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Genome editing in plant cells by zinc finger nucleases

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Gene targeting is a powerful tool for functional gene studies. However, only a handful of reports have been published describing the successful targeting of genome sequences in model and crop plants. Gene targeting can be stimulated by induction of double-strand breaks at specific genomic sites. The expression of zinc finger nucleases (ZFNs) can induce genomic double-strand breaks. Indeed, ZFNs have been used to drive the replacement of native DNA sequences with foreign DNA molecules, to mediate the integration of the targeted transgene into native genome sequences, to stimulate the repair of defective transgenes, and as site-specific mutagens in model and crop plant species. This review introduces the principles underlying the use of ZFNs for genome editing, with an emphasis on their recent use for plant research and biotechnology.

Gene targeting in plants: a brief overview

Gene targeting (GT) is one of the most sought after technologies for plant research and biotechnology [1–4]. From a broader perspective, GT can be viewed as any method that can lead to permanent site-specific modification of the plant genome (or the genome of any other species). Developing reliable, efficient and reproducible tools for GT in plant species will have a profound impact on basic plant research and biotechnology because it will potentially accelerate the rate of functional gene analysis and the introduction of novel traits into commercially important crop plants. In the late 1970 s, GT was developed as a powerful tool for functional genetic studies in the budding yeast *Saccharomyces cerevisiae* [5], and was later adapted for various other model organisms and cell lines, including *Drosophila*, various fungal species, mouse embryonic stem cells and human cell lines [6–11]. GT in yeast (and in principle, also in other species, including plants) is made possible by homologous recombination (HR) between a foreign transforming (donor) DNA molecule and a partially homologous sequence on the chromosomal (target) DNA of the target cell (reviewed in Refs [1–3,12]). Nevertheless, the implementation of HR-based GT technology, which relies not only on the design of the donor DNA molecule, but also on the DNA-repair machinery of the target cell, has been difficult, if not impossible, to achieve in plant species because DNA repair is typically effected by non-homologous end joining (NHEJ) and not by HR [13,14]. Indeed, most foreign DNA molecules, which are often delivered by *Agrobacterium*-mediated genetic transformation [15] into plant cells, and are most likely to integrate via NHEJ [16–18], are destined to random rather than site-specific integration.

The fact that plants often incorporate foreign DNA using the NHEJ rather than the HR DNA-repair pathway poses a biological barrier to the development of reproducible and practical tools for efficient recombination between donor and target DNA in plant cells [13]. Efforts have been made to develop methods for GT by either enhancing the rate of HR in plant cells by modifying the plant DNA-repair machinery (e.g. Refs [19–21]), or by devising novel selection schemes for the detection and recovery of the rare HR-dependent GT events in plant cells (e.g. Refs [22–24]). Although these reports showed that HR-dependent GT in model and crop plants is feasible, we have yet to witness the widespread application of such methods with other genes, or other model species and crop plants.

HR-dependent GT can potentially be enhanced by the induction of genomic double-strand breaks (DSBs) in the target cell genome. Expression of naturally occurring rare-cutting restriction enzymes such as I-*Sce*I can induce HR-mediated repair and/or targeting of donor DNA molecules in transgenic plants previously engineered to carry sites for the corresponding rare cutters [25–27]. Moreover, studies have shown that DSBs induced by rare-cutting restriction enzymes can also be repaired via NHEJ, leading to site-specific mutagenesis and the trapping of foreign DNA molecules that share no homology to with the target site [28–30]. Key to the development of DSB-dependent GT technologies in plant cells (or in any species) is the development of novel rare-cutting restriction enzymes capable of inducing genomic DSBs at specific genomic locations. One such class of enzymes are the ZFNs – synthetic restriction enzymes that can be specifically designed to cleave virtually any long stretch of double-stranded DNA sequence (for recent reviews reports see Refs [31–33]). A great deal of effort has been invested in introducing ZFNs as a tool for GT in whole animals and in cell lines, and novel ZFNs have been successfully used for GT experiments in *Drosophila* [34], *Xenopus* [35], *Caenorhabditis elegans* [36], zebrafish [37] and human cell lines [38], among others. ZFN-mediated GT in whole animals, animal cell lines and human cell lines, leads to a variety of outcomes, among them the precise correction of endogenous and artificial genes [38], site-specific mutagenesis [34], gene disruption [37,39], targeted gene addition [40], and even targeting of mitochondrial DNA [41]. By contrast, the

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use of ZFN technology for plant research and biotechnology has been limited. Nevertheless, several key publications have demonstrated the power of ZFNs as a novel tool for genome editing in model and crop plants [42–49]. In this review, we introduce the reader to key reports describing successful GT experiments in plants, while discussing the novel strategies and selection methods deployed in these studies. We next shift our discussion to ZFNs and their use as a novel tool for GT in plants. Methods for the construction of novel ZFNs, assays for their validation in plant cells, and examples of their use for genome editing in model and crop plants are presented.

Homologous recombination-based gene targeting in plants

Various reports have been published during the past two decades on the harnessing of the HR DNA-repair machinery to achieve GT events in plants. In a pioneering work [50], a selection- marker gene repair strategy was used to demonstrate the possibility of obtaining transgenic plants with site-specific modifications of their genome. Transgenic tobacco (*Nicotiana tabacum*) plants were produced that carried a truncated, non-functional, bacterial APH(3′)II gene (coding for neomycin phosphotransferase). Retransformation of these plants with a linearized plasmid construct carrying complementing parts of the truncated selection gene and regions of homology to the target sequence led to HR-mediated restoration of the mutated APH(3′)II, which was monitored by regeneration of kanamycin-resistant plant cells. The GT frequency, estimated relative to random integration of a fully functional APH(3′)II gene, was 0.5×10^{-4} to 4.2×10^{-4}, significantly lower than the frequency of 10^{-2} or more that had been reported for HR-mediated GT in embryonic stem cells [51]. Reconstruction of a defective selection marker in transgenic plants by HR was later used to investigate the suitability of transferred DNA (T-DNA) molecules (which are delivered into plant cells during *Agrobacterium*-mediated genetic transformation, [15] as a substrate for HR-mediated GT in tobacco plants [52]. Here too, the estimated GT frequency was relatively low, in the order of 10^{-4}. Comparably low GT frequencies were also reported when a different selection marker [a mutated hygromycin phosphotransferase gene (*hpt*)] and a different plant species (*Arabidopsis thaliana*) were used in direct DNA transfer experiments [53], which indicated that the nature of the target gene and the transformation method might not affect the rate of GT frequency in these model plants.

Selection-based methods were not confined to targeting a mutated transgene; they were also used to target the genes coding for ALS (acetolactate synthase) in tobacco [54]. There are two ALS-encoding genes in tobacco (*SurA* and *SurB*), and previous data have shown that a single amino acid change in either one of these proteins results in resistance to the herbicide chlorsulfuron [55]. In the study [54], T-DNA molecules encoding a mutated ALS coding sequence were used to demonstrate that it was feasible to obtain HR-mediated GT events, as determined by recovery of chlorsulfuron-resistant tissue, at a rate of 8.4×10^{-5} in tobacco cells.

Disruption of other native sequences that could not be directly selected by resistance to selection agents was also reported [56]. In this study, the TGA3 [a basic leucine zipper (bZIP)-like transcription factor] locus was targeted in *Arabidopsis* plants. For their GT experiments, the authors developed a vector in which a *neo* selection gene was flanked by two genomic regions of the TGA3 locus. Replacement of the bZIP region of TGA3 could potentially be detected by regeneration of kanamycin-resistant calluses from *Agrobacterium*-infected *Arabidopsis* roots. However, kanamycin-resistant calluses could also be obtained following random integration of the GT vector. To distinguish between GT and ectopic insertions, the authors included a GUS-encoding gene outside the region of homology. More than 2500 kanamycin-resistant calluses were produced, leading to the recovery of a single callus line with a confirmed GT event, which demonstrated the feasibility of targeting non-selectable loci in plants. The disruption of another native sequence (the *AGL5* MADS-box gene) in *Arabidopsis* soon followed, this time with the regeneration of a whole mutant plant [57]. Here again, the use of a kanamycin-resistance gene, flanked by regions of homology to the target locus, proved useful for producing transgenic plants with a targeted AGL5. The authors identified a single mutant by PCR-based screening of 750 kanamycin-resistant lines that had been obtained by the flower-dip transformation method. The exact frequency of this HR-mediated GT was not determined. Mutant *Arabidopsis* lines were also obtained by targeting the gene encoding protoporphyrinogen oxidase [58], and mutant rice (*Oryza sativa*) plants were obtained by replacing the ALS-encoding gene with a novel mutant form, rendering them resistant to bispyribac [59]. These studies and several others (e.g. Refs. [60–62]) indicated that the overall range of HR-dependent GT frequency in plant cells is between 10^{-7} and 10^{-5}, which is extremely low in terms of obtaining reliable and reproducible targeting in model plants, and probably also in crop plants (for further reading, see Refs [3,13,63–66]).

Homologous recombination enhancement and strong selection

Two main approaches have been proposed to address the relatively low and variable frequency of HR-dependent GT in plants: modulating the DNA-repair machinery and applying novel and strong selection schemes. The first approach, which was probably inspired by the high efficiency of HR-mediated GT in bacterial and yeast cells, was to modulate plant DNA-repair machinery by expressing heterologous genes or modulating plant DNA-repair genes. In a pioneering work [19], overexpression of RecA (a key recombination gene from *Escherichia coli*) in transgenic tobacco plants was reported to stimulate sister chromatid exchange. Nevertheless, RecA overexpression did not result in significant enhancement of GT in transgenic plants. Similarly, overexpression of RuvC (a protein involved in resolving Holliday junctions in *E. coli*) increased somatic crossovers between genomic sequences, intrachromosomal recombination and extrachromosomal recombination in transgenic plants [67]; whether RuvC can be used to enhance GT in plant cells remains to be

determined. More promising results were obtained by overexpressing *RAD54* from yeast (a member of a yeast superfamily of chromatin-remodeling genes) in *Arabidopsis* plants [21]. Using a unique seed-based GT assay in which GFP fusion protein is activated upon HR-dependent GT of the *Arabidopsis* seed-expressed *cruciferin* gene, an up to two orders of magnitude increase in HR-dependent GT frequency (up to 10^{-1}) was observed in *RAD54*-transgenic *Arabidopsis* [21]. This impressive increase in HR-dependent T-DNA integration resulted in the production of an extremely large number of targeted plants (>550 independent lines) and greatly improved the prospects of using heterologous genes as a means of achieving efficient GT in plants [21].

The plant DNA-repair machinery can also be modulated by silencing or overexpressing native plant genes. Indeed, several reports have shown that DNA-repair mutants and transgenic plants exhibit altered DNA repair and maintenance responses. These include, for example, decreased intrachromosomal HR in MIM mutant plants [which are hypersensitive to methyl methanesulfonate (MMS), irradiation and mitomycin C] [68], increased intrachromosomal HR in MIM-overexpressing plants [20] and increased levels of intrachromosomal HR in *rad50* [69] and chromatin assembly factor 1 (CAF-1) [70] mutants. Nevertheless, whether knocking down or overexpressing these proteins and others will enhance GT in plant species still needs to be examined.

The second approach developed to cope with the low natural frequency of GT by HR was to use strong and novel positive–negative methods for selection of the rare GT events. The positive–negative selection approach was originally developed for research using mouse embryo-derived stem cells as a useful strategy for enhancing the selection of mutants in non-selectable genes [71]. In a positive–negative GT vector, a positive selection marker [e.g. neomycin phosphotransferase II (*nptII*) or *hpt*] is flanked by two regions of homology to the target gene, and copies of a negative selection marker (e.g. *codA* or DT-A) are placed outside the targeting sequence. Applying positive selection allows the detection of both random and GT events, whereas negative selection eliminates the random integration events. A positive–negative selection scheme, using a combination of *nptII* and *codA*, was first used in an attempt to target the *Gln1* and *Pzf* genes in *Lotus japonicus* [72]. The authors developed a series of GT vectors and used them to obtain 185 5-fluorocytosine-resistant calluses from a total of 18,974 primary transformation events. Although they paved the way for the use of the positive–negative strategy for GT in plants, the authors did not provide conclusive evidence of the success of GT events among the 5-fluorocytosine-resistant calluses. In another report, the authors [73] attempted to target the chalcone synthase (CHS) encoding gene and an artificial *htp* gene in *Arabidopsis* cell suspension culture by using a positive–negative GT vector in which a single *codA* was placed next to the T-DNA right border region. Here too, the authors obtained many doubly selected calluses (4379 obtained from 109,475 primary transformants), but no detectable GT events. The high proportion of doubly selected calluses was attributed to deletions of the right-border region of the T-DNA molecule, suggesting that re-engineering the GT vector might assist in overcoming the possible problem of T-DNA truncation during integration.

The first successful report on the use of a positive–negative GT vector for GT in plants involved targeting of the alcohol dehydrogenase (ADH) encoding gene in *Arabidopsis* root culture [74]. Of 6250 transformants, 39 surviving calluses were obtained and a single transgenic line with an ectopic targeted ADH was recovered [74]. The positive–negative strategy has proven useful for HR-dependent GT in rice: *Waxy* and *Adh2* genes were targeted using *hpt* and DT-A [22,23,75]. Several fertile mutant rice plants that were heterozygous at the targeted locus were obtained, and in a recent report, a positive–negative strategy was used for the production of rice plants with knock-in targeting of a methyltransferase (MET1a) encoding gene [24]. Thus, not only do these reports [22–24,74,75] demonstrate that a positive–negative strategy can be used to select for and regenerate transgenic plants with targeted genes, but they also show that this technology can be applied to a crop plant and for the production of mutant lines with true GT events. Nevertheless, although progress has been made in harnessing heterologous genes and novel selection schemes to obtain GT in model and crop plants, genome engineering in plant species remains challenging [3,4,13,63–66,76]. The recent progress made in the use of ZFNs as a tool for inducing DSBs in genomes of animal cells, including human cells, could provide not just an alternative, but also a means complementary to the above-described methods for achieving GT in plant cells.

Enhancement of gene targeting by genomic double-strand breaks

Induction of DSBs at specific genomic locations has been shown to stimulate HR-dependent repair in a wide range of animal and plant species (reviewed in Refs [4,77,78]). Studies have shown that induction of DSBs by X-ray [79] or by excision of transposable elements [80] can greatly enhance intrachromosomal HR in plant cells. DSBs, and by implication the enhancement of HR, can also be induced by expressing naturally occurring rare-cutting endonucleases. Expression of HO and I-*Sce*I endonucleases, for example, has been shown to enhance intrachromosomal HR in tobacco and *Arabidopsis* plants [26,81,82]. Furthermore, expression of I-*Sce*I has been shown to increase extrachromosomal HR by up to 100-fold in plants [83] and by >1000-fold in mammalian and human cells and embryonic stem cells [84–86]. However, DSBs induced by rare-cutting endonucleases, transposable elements and other means are often repaired by NHEJ [13,77] which, in the absence of regions of homology, might lead to site-specific mutagenesis [28]. Furthermore, both I-*Sce*I- and I-*Ceu*I-induced DSBs have led to NHEJ-mediated integration of foreign DNA molecules into the break sites [28–30].

The potential of using rare-cutting endonucleases as a tool for NHEJ-mediated site-specific integration and targeted mutagenesis and for increasing the rate of HR-mediated gene replacement in plants is clear [78,87]. Nevertheless, the limited number of naturally occurring rare-cutting endonucleases and the difficulties involved in

reengineering these enzymes for novel DNA-target specificities [87] greatly limit their use for GT experiments in plants (as well as in other species). ZFNs, which are novel rare-cutting restriction enzymes, could provide a viable alternative to rare-cutting endonucleases for genome editing in plant cells. Indeed, ZFNs have been successfully used for gene replacement and targeted mutagenesis in both scientifically important model plants and economically important crop plants, as we describe below.

Zinc finger nucleases: structure, design and assembly

ZFNs are synthetic restriction enzymes that can be specifically designed to cleave virtually any long stretch of double-stranded DNA sequence (for recent reports see Refs [31–33]). A generic ZFN monomer is made by fusing two domains: an artificially prepared Cys_2-His_2 zinc finger domain and the non-specific DNA cleavage domain of the *Fok*I DNA restriction enzyme. Dimerization of the *Fok*I domain is crucial for its enzymatic activity. Thus, digestion of target DNA can be achieved when two ZFN monomers bind to their respective DNA target sequences and properly align with each other in reverse configuration. The two ZFN monomers are typically designed such that they will flank a 5- to 6-bp-long sequence within the DNA target sequence, allowing the *Fok*I dimer to digest within that spacer sequence. A typical zinc finger domain is composed of 3–4 individual fingers, each capable of recognizing an approximately 3-bp-long sequence. Thus, a heterodimer ZFN, composed of two 9-bp-long DNA-binding domains, will recognize a ~24-bp target sequence that, statistically, will compose a unique site in the *Arabidopsis* genome (and in those of other organisms as well).

Several methods have been described in the literature for the construction of novel ZFNs (reviewed in Refs [88–90]). In the 'modular assembly' method, for example, ZFN DNA-binding domains are assembled from a collection of zinc fingers with known DNA-binding specificities. Several web-based tools [91,92] and archives of zinc finger modules [93–98] have been developed to facilitate the design and assembly of novel ZFNs by this method. Other methods rely on combining the assembly of large random multi-finger libraries with specific screening methods (e.g. phage display) for the detection and isolation of specific DNA-binding domains [99–101]. ZFNs have also been developed by companies (Sangamo BioSciences, www.sangamo.com), and vectors containing corresponding DNA can be purchased (Sigma-Aldrich®; www.sigmaaldrich.com) [89]. More recently, the Oligomerized Pool ENgineering (OPEN) strategy, which involves a collection of zinc finger pools and an in vivo-based selection method, has been developed as a simple and robust 'open source' alternative to the abovementioned methods [45].

Validating and monitoring zinc finger nuclease activity in living cells

Regardless of the method selected for the design and assembly of novel ZFNs, their activity needs to be properly validated and monitored before they are used for GT experiments in target organisms [45,102]. Several assays have been developed to assess the binding and/or digestion activity of novel ZFNs *in vitro* and *in vivo*, which are then used in GT experiments in plants and other species. In one assay, for example, a modified *in vitro* transcription–translation assay and crude protein extracts were used to study the cleavage properties of novel ZFNs [103]. To facilitate the use of their assay, the authors [103] designed protein expression vectors that were suitable for the construction of zinc finger protein-*Fok*I fusions in a single cloning step and used them in conjunction with pUC18 vectors carrying the ZFN target sites as substrates for the *in vitro* digestion assay. The authors used their assay to demonstrate that only specific pairs of ZFNs, and not single monomers, are capable of digesting their corresponding target sequences.

In a different approach, newly assembled zinc finger proteins were validated using a bacterial two-hybrid reporter assay. The assay was part of an extensive set of reagents and protocols developed for the engineering of ZFNs by modular assembly [98]. In their bacterial two-hybrid reporter assay, the binding activity of a newly assembled zinc finger protein can be measured by activation of a *lacZ* reporter gene in live bacterial cells following co-expression of a zinc finger protein-Gal11P and alpha-Gal4 fusion proteins. Here again, the authors developed a comprehensive set of vectors that facilitated the modular assembly of novel zinc finger proteins and the construction of bacterial two-hybrid expression vectors. The authors also developed a set of cloning vectors suitable for the construction of plant and animal ZFN expression vectors from bacterial two-hybrid expression vectors, which are expected to facilitate the use of the validated enzymes in actual targeting experiments.

In a more direct approach to measuring ZFN digestion activity in living cells a *gfp* GT repair assay was developed [85]. In this assay, a mutated *gfp* gene was first integrated into a human embryonic cell line and was then repaired via HR by co-delivery of a ZFN expression plasmid and a *gfp* repair construct. A chromosomal reporter repair system was also developed in yeast, where the intrachromosomal HR-based repair of a chromosomally embedded disrupted alpha galactosidase encoding yeast gene (*MEL1*) could be detected upon transient expression of ZFNs in living yeast cells [39]. This assay was later modified to allow the detection of ZFN- and HR-dependent GT in yeast by activation of a mutated *lacZ* reporter gene [44]. The *gfp* and *MEL1* repair assays were successfully used for the initial assessment of ZFNs developed by Sangamo BioSciences targeting the inositol-1,3,4,5,6-pentakisphosphate 2-kinase (IPK)-encoding gene (*IPK1*) in maize (*Zea mays*) plants [49], and the *lacZ* repair assay was instrumental for analysis of ZFNs developed for targeting the ALS-encoding genes (*SuRA* and *SuRB*) in tobacco using the OPEN system [44]. These experiments indicated that non-plant-based assays can be useful for functional analysis of ZFNs destined for use in GT experiments in plant cells. Nevertheless, expanding the use of ZFN technology for GT to a wide range of plant species that differ in the tissues or organs used for transformation and in their transformation efficiencies could be greatly enhanced by plant-specific assays for ZFN validation and analysis. Indeed, three reporter-repair-based assays (Figure 1) have been developed to measure and assess the activity of ZFNs in model plant species [42,46,48].

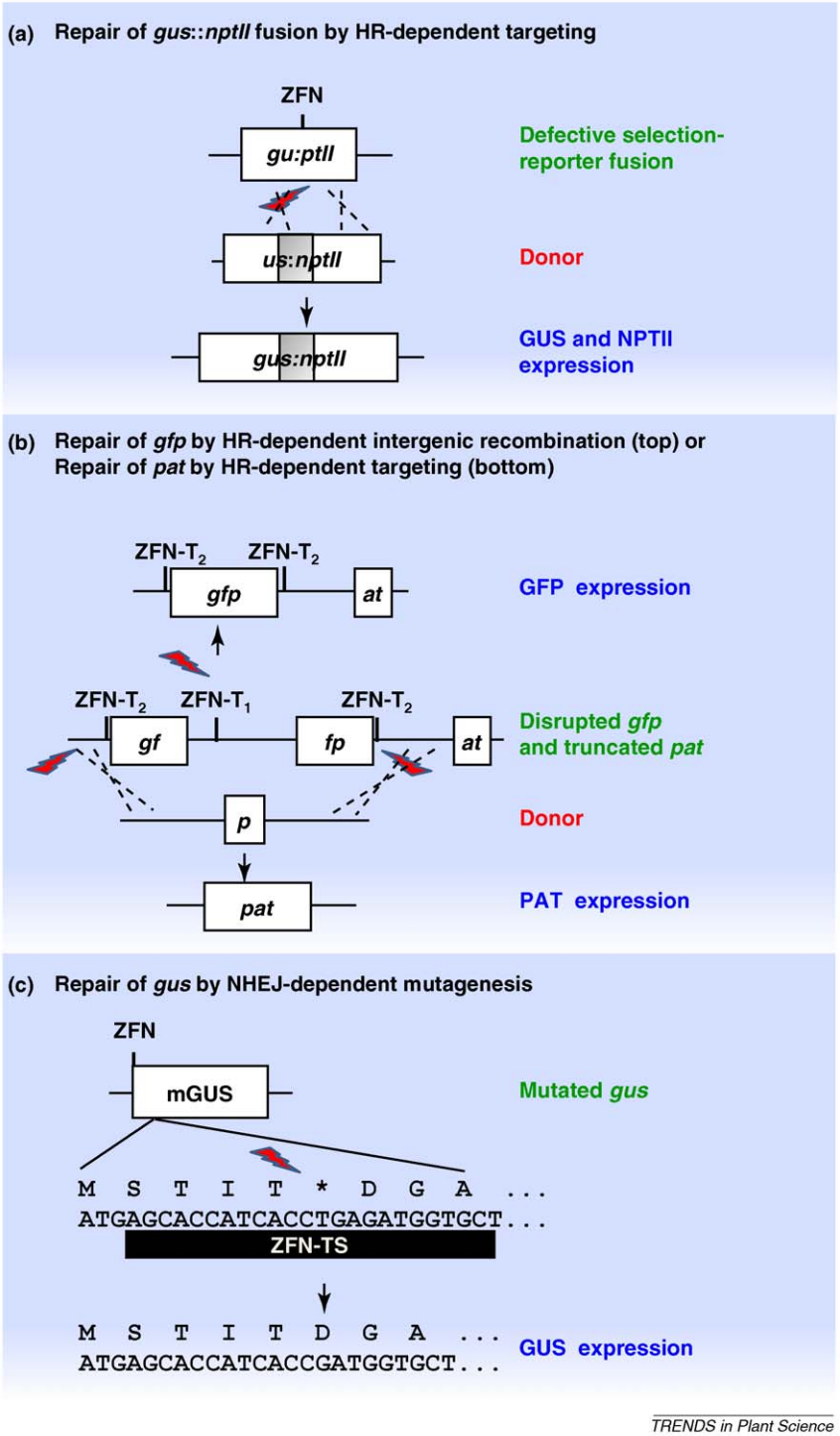

Figure 1. Recombination and transgene-repair assays for the validation and monitoring of zinc finger nuclease (ZFN) activity in plant cells. **(a)** Selectable and screenable transgene-repair assay. ZFN activity is monitored by reconstructing a non-functional *gus::nptII* translational fusion that is missing sections of the GUS and NPTII coding sequences. Target cells are transfected with a donor DNA molecule, and HR-dependent repair of the *gus::nptII*-defective locus gives rise to GUS-expressing and kanamycin-resistant cells. **(b)** Selectable or screenable transgene-repair assay. In this dual-function assay, ZFN1 activity is monitored by GFP expression, which is restored upon recombination between two repeating parts of a disrupted *gfp* coding sequence. ZFN2 activity is monitored by bialaphos resistance upon HR-mediated complementation of the 3′-fragment of the *pat* gene by donor DNA that carries the 5′-fragment of the *pat* gene and regions of homology to the transgene. **(c)** Screenable transgene-repair assay. ZFN activity is monitored by reconstruction of a disrupted *gus* gene by non-homologous end joining (NHEJ)-mediated mutagenesis of a premature stop codon that was engineered within the ZFN target site (ZFN-TS). Assays are described in Refs [42,46,48].

In the first assay (Figure 1a) designed to enable the use of both screenable and selectable phenotypes to measure ZFN-mediated HR in plant cells, a *gus*::*nptII* fusion marker gene, which upon expression in plant cells confers resistance to kanamycin and expression of the GUS visual reporter gene, was used [46]. The GUS::NPTII fusion marker was rendered non-functional by deleting parts of the GUS and NPTII coding sequences and replacing the deleted part with a recognition site to the Zif268 ZFN [104]. A donor DNA molecule, suitable for HR-mediated repair of

the mutated *gus*:*nptII,* was also produced. The donor DNA was identical to a long stretch of DNA on the target sequence, and contained the missing part of the mutated *gus*:*nptII* sequence (Figure 1a). Transgenic tobacco plants carrying the mutated *gus*:*nptII* were produced. Protoplasts from these transgenic plants were electroporated with the donor DNA together with a second DNA molecule carrying a Zif268 ZFN-expression cassette. HR-mediated repair of the mutated *gus*:*nptII* could be measured following the regeneration of kanamycin-resistant and GUS-expressing calluses from the transfected protoplasts. This assay has been useful for estimating the overall GT frequency in several transgenic plants and allows the regeneration of mutant plants from mutated calluses, as described further on. The simplicity of this assay enables its potential use for analysis of ZFN activity in any system that is amenable to co-transformation and regeneration of transgenic tissues.

In a slightly different strategy a second, intrachromosomal reporter gene correction assay (Figure 1b) was developed for validating ZFN activity in the tobacco BY2 cell line [48]. In their assay, the authors constructed a vector that carried two fragments of the *gfp* coding sequence separated by a large heterologous DNA carrying a semi-palindromic ZFN-recognition site. Tandem repeats within the fragmented *gfp* coding sequences served as the substrate for intrachromosomal HR upon digestion of the spacer DNA by ZFNs (e.g. ZFN-1, Figure 1b). Transgenic BY2 cells carrying the *gfp*-repair construct were produced and green fluorescent foci could be observed upon retransformation of these cells with the ZFN-expressing constructs. PCR analysis of *gfp*-expressing tissues indicated that the disrupted *gfp* gene had indeed been corrected by HR-directed repair. The same *gfp*-repair construct also contained two additional identical ZFN sites (e.g. ZFN-2, Figure 1b), which flanked the entire disrupted *gfp* cassette and the 3′-end of the selectable marker phosphinothricin N-acetyltransferase (*pat*). This design enabled not only the monitoring of the intrachromosomal recombinations, but also the detection of HR-mediated site-specific integration following removal of the entire *gfp* construct, as described further on. An advantage of this assay is that it relies on intrachromosomal recombination and thus requires only the delivery of the ZFN-expression vector without the need for a donor DNA to assess the activity of novel ZFNs.

A third reporter gene correction assay that relies on the error-prone NHEJ repair pathway of plants was recently developed for use in plant cells [42]. In this assay, a mutated *gus* gene is engineered to carry a stop codon within the spacer of a ZFN target site (Figure 1c), leading to premature termination of translation of the reporter gene in plant cells. Digestion and misrepair of the DSBs by the NHEJ repair pathway could lead to deletion and/or mutation of the stop codon and reconstruction of the *gus* open reading frame. Indeed, co-delivery of the mutated *gus*-expression cassette with a ZFN-expression cassette from a single T-DNA molecule led to GUS expression in transiently infected tobacco leaves. One advantage of this assay is that it does not require the regeneration of transgenic tissues and thus can potentially be applied to every plant species that is amenable to *Agrobacterium*-mediated transient transformation. In addition, it relies on the use of the GUS reporter gene, which is sensitive and useful for monitoring gene expression in a wide number of plant species [105,106]. However, the digestion of T-DNA molecules might not correspond to the ability of ZFNs to target genomically embedded sequences. A second assay was therefore developed in which the mutated *gus* gene is first used in stable transformation and regeneration of transgenic tobacco calluses, and successive infection of the transgenic tissues with the ZFN-expression cassette then enables ZFN activity to be evaluated against integrated DNA molecules [42].

Building the components of the mutated *gus* reporter repair assay into a flexible and versatile plant-transformation assembly system [107] enabled its use to be expanded to include analysis of ZFN activity in whole plants [42]. Transformation vectors were constructed to carry two constitutive expression cassettes for the mutated *gus* reporter and the plant selection gene and a third, heat-shock-inducible ZFN-expression cassette. Transgenic *Arabidopsis* plants carrying all three independent cassettes were produced and their induction by heat shock allowed the detection of ZFN activity in different organs and developmental stages [42]. It also allowed the recovery of mutated *Arabidopsis* seedlings as we describe further on. The ability to customize the above described whole plant mutated GUS repair assay with different promoters and inducible systems should potentially facilitate its use for analyzing ZFN activity in other model and crop plants.

Relying on the error-prone NHEJ repair pathway as a tool for evaluating ZFN activity in living cells was also instrumental during the development of the OPEN system, where disruption of a chromosomally embedded *egfp* gene was used to evaluate the activity of several ZFNs by monitoring decreases in EGFP expression in human cell cultures [45]. Monitoring ZFN-mediated mutagenesis by NHEJ can also be achieved by direct molecular analysis. The CEL 1 nuclease mismatch assay, for example (Figure 2a), allows the detection of NHEJ-mediated misrepaired alleles and has been used to analyze the various ZFNs used in GT experiments in animal cells, including human cell lines [45,108–110].

Direct molecular analysis was also used in the pioneered study on targeted mutagenesis using ZFNs in *Arabidopsis* [47]. The authors devised a strategy to detect ZFN-mediated mutations within an artificial target site that was incorporated into the genome of *Arabidopsis* plants. To this end, the authors incorporated an *Eco*RI-recognition site into the ZFN target site that allowed enriching for plant DNA molecules lacking this site by digestion of total plant DNA with *Eco*RI before its analysis by PCR and DNA sequencing (Figure 2b). This is a powerful method for manual selection of putative mutated *Arabidopsis* plants and for estimating the overall efficiency of ZFN-mediated targeted mutagenesis in this model plant [47]. A similar strategy was also used to detect single-nucleotide changes that could be responsible for reactivating GUS expression in the mutated *gus* reporter repair assay [42].

High-throughput sequencing technologies have also been used as a tool for rapid measurement of ZFN activity in large populations of plant cells. Pyrosequencing was

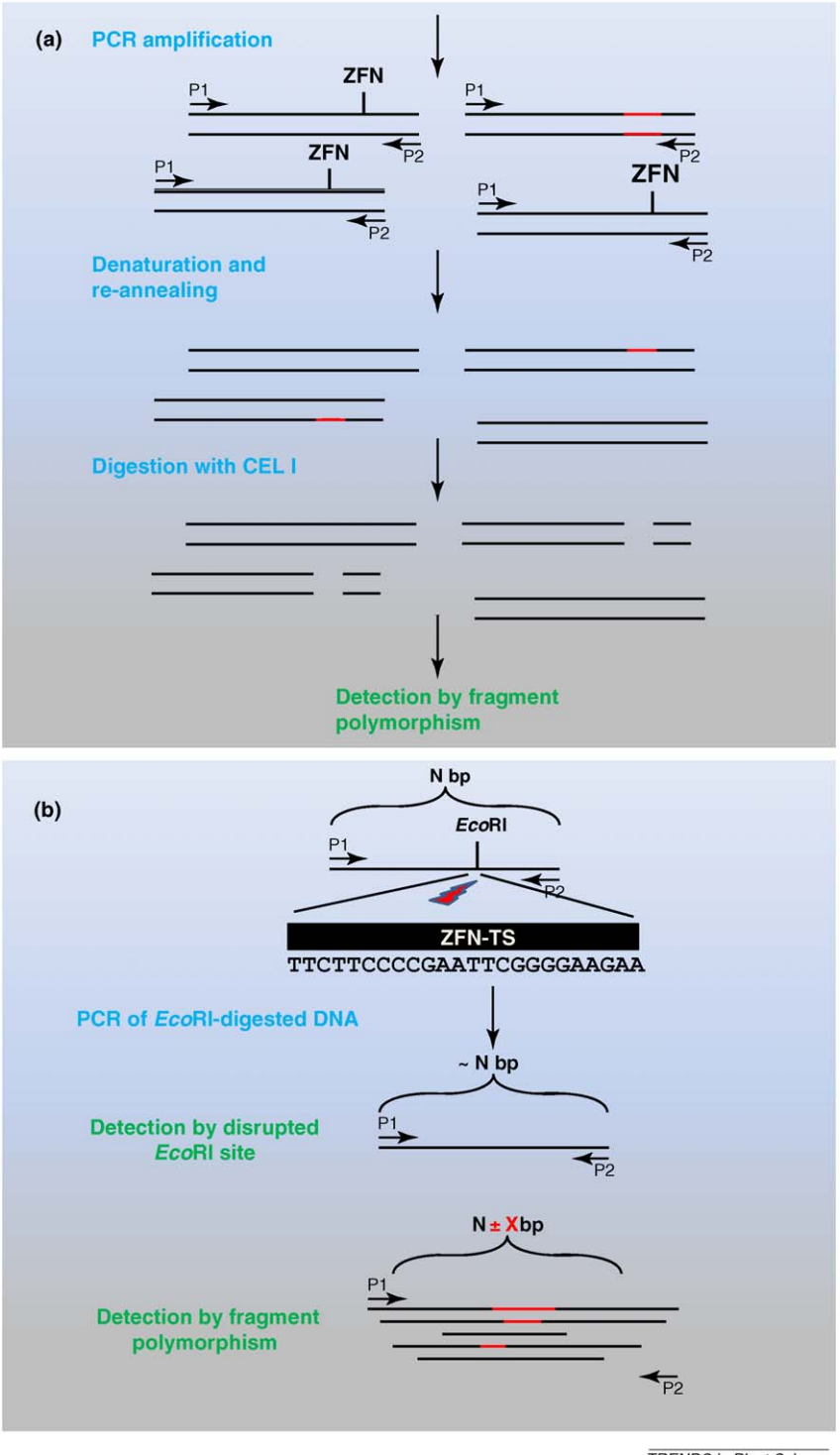

Figure 2. Molecular assays for validating and monitoring zinc finger nuclease (ZFN) activity in living cells. **(a)** CEL I endonuclease assay. ZFN activity is detected by PCR amplification of ZFN-treated plant cells and detection of wild-type::mutant hybrid DNA molecules by CEL I digestion. **(b)** Detection of fragment polymorphism by PCR amplification of digested ZFN-treated plant DNA. In this assay, ZFN activity is detected by PCR amplification of *Eco*RI-digested total DNA, and cloning and sequencing of *Eco*RI-insensitive products that arise from elimination or disruption of the *Eco*RI site, which was engineered within the ZFN target site (ZFN-TS). Assays are described in Refs [45,47].

used to analyze the functionality of several ZFNs that were developed to target the *SuRB* and *SuRA* genes in tobacco [44]. Tobacco protoplasts were infected with ZFN-expression constructs, pooled together, and their *SuRB* and *SuRA* genes were PCR-amplified and pyrosequenced. Because NHEJ-mediated repair of DSBs often results in insertions and/or deletions at the break site, size polymorphism could be detected in the sequencing results. The authors [44] used this technique to compare the targeting efficiency of ZFN pairs that were designed by modular assembly and by the OPEN system, and to select ZFNs that would later be used for HR-dependent co-targeting of the two *SuR* genes

in tobacco, as described further on. Pyrosequencing was also used to validate the activity of ZFNs (which were previously assayed by the mammalian GFP repair assay [85] and the yeast *MEL1* gene repair assay [39]) in cultured maize cells [49]. Taken together, the above tools and assays, as well as the construction and validation services provided by some companies (e.g. Sigma-Aldrich), could potentially facilitate the construction and validation of novel ZFNs for GT experiments in different model and crop plants.

Plant genome editing by zinc finger nucleases

Different strategies can potentially be used when using ZFNs to modify the genome of plant species. Targeting a given native genome sequence (which is not typically composed of a palindrome-like sequence) requires the delivery and expression of at least two ZFN monomers in the same cell (Figure 3). ZFN expression can lead not only to site-specific mutagenesis, but also to gene stacking and/or gene replacement, depending on the presence and structure of the donor DNA and the plant DNA-repair machinery (Figure 3). The use of ZFNs as site-specific mutagens was initially tested in transgenic *Arabidopsis* plants which were engineered to carry a semi-palindromic target site for a well-defined QQR ZFN [35,47]. Expression of the QQR coding sequence was controlled by a heat-shock promoter and site-specific mutagenesis was monitored by PCR (Figure 2b). The controlled expression of the QQR ZFN by heat induction enabled restricting its expression to specific developmental stages in the transgenic *Arabidopsis* plants. Ten-day-old T_2 plants from single-locus lines were heat-shocked and analyzed for the presence, type and transmission of QQR-induced mutations. PCR amplification of *Eco*RI-digested DNA from the heat-shocked plants revealed that QQR did indeed induce site-specific mutations within its target site. By calculating the number of DNA molecules with disrupted *Eco*RI that were amplified from undigested plant DNA, the authors also estimated that QQR, when expressed under a heat-shock promoter, can induce mutations at a frequency as high as 0.2 mutations per gene. This is a high number relative to the extremely low HR-dependent frequencies (i.e. 10^{-7} to 10^{-4}) previously reported for plant cells (reviewed in Ref [63]).

Further analysis of mutated target sites revealed that most of the QQR-induced mutations could potentially produce a functional gene knockout (if generated within a coding sequence), and that nearly 10% of the T_2 heat-shock-induced plants transmitted the QQR-induced mutations to their progeny (probably as a result of mutations in the L2 cells of the shoot apical meristem). These observations suggest that the heat-shock-controlled expression of ZFNs could potentially be adapted for efficient generation of functional gene knockout in *Arabidopsis*.

Heat-shock-induced expression was also instrumental in the development of a set of tools for the characterization and expression of ZFNs in plant cells [42,111], where the mutagenic effect of QQR was measured by activation of a mutated *gus* gene in transgenic *Arabidopsis* plants (Figure 1c). The *gus* reporter activation assay offers a simplified visual alternative to PCR-based detection of ZFN-mediated mutagenesis and has proved useful for visualizing ZFN activity in various organs of transgenic *Arabidopsis* plants and in transformed tobacco tissues [42]. Placing the ZFN recognition site three codons upstream of the *gus* initiation codon might hinder the detection of large deletions and off-frame mutations; nevertheless, the assay was sensitive enough to detect the occurrence of small, in-frame deletions and insertions. More importantly, it was also useful for detecting single-nucleotide replacement at the break site [42], suggesting that ZFNs can also be used for precise and localized genome editing in plant cells.

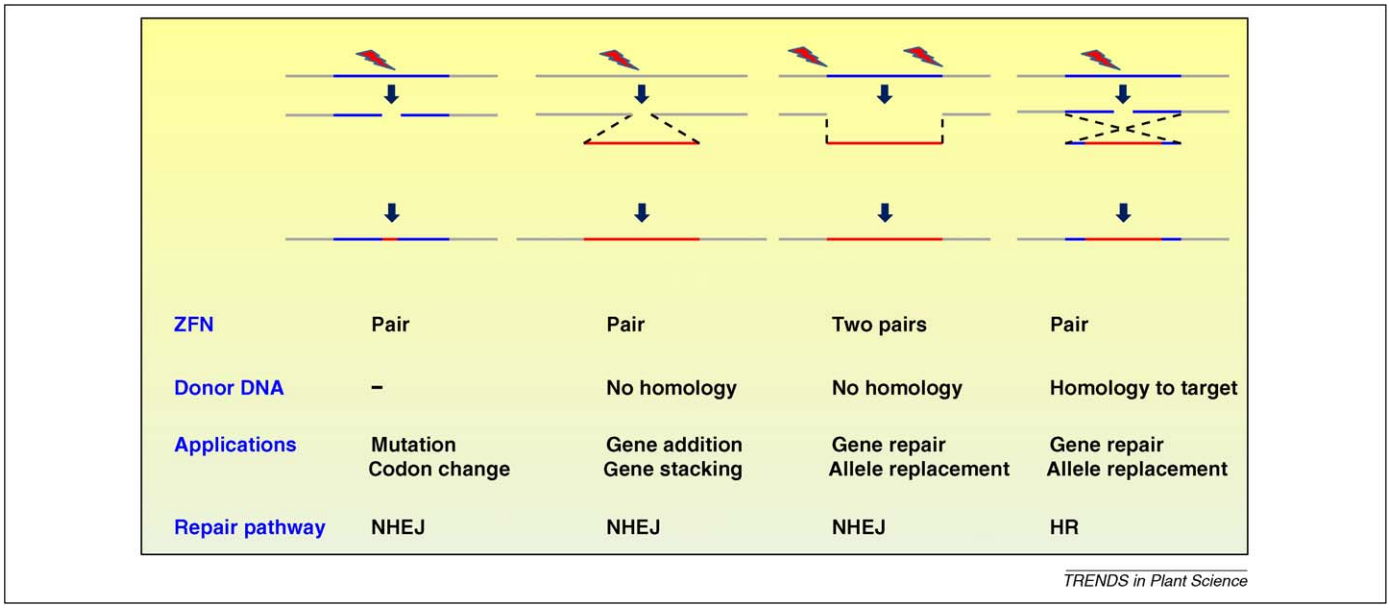

Figure 3. Strategies for zinc finger nuclease (ZFN)-mediated gene targeting (GT) in plant cells. Expression of a single or double pair of ZFNs can lead to one or two double-strand breaks (DSBs) in the target genome. Two types of donor DNA molecules (with or without homology to the target site) can be driven to integration by non-homologous end joining (NHEJ) or homologous recombination (HR) and can potentially be used to achieve a variety of outcomes: gene addition, gene stacking, gene repair and allele replacement. In the absence of donor DNA, NHEJ-driven repair of the break site can lead to site-specific mutagenesis.

Single-nucleotide changes and single- and double-codon deletions were also reported in a study aimed at evaluating the use of ZFNs for GT in *Arabidopsis* using flower-dip transformation (the most commonly used transformation method for *Arabidopsis* plants [43]). A transgenic *Arabidopsis* line with a single target locus was produced. The target locus was engineered to express the phosphinothricin acetyltransferase (encoded by *pat*) selection marker, the *gfp* reporter gene, a unique recognition site for two ZFN monomers (designated PTFFOK and E2CFOK) and an *Eco*RI restriction site that facilitated the isolation of mutated genomic fragments from transgenic *Arabidopsis* plants by PCR amplification (Figure 2b). Target transgenic plants were subsequently transformed with the PTFFOK and E2CFOK ZFNs, which were driven by various promoters. Tissue-specific, chemically induced and constitutive expression of the PTFFOK and E2CFOK ZFNs resulted in a wide variety of mutations that ranged from single-nucleotide changes and single- and double-codon deletions to large deletions and/or insertions [43]. Interestingly, the authors reported that co-expression of PTFFOK and E2CFOK under the control of the constitutive CaMV 35S promoter resulted in mutations in only 2% of the cells of the doubly transformed plants and that these plants did not exhibit an aberrant phenotype, as one would expect from overexpression of ZFN in transgenic tissues [43]. The exact cause for this relatively low mutation efficiency is still unknown, but it can potentially be attributed to transgene silencing, ZFN instability, rapid repair of the genomic DSBs or the specific genomic position of the PTFFOK/E2CFOK target site. Nevertheless, it led the authors to suggest that a third round of transformation with a donor DNA might result in HR-mediated GT events at unaffected sites. Realizing that expression of the PTFFOK and E2CFOK ZFNs under the control of the Rps5 promoter (which is active in dividing cells and early embryos) resulted in an even lower rate of mutated cells as compared with the 35S promoter, the authors used Rps5-ZFN-transgenic plants, which were homozygous for the target locus, for their HR-dependent GT experiments [43]. The donor DNA was engineered with partial homology to the target locus and carried a functional *hpt* selection cassette, which allowed the detection of both random and putative GT events following its delivery by flower-dip transformation into Rps5-ZFN/target-transgenic *Arabidopsis*. Over 3000 hygromycin-resistant transgenic *Arabidopsis* plants were produced and screened by PCR for possible GT events. Three targeting events were isolated and characterized. Two of them were classified as 'true' GT events and they were likely the result of HR between the donor and the target locus. A third event was classified as an ectopic GT event and it was suggested to be derived from HR between the donor and target locus followed by release and transfer of the recombinant T-DNA to a different genomic location. Interestingly, all three targeted lines remained heterozygous for the targeted locus. They also carried randomly integrated full and/or partial T-DNA molecules within their genomes. Nevertheless, given the high GT frequency of 10^{-3} and the possibility of segregating out the randomly integrated T-DNAs and the ZFN-expressing cassettes, this study demonstrated that a combination of stably expressed ZFNs and *Agrobacterium*-mediated floral-dip transformation can potentially be used not only for site-specific mutagenesis but also for HR-based gene-replacement experiments in *Arabidopsis*.

HR-dependent GT has been achieved in other plant species as well (i.e. tobacco and maize). In a pioneering work, targeted tobacco plants regenerated, following the co-delivery of donor and ZFN-expressing DNA molecules, into transgenic tobacco protoplasts [98]. More specifically, 12 transgenic tobacco plants were produced carrying a non-functional *gus::nptII* fusion marker (Figure 1a) that also carried a recognition site for the ZFN Zif268 at different genomic locations. One double-locus- and nine single-locus-transgenic plants with relativity high levels of *gus::nptII* transcript (as determined by reverse transcription-PCR) were selected for further GT experiments. Transgenic protoplasts were co-transformed with Zif268-expressing and donor DNA molecules, and were cultured to recover and regenerate kanamycin-resistant calluses and whole plants that were subsequently tested for GUS expression. Interestingly, the average frequency of GUS-expressing calluses (1.0×10^{-3}) was lower than that of the kanamycin-resistant calluses (4.5×10^{-3}). This discrepancy was explained by the possible low sensitivity of the histochemical GUS assay relative to antibiotic selection pressure. Furthermore, kanamycin-resistant callus was also detected when protoplasts were transformed with only donor DNA, albeit at a much lower frequency than the accepted rate of regular transformation experiments (which was about 14-fold higher). Regeneration of such kanamycin-resistant (but GUS-negative) calluses might have resulted from random promoter trapping by the donor DNA, which even though engineered to carry only 457 bp of the *gus* coding sequence, still carried a complete *nptII* coding sequence. More important was the observation that the frequency of the kanamycin-resistant calluses was more than threefold higher when Zif268-expressing and donor DNA were co-transformed compared with when only donor DNA was used for transformation. Further calculation led to the estimation that 1–2 of every 10 doubly transformed protoplasts resulted in a HR event and that on average there was 1 HR event per 5.9 NHEJ events.

HR-dependent GT events were observed in all of the different target transgenic tobacco lines, which indicated that various chromosomal sites are amenable to targeting in tobacco protoplasts. Interestingly, the frequency of GT did not correlate with the target gene expression, which suggests that similar to the randomness of NHEJ-mediated T-DNA integration [16,112], various chromosomal locations might be amenable to GT by HR. PCR and Southern blot analyses were performed on selected targeting events derived from three parent lines and revealed a variety of targeting and random T-DNA integration events. Thus, for example, the targeting of not only one but both alleles in a homozygous target plant, the random integration of not only the donor but also the ZFN-expressing T-DNA molecule, and genomic rearrangements at some of the targeting sites, were observed. The mechanisms by which such rearrangements occur are still unclear, but it could be that such events were initiated by strand invasion from one side of the T-DNA but resolved by NHEJ

on the other. Overall, out of 26 GT events, five exhibited accurate HR-mediated gene-replacement events, bringing the efficiency of precise HR-dependent GT in tobacco protoplasts to 20% of the total targeting events.

HR-mediated GT was also reported in BY2 tobacco cells, where a targeted transgene was directed into not only the pre-integrated defective reporter construct, but also an endogenous locus in tobacco cells [48]. For targeting of a transgenic locus, BY2 tobacco cells were engineered to carry a disabled *gfp* gene (Figure 1b). Reconstitution of functional *gfp* by ZFN-T_1-mediated chromosomal recombination indicated that ZFNs can facilitate HR in transgenic BY2 cells (Figure 1b). HR-dependent GT and replacement of the disrupted *gfp* with a functional *pat* expression cassette was achieved by co-transfection of the ZFN-expression construct and a donor DNA that shared homology to the transgene target sequence and carried a plant promoter and the 5′-fragment of the *pat* gene. Digestion of either two (ZFN-T_2) or just one (ZFN-T_1) location within the target site (Figure 1b) resulted in successful recombination between the donor DNA and the target, and allowed the regeneration of bialaphos-resistant cells that no longer carried the disrupted *gfp* transgene. Sequencing analysis revealed precise recombination between the 5′- and 3′-parts of the *pat* gene, and Southern blot analysis showed that 18 out of 22 bialaphos-resistant isolates exhibited a hybridization pattern consistent with accurate HR-dependent gene-replacement events.

Co-transfection of ZFN-expression construct and a donor DNA was also used to target a native genomic sequence in BY2 cells and tobacco plants. The endochitinase gene *CHN50* was selected because of its high expression level in stationary-phase tobacco cell suspension culture, which led the authors [48] to suggest that methods for its replacement with genes of interest can be used for the production of recombinant proteins in tobacco cell culture. A pair of CHN50-specific ZFNs targeting the 3′-end of the second exon of the gene were designed and constructed by Sangamo BioSciences (designated ZFN-CHN50-L and ZFN-CHN50-R), and a dual ZFN monomer-expression cassette was assembled by flanking the 2A sequence [48] with the ZFN-CHN50-L and ZFN-CHN50-R coding sequences. The donor DNA molecule was engineered to carry a functional *pat* gene driven by a constitutive promoter that was flanked by 750 bp of sequence homologous to *CHN50*. GT experiments were performed by *Agrobacterium*-mediated co-transformation of BY2 cells and tobacco leaf disks. PCR analysis revealed that 12 of 231 bialaphos-resistant BY2 isolates and five of 46 bialaphos-resistant transgenic plants produced DNA fragments that would be expected from true targeting events. Sequence analyses confirmed the nature of the HR-mediated integration of the donor DNA into the *CHN50* target site. Although Southern blot analysis of several targeted bialaphos-resistant tissues revealed that they also carried randomly integrated donor DNA, the data presented by the authors clearly showed that ZFNs can be used for HR-mediated GT in a native tobacco locus.

Native genome editing in tobacco cells, albeit via NHEJ, was recently reported as part of the development of the OPEN system, used to construct ZFNs for targeting native sequences in human cell lines and in tobacco plants [45]. A pair of ZFNs, designated SR2163 ZFNs, capable of targeting position 2163 on both copies of the ALS-encoding genes in tobacco (*SuRA* and *SuRB*) were constructed and delivered into tobacco protoplasts together with a construct coding for *nptII* expression: 66 kanamycin-resistant plantlets were obtained, three of which exhibited a single-base deletion within the *SuRA* coding sequence, as determined by PCR and sequencing analysis. One plant was mutated in both *SuRA* alleles, and no mutants in *SuRB* were detected.

Targeting of the *SuRB* allele was later achieved by using a different set of ZFNs that were designed to target positions 815 and 1853 of the coding sequences of the *SuR* genes and were designated ZFN815 and ZFN1853, respectively [44]. High-throughput pyrosequencing of DNA isolated from tobacco protoplasts transfected with ZFN815 or ZFN1853 revealed that both *SuR* genes were mutagenized by these enzymes. Interestingly, ZFN815, which was specifically designed by modular assembly to target the *SuRB* locus, targeted *SuRA* with higher frequency, even though the target site on *SuRA* differed by two nucleotides from that on *SuRB*. This observation indicated that not only was ZFN815 less specific, but also that other factors (e.g. chromatin structure and DNA methylation) might be affecting the accessibility and activity of ZFNs on their target sites.

The ability of ZFN815 to stimulate HR-mediated GT to *SuR* loci was investigated using various types of donor DNA molecules that were engineered to carry missense mutations that conferred resistance to different herbicides, as well as silent mutations that enabled spontaneous mutations to be distinguished from recombination-generated mutations in putative targeting events. Co-delivery of donor DNA with ZFN815 into tobacco protoplasts resulted in the regeneration of herbicide-resistant calluses for all three types of donor DNA molecules, with an estimated rate of ZFN-induced herbicide resistance of 2.4–5.3%. PCR and sequence analyses of randomly selected resistant calluses revealed that 0.2–4.0% of the targeting events resulted from HR-dependent recombination between donor DNA molecules and *SuR* loci, and that the remainder resulted from spontaneous mutations and/or somatic variations. Interestingly, >2% of the GT events were obtained at a distance of >1300 bp from the ZFN cleavage site, which suggests that GT can potentially be obtained even if ZFNs cannot be designed for the desired site. This notion is further supported by observations of GT events being obtained even at a distance of 3000 bp from the ZFN-induced DSBs [48].

Overall, the high GT frequency of both *SuR* alleles when ZFN815 and donor DNA molecules were co-delivered into tobacco protoplasts was consistent with the pyrosequencing data. These observations demonstrated that a pair of ZFNs, capable of targeting two slightly different sequences, can potentially be used to target several closely related genes across plant genomes. Indeed, among 47 different herbicide-resistant calluses produced via different targeting experiments (which included the use of other ZFNs and donor DNA molecules), 19 were modified at multiple *SuRA* loci (including mutations induced by

NHEJ). Furthermore, 10 *SuRA* and *SuRB* double mutants regenerated from herbicide-resistant calluses, clearly demonstrating that ZFNs can be used to engineer whole plants.

Targeting of native genome sequences cannot always rely on direct selection of targeting events, and given the high frequency of *SuRA* and *SuRB* targeting, the authors [44] suggested that indirect selection for putative targeting events could be applied for GT in tobacco. To test this idea, tobacco protoplasts were co-transformed with plasmids encoding ZFN815, a donor DNA and a functional kanamycin-resistance-encoding cassette. Of approximately 1000 kanamycin-resistant cells, two were modified by the donor DNA and were resistant to herbicide. A similar approach, using PCR-based screening, was taken for the detection of GT events among a population of >3000 transgenic *Arabidopsis* plants [43]. These experiments showed that indirect selection methods and/or molecular screening can potentially be harnessed for the identification of ZFN-mediated GT events in non-selectable target genes in plant genomes.

ZFN-mediated GT is not limited to model plants, and in a recent report [49] the technology was used to produce mutated maize plants with an altered phytate-biosynthesis pathway. The *IPK1* gene, which encodes the enzyme that catalyzes the final step in phytate biosynthesis, was selected for targeting experiments. *IPK1*, one of two *IPK* paralogs in maize that share 98% sequence identity in their coding sequences (the second is referred to as *IPK2*), was selected for targeting based on its expression pattern. Four ZFN pairs (designated ZFN8, ZFN12, ZFN15 and ZFN16) were designed to target a specific region in exon 2 of *IPK1*. Initial evaluation of ZFN-mediated GT in maize cell culture revealed that transient expression of ZFN12 leads to site-specific mutagenesis (i.e. deletions or insertions) at the target sites, as determined by deep sequencing analysis. Two donor DNA molecules carrying the *pat* selectable marker and regions of homology to the *IPK1* target site were constructed. The first molecule, designated the autonomous donor, carried a fully functional *pat*-expression cassette, whereas the second molecule, designated the non-autonomous donor, carried a promoterless *pat* and hence required the *IPK1* promoter to be trapped in a successful GT event. All *IPK1* ZFNs were capable of inducing site-specific integration of either the autonomous or non-autonomous donor into maize cell culture, as determined by the regeneration of herbicide-resistant calluses. Molecular analysis revealed insertion in both, or just one of the *IPK1* alleles in herbicide-resistant calluses and sequencing data confirmed the HR-mediated nature of the insertion events. However, even though the non-autonomous donor yielded a lower number of herbicide-resistant events than the autonomous donor, the frequency of targeted events (out of the total targeted and random integration events) obtained with the non-autonomous donor was higher than that with the autonomous donor. More interesting was the observation that whereas multiple random integration events were observed in genomes of calluses obtained from non-targeted events (i.e. most likely to be derived from integration by NHEJ), a single, site-specific integration of the *pat* gene was observed in calluses obtained from targeted events (i.e. those derived from integration by HR). Although the exact mechanism by which donor DNA molecules are preferentially directed to HR and not NHEJ in maize cell culture is still unknown, it is clear that ZFNs can be used to control the integration of foreign DNA into specific locations without the addition of foreign DNA molecules into random genomic sites.

ZFN-mediated genome modification can potentially lead to off-site targeting [108] and a full analysis was conducted to examine whether *IPK1*-targeted plants, which regenerated from mutated maize calluses, carried potential off-target genome modifications. Interestingly, sequence analysis revealed that *IPK2* remained intact in five independent *IPK1*-targeted plants, and although the possibility that *IPK2* was digested, but was then accurately repaired cannot be ruled out, these data demonstrate that ZFNs can be designed and used to target their intended paralog. Furthermore, SELEX/genotyping analysis, a method that has been successfully used to indentify rare off-target genome-modification events in human cells [113], revealed that the five most probable off-target ZFN binding sites in the maize genome were true to type.

Multiple independent fertile maize plants were recovered and were either self-pollinated or crossed to an inbred maize variety [49]. Genotyping and segregation analysis of progeny from various crosses revealed that the *IPK1* mutant allele segregated in a Mendelian fashion and was transmitted via sexual reproduction. Furthermore, biochemical analysis revealed that a significant number of seeds obtained from segregating populations of *IPK1*-mutant lines exhibit high phytate accumulation and low inorganic phosphate accumulation, as would be expected from reduction or disruption of the *IPK1*-encoded inositol-1,3,4,5,6-pentakisphosphate 2-kinase enzyme. Taken together, this pioneering report [49] clearly demonstrates that ZFNs can be used for precise genome modification of not only model plants, but also those that are economically important.

Future prospects

Reliable and reproducible precise genome-modification methods have eluded plant biologists and biotechnologists for many years. However, the remarkable progress made in the use of ZFNs for genome editing in animals, animal cells and human cells (see reviews in Refs [31,78,88,114]), and their recent application for precise editing of model and crop plants, heralds the dawn of a new era for basic plant research and biotechnology. Extensive research is required to extend the use of ZFN technology beyond proof-of-concept studies to the targeting of a wide range of native sequences in a variety of model and crop species. The application of ZFN technology to other plant species and target sequences relies not only on the development of novel ZFNs but also on the adaptation and modification of existing tools and methods for ZFN delivery, expression and validation in plant cells and on the design of strategies for the identification and selection of putative targeting events.

Obtaining functionally active ZFNs is a key step in the deployment of this technology for any GT experiment.

Prospective users can potentially construct their own enzymes, in house, using publicly available information and reagents [33,45,92,93,97,98,103]. Both modular assembly and the OPEN system have yielded enzymes that have been used in various eukaryotic cells, including plants, and although the OPEN system is more technically challenging and demanding, its reliance on selection processes is proposed to yield enzymes with higher activity compared with enzymes yielded using modular assembly [44,45,115]. Users who plan to construct their own enzymes are advised to choose the method that is best suited to their research [116,117]. ZFNs are also commercially available from Sigma-Aldrich, but as pointed out by Porteus [4], their market is restricted to laboratories conducting animal research due to licensing agreements with Sangamo BioSciences and Dow Agrosciences (www.dowagro.com). Users can also seek to draw up an agreement with Sangamo BioSciences and Dow Agrosciences to obtain functionally active ZFNs from their laboratories.

A wide range of validation methods have been developed to assist with the screening process for active ZFNs. Plant biologists can adapt existing methods and analyze the activity of novel ZFN monomers by activation of reporter and/or selection genes, by PCR and/or by deep sequencing of transgenic and/or native target sequences. However, the co-delivery of two ZFN monomers, with or without donor DNA, into the same target cell, can be technically challenging in different plant species, where the transformation efficiency of several independent molecules (often delivered by *Agrobacterium*-mediated genetic transformation) is highly variable and typically low [118–123]. Launching the donor sequence and the ZFN-expressing cassettes from a single vector has been shown to be useful for GT experiments in plants [49], and although we cannot preclude the possibility that two DNA molecules were actually present in the same transformed cell, this strategy can potentially be used as an alternative for co-transformation experiments. Alternatively, combining the use of ZFN-expressing transgenic plants with a second transformation cycle with a donor DNA has also been shown to be useful for GT experiments in plants [43]. The availability of an assortment of vectors [118,119,124,125], some of which have been designed for maximum compatibility with a generalized plant-expression system [42,118,126], could potentially facilitate the assembly of multi-ZFN plant transformation and donor vectors for GT experiments in these organisms.

Not only is targeting efficiency likely to depend on the activity of the ZFNs, but also on their expression level within the target cell, the location of the targeting sequence in the genome and the developmental and physiological stage of the targeted cell. Interestingly, heat-shock induction and overexpression of ZFNs in transgenic plants have not led to high targeting efficiency in *Arabidopsis*, and detection of rare targeting events in a non-selectable locus required extensive PCR screening [42,43,47]. Improved expression techniques and novel selection methods could result in higher targeting efficiency and more efficient detection of targeting events in plant cells. No less important are a better understanding of the biological processes by which DNA molecules are targeted to integration in plant cells and the potential revelation of factors that contribute to the accessibility of ZFNs to specific genomic locations; these could further contribute to the establishment of reproducible and efficient GT procedures for plant species.

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