an additional challenge.

To overcome this, a specific type of GT technique was developed: 'in planta' GT should be applicable to all transformable plant species, even if the transformation efficiency is extremely low (Fauser *et al.*, 2012). *In planta* GT relies on the principle that the targeting reaction takes place during plant development. GT occurs *in vivo* in all cells; if it occurs in reproductive tissues, the event will be transferred to the next generation. As a result, clonal seeds containing the GT event may be directly identified and harvested. Indeed, large-scale tissue culture and regeneration become obsolete with this technique. The basic principle of the *in planta* GT technique is shown in Figure 6. GT is achieved by simultaneous induction of one DSB in the target locus and two DSBs in a transgene sequence that harbours the targeting vector. The transgenic DNA is constructed in such a way that it carries a targeting vector with sequences homologous to the target locus, which are flanked by two recognition sites for a custom-made endonuclease that cuts the locus of interest. This vector is activated by excision. GT may be achieved via controlled expression of a single site-specific endonuclease. Although the pilot experiments were performed in *Arabidopsis* with the scorable marker β -glucuronidase and I-SceI, the method should be applicable to any endogenous locus and synthetic nuclease. For various target/donor combinations, up to one GT event per 100 seeds may be recovered (Fauser *et al.*, 2012). The molecular analysis of recombinant lines indicated that, in nearly all cases, HR occurred at both ends of the DSB. Additionally,

(a) Integration of a transgene



(b) Precise modification of the target locus

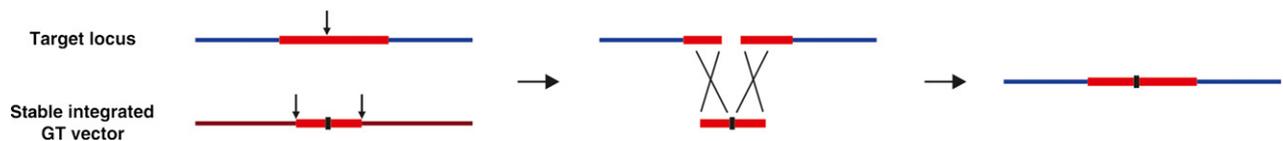


Figure 6. *In planta* GT used for transgene insertion and precise genomic modifications. An expression construct containing a synthetic nuclease that only cuts once within the genome of interest (vertical arrow) and a GT vector are simultaneously or sequentially integrated. The GT cassette harbours at least the GT vector itself and two recognition sites for the nuclease flanking the GT vector. As soon as the nuclease is expressed, cutting occurs at the target site and within the GT cassette. The GT vector is then released and is free to recombine with the DSB at the target locus. The GT vector may either be designed to integrate a transgene (a) or to precisely modify the target locus, e.g. for a pre-determined amino acid exchange (b).

no additional copies of the vector were integrated elsewhere in the genome. This is most likely because only one copy of the target vector is set free per transgene within the genome. The number of unwanted random integration events is therefore minimized in comparison with classical GT approaches, where multiple copies of a vector are often transferred into a single cell. As the transgenic donor locus may be segregated from the targeted integration site, a plant may be obtained that carries only the designed change in the target without any additional transgenic sequences being inserted.

FROM GENOME MODIFICATION TO A SYNTHETIC PLANT GENOME

By use of synthetic nucleases, we are able to introduce subtle changes into plant genomes by initiating natural repair mechanisms. For example, NHEJ may be used for the induction of mutations, and HR allows us to modify any target in a precise manner. Foreign genes may be inserted either via NHEJ or HR into any site of interest that is activated by a DSB. In principle, any synthetic nuclease that induces a unique specific DSB is sufficient for these purposes.

This is obviously not the end of the story. More than one site-specific DSB may be induced simultaneously using artificial nucleases, especially by the CRISPR/Cas system. Thus significant changes in the plant genome are within our reach. Deletions, inversions and the exchange of genomic sequences between chromosomes and chromosome arms are possible in principle. Based on induction of several site-specific DSBs, proof-of-concept experiments for manipulation of plant genomes have already been reported, as described below.

Using two DSBs in more or less close proximity, the sequences between the respective sites may be deleted from the plant genome (Petolino *et al.*, 2010; Siebert and Puchta, 2002). To achieve deletions in the genome, two types of repair reactions may be used. Two broken ends may be joined after elimination of the internal sequence

via NHEJ. Depending on the availability of direct repeats in the genome, annealing of repeated sequences via SSA mechanisms may be used to obtain a deletion with a junction that may be predicted beforehand. A classic application of this technique is removal of selection markers used for transformation. Of course, any type of unwanted natural sequence may be removed from plant genomes in this way. An especially large deletion may result in a lack of viable progeny. When two DSBs are utilized, besides a deletion, inversion of the intervening sequence may be achieved (Figure 7). This has already been demonstrated in mammalian cells (Lee *et al.*, 2012).

In addition to deleting, inverting and inserting sequences, artificial DSBs may also be used to exchange sequences within a plant genome, such as the exchange of chromosome arms (Figure 7). This has been previously demonstrated in tobacco harbouring two unlinked transgenes, each carrying an endonuclease restriction site and parts of kanamycin resistance gene that includes an additional intron. The kanamycin resistance gene was restored by joining two previously unlinked broken ends, either via SSA or NHEJ. Indeed, both types of events were recovered. Despite the fact that no selection was applied for joining of the two ends, the respective linkage was detected in most cases. This demonstrates that the respective exchanges were reciprocal (Pacher *et al.*, 2007). The frequencies obtained indicate that DSB-induced translocation is up to two orders of magnitude more frequent in somatic cells than DSB-induced ectopic gene conversion (Puchta, 1999). The reciprocal exchange of chromosome arms may be achieved via induction of one DSB per chromosome. The exchange of sequences between two chromosomal locations should be possible by inducing four DSBs in total, two at each end of sequences to be exchanged. Although such an experiment has not yet been reported, Weinthal *et al.* (2013) performed a ZFN-induced reaction in which a chromosomal marker flanked by two ZFN recognition sites was replaced by a marker that was flanked by the same recognition sites within a transiently

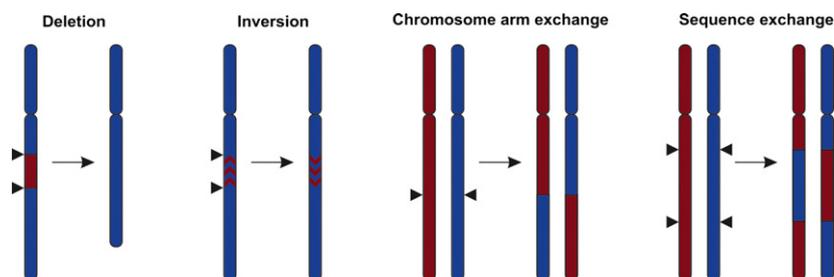


Figure 7. Applications for genomic engineering in plants. The simultaneous induction of two or more DSBs may be used in various methods of genome engineering. Now that different types of synthetic nucleases are available, it should be possible to induce DSBs at almost any site of interest and delete any given genomic sequence (black arrowheads). Induction of two DSBs within a chromosome may lead to deletions or inversions. Induction of two DSBs on different chromosomes may lead to the exchange of chromosome arms. Induction of four DSBs may be used to exchange sequences between chromosomes.

transformed T-DNA molecule. Taking successful establishment of the *in planta* GT technique into account (Fauser *et al.*, 2012), this newly developed NHEJ-mediated gene exchange should not only be applicable to donor sequences on T-DNAs, but also on chromosomes.

The ways that synthetic nucleases may be applied for modification of genomes is growing. Using efficient GT approaches, we are able to introduce multiple genes into a specific genomic region. In future studies, these techniques may make it possible to re-synthesize whole pathways or express multiple genes that behave as a single locus. DSB-induced plant genome engineering may now be combined with the site-specific recombinase technology already established in plants (Wang *et al.*, 2011). It is tempting to speculate that chromosome engineering, and, in the long run, construction of synthetic plant genomes through DSB-mediated manipulation techniques will become a possibility.

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