ZFN-induced mutagenesis and gene-targeting in Arabidopsis through Agrobacterium-mediated floral dip transformation.

Pater, B.S. de; Neuteboom, L.W.; Pinas, J.E.; Hooykaas, P.J.J.; Zaal, E.J. van der

Citation

Pater, B. S. de, Neuteboom, L. W., Pinas, J. E., Hooykaas, P. J. J., & Zaal, E. J. van der. (2009). ZFN-induced mutagenesis and gene-targeting in Arabidopsis through Agrobacterium-mediated floral dip transformation. *Plant Biotechnology Journal*, 7(8), 821-835. doi:10.1111/j.1467-7652.2009.00446.x

Plant Biotechnology Journal

Plant Biotechnology Journal (2009) **7**, pp. 821–835 doi: 10.1111/j.1467-7652.2009.00446.x

ZFN-induced mutagenesis and gene-targeting in Arabidopsis through *Agrobacterium*-mediated floral dip transformation

Sylvia de Pater*, Leon W. Neuteboom, Johan E. Pinas, Paul J. J. Hooykaas and Bert J. van der Zaal

Department of Molecular and Developmental Genetics, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

Received 2 July 2009;
Revised 23 July 2009;
Accepted 28 July 2009.
**Correspondence* (fax +31 715274900; e-mail b.s.de.pater@biology.leidenuniv.nl)

Summary

Zinc-finger nucleases (ZFNs) are artificial restriction enzymes, custom designed for induction of double-strand breaks (DSBs) at a specific locus. These DSBs may result in site-specific mutagenesis or homologous recombination at the repair site, depending on the DNA repair pathway that is used. These promising techniques for genome engineering were evaluated in Arabidopsis plants using *Agrobacterium*-mediated floral dip transformation. A T-DNA containing the target site for a ZFN pair, that was shown to be active in yeast, was integrated in the Arabidopsis genome. Subsequently, the corresponding pair of ZFN genes was stably integrated in the Arabidopsis genome and ZFN activity was determined by PCR and sequence analysis of the target site. Footprints were obtained in up to 2% of the PCR products, consisting of deletions ranging between 1 and 200 bp and insertions ranging between 1 and 14 bp. We did not observe any toxicity from expression of the ZFNs. In order to obtain ZFN-induced gene-targeting (GT), Arabidopsis plants containing the target site and expressing the ZFN pair were transformed with a T-DNA GT construct. Three GT plants were obtained from ~3000 transformants. Two of these represent heritable true GT events, as determined by PCR, Southern blot analysis and sequencing of the resulting recombined locus. The third plant showed an ectopic GT event. No GT plants were obtained in a comparable number of transformants that did not contain the ZFNs. Our results demonstrate that ZFNs enhance site-specific mutagenesis and gene-targeting of *Agrobacterium* T-DNA constructs delivered through floral dip transformation.

Keywords: Arabidopsis, double-strand break, floral dip transformation, gene-targeting, mutagenesis, zinc-finger nucleases.

Introduction

Genetic modification of plants is now routinely performed. Transformation can be done by various methods and vectors including *Agrobacterium tumefaciens*. It has been observed that transgenes integrate at fairly random positions and in variable copy numbers in the plant genome through non-homologous recombination (NHR). This may cause position effects (like silencing of transgenes) and unintended mutations of genes at the integration site. Therefore, it would be an advantage if integration could be targeted to a specific locus. Such gene-targeting (GT) would also be of great advantage for the modification or inactivation of genes in the plant genome. GT can be achieved by homologous recombination (HR). This process is efficient in yeast but very rare in most higher eukaryotes, like animals and plants. Estimates of GT frequencies in several different plant species vary from 10^{-4} to 10^{-6} (Paszkowski *et al.*, 1988; Lee *et al.*, 1990; Offringa *et al.*, 1990; Halfter *et al.*, 1992; Hrouda and Paszkowski, 1994; Miao and Lam, 1995; Risseeuw *et al.*, 1995; Hanin *et al.*, 2001). Gene-targeting frequency can be increased by the introduction of a targeted DNA double strand break (DSB) near the site of the desired recombination event. In plants, this was demonstrated for the first time through the use of the rare cutting meganuclease I-*Sce*I, resulting in an increase in GT frequency by two orders of magnitude (Puchta *et al.*, 1996). In order to create a DSB at a predetermined site in the genome, zinc-finger nucleases (ZFNs) are rapidly emerging as the tools of choice (Porteus and Carroll, 2005; Camenisch *et al.*, 2008). The current generation of ZFNs combines the nonspecific cleavage domain of the *Fok*I restriction enzyme and a specific DNA binding domain with several C_2H_2 zinc-fingers (ZFs) to provide cleavage specificity. Efficient cleavage of the target site requires dimerization of the *Fok*I cleavage domain (Bitinaite *et al.*, 1998; Smith *et al.*, 2000; Mani *et al.*, 2005). Therefore, two ZFN subunits are typically designed to recognize the target sequence in a tail-to-tail configuration and the DSB is then introduced within a spacer sequence which is located between the binding sites of the two polydactyl zinc-finger (PZF) domains.

Each individual ZF present within a PZF consists of a stretch of ~30 amino acids, stabilized by a zinc ion that binds a particular three-base DNA sequence (triplet). At the present date, a series of ZF modules has been created for recognition of most of the 64 possible triplets (Sega *et al.*, 1999; Dreier *et al.*, 2001, 2005; Liu *et al.*, 2002). Although not all interactions are robust enough for this purpose, the availability of the ZF lexicon and the recently published ZFN selection method OPEN (Maeder *et al.*, 2008) in principle allows the construction of effective ZFNs. A minimum of three ZFs are required per PZF domain, but more complex PZF domains recognizing longer target sites usually possess increased DNA binding specificity as well as higher affinity for the cognate recognition site. In previous work, we developed a convenient repetitive cloning method that facilitates the construction of extended PZF domains. Different types of PZF designs were critically evaluated for their ability to bind to chromosomal chromatin-embedded DNA sequences in the yeast *Saccharomyces cerevisiae* (Neuteboom *et al.*, 2006). Subsequently, it was also demonstrated that PZF domains constructed via this method were highly effective in Arabidopsis, enabling novel mutant screens (Lindhout *et al.*, 2006) and *in vivo* labelling of chromosomal target sites (Lindhout *et al.*, 2007).

For site directed mutagenesis (SDM) in animals and plants, one can exploit the fact that DSBs are mainly repaired via non-homologous end-joining (NHEJ). In contrast to HR, no homologous sequences are required for this particular DNA repair pathway. Therefore, NHEJ is intrinsically error prone. When active ZFNs are present, the cycle of cutting and repairing the ZFN target site continues until an imperfect NHEJ-mediated repair event results in a mutation or footprint within the target site, which prevents recognition or subsequent cleavage by the ZFNs. Such a NHEJ-based mutagenesis strategy was developed in Drosophila (Bibikova *et al.*, 2002) and was also shown to be an efficient mutagenesis method in Arabidopsis, tobacco and maize (Lloyd *et al.*, 2005; Maeder *et al.*, 2008; Shukla *et al.*, 2009; Tovkach *et al.*, 2009).

In addition to SDM via the generation of site-specific DSBs and their subsequent imperfect repair, ZFNs have recently also been instrumental for precise GT, a process where a DNA sequence inserts at the induced DSB site, preferably via a double HR event. This has by now been demonstrated in Drosophila embryos (Beumer *et al.*, 2006), in human cells (Urnov *et al.*, 2005; Lombardo *et al.*, 2007; Moehle *et al.*, 2007), in zebrafish (Doyon *et al.*, 2007) as well as in plant protoplasts and plant cell suspensions. As for the plant experiments, ZFN-induced GT was demonstrated by precise repair of defective reporter genes by means of integration of specific DNA constructs at the ZFN target site (Wright *et al.*, 2005; Cai *et al.*, 2008). Very recently, it was demonstrated that ZFN technology can be instrumental for HR-mediated targeted integration (Shukla *et al.*, 2009) and mutation (Townsend *et al.*, 2009) of endogenous genes in maize and tobacco. In most studies, the GT repair constructs and ZFNs expression constructs were co-delivered to cell suspensions (Cai *et al.*, 2008; Shukla *et al.*, 2009) or protoplasts (Wright *et al.*, 2005; Townsend *et al.*, 2009) via a variety of direct DNA transformation procedures. For plants, it will be of great interest to further develop ZFN-induced GT by means of DNA transfer via the widely used bacterial vector *Agrobacterium tumefaciens*. Thus far, in respect of ZFN-induced GT, *Agrobacterium* has been used only for tobacco cell cultures (Cai *et al.*, 2008).

Here we report the use of ZFNs to create DSBs for the introduction of site-specific mutations and to enhance the frequency of gene-targeting in Arabidopsis by means of the simple floral dip method (Clough and Bent, 1998). A reporter-based assay was developed exploiting PZF domains that had been shown to bind to chromatin-embedded DNA. The functionality of the ZFNs was first tested in a yeast-based test system. Subsequently, footprints in the ZFN target sequence were detected in Arabidopsis plants expressing the ZFNs. It was shown that floral dip transformation of such plants with an incoming homologous T-DNA GT construct can indeed lead to precise GT events.

Results

ZFN functional assay in yeast

For the production of DSBs, we made use of the PTF and E2C PZF DNA binding domains that were previously shown to bind to their target site in chromatin-embedded DNA (Neuteboom *et al.*, 2006). These PZFs were coupled to the *Fok*I nuclease domain with a 21 amino acid linker, having about the same length as a linker used previously (Smith *et al.*, 1999) (Figure 1a). To determine the functionality of PTFFOK and E2CFOK ZFNs for DSB formation in chromatin-embedded DNA, these ZFNs were expressed in yeast and an artificial chromosomal target site, with a 6 or 16 bp spacer between the PZF recognition sequences, were analysed for the presence of footprints. These spacers lengths were chosen since Smith *et al.* (2000) demonstrated that 6 bp to 35 bp spacers were cleaved by ZFNs possessing the long amino acid linker in between the PZF and the *Fok*I cleavage domain mentioned above. When also our ZFNs would efficiently digest longer target sites in addition to sites with a 6 bp spacer, it was reasoned that the use of a 16 bp spacer would reveal this. An *Eco*RI restriction site was included in the spacer sequences (Figure 1b), which may be lost upon imperfect repair of the ZFN-induced break. The 16 bp spacer also contained a *Pvu*II and a *Spe*I restriction site. Expression of the ZFNs was confirmed by Western blotting using anti-FLAG antibodies (results not shown). Only when both PTFFOK and E2CFOK were present and when 6 bp separated the recognition sites, PCR products were found that were lacking the *Eco*RI site. The sequences of cloned *Eco*RI resistant PCR products exhibited deletions ranging from 1 to 26 bp (Figure 1c). These results showed that the ZFNs constructed for our study are functional for DSBs formation in chromatin-embedded yeast DNA, recognizing a target site with 6 bp spacing rather than 16 bp.

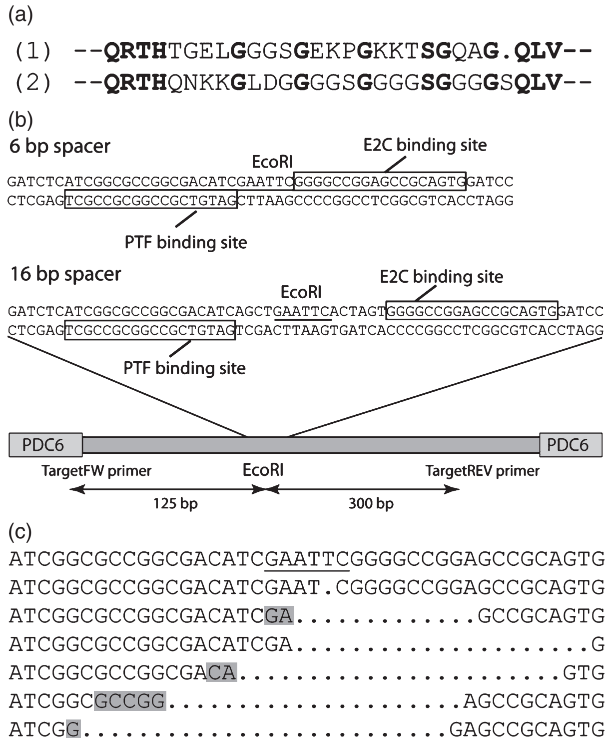
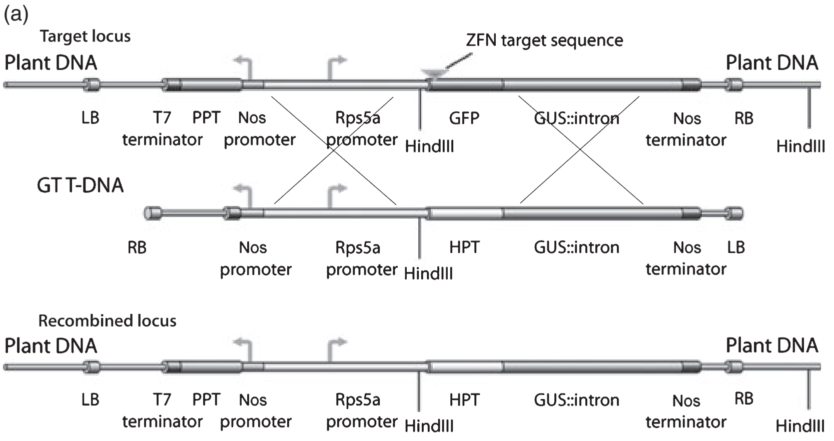


Figure 1 Outline of the yeast ZFN test system. A. Linker sequences present between the PZF domains and the *Fok*I nuclease domain of the ZFNs used here (1) compared with the linker used by Smith *et al.*, 1999 (2). B. The ZFNs target locus was inserted in the *PDC*6 locus, containing the target sequences (boxed) for the PTF and E2C PZF domains in a tail-to-tail orientation with 6 or 16 bp spacing containing an *Eco*RI site (underlined). C. Footprints in the yeast target locus. Deleted nucleotides are indicated by dots. Nucleotides which can be placed left or right of the deletion are shown in grey.

ZFN-induced mutagenesis in Arabidopsis plants

For ZFN-induced mutagenesis in Arabidopsis, an assay was developed utilizing PTFFOK and E2CFOK and their target sequences. First, a T-DNA containing the ZFNs target sequences with 6 bp spacing was introduced in Arabidopsis (Figure 2a). One plant line was selected containing one full-length copy of the T-DNA. By means of TAIL-PCR, the integration site of the T-DNA was found to be located on chromosome 4 between At4g22750 and At4g22760 (Figure 2b). Both flanking regions were analysed by PCR and sequencing. Constructs encoding PTFFOK and E2CFOK both under control of the Rps5a promoter, which is primarily active in meristematic cells and early embryos (Weijers *et al.*, 2001), the constitutive 35S promoter, or the tamoxifen-inducible 35S promoter (pINTAM construct; Friml *et al.*, 2004) were stably integrated in the homozygous target plant line. The presence of both ZFN genes was checked by PCR using primers amplifying the coding region of both ZFNs and subsequent *Hin*dIII digestion of the DNA fragment (Figure 3). Most plant lines contained both ZFNs as determined by the presence of five DNA fragments. A selection of these plant lines was analysed by RT-QPCR for expression of the ZFNs. Relative expression levels were determined with primers in both the E2C and PTF PZF domains, and showed similar expression levels of both ZFNs (results not shown). To compare expression levels of ZFN constructs with different promoters, primers in the *Fok*I nuclease domain were used (Figure 4). As expected, the relative expression levels were low in lines



(b)

```
CGTTGTGTTTGGAGTAGGCCCAATTTTCTTTCTTTAATTGTTTAAATGGTGCATACAAATTTCTGTTTTT
TTTTTTAAGAAAAAAAACTTTATTATACACTTGGATATACACATATATATAAAGACACGGATATACATAC
ACGAGTTTTGTGGAATGTGTCTATGAATTTTTATGACACCATTATACTATAGAGATAGCAAAAGGTTGAA
ATAAATTGAGGCTGTTGGCCAATTGTATTTAAGTAATAGAATGGTATTACATCATCACGAAAAGTGACGT
TACATTTGAAATTATAAAGTCTTACAATTTTTTCGTCATGACAATAAATGATATTTAAATGTAATAATAC
ATATTTTTTAAGTTTCAAGTATACATAAAAAAGGGTCACAAACGATCGTTGTTAAATACTTGGTGGAAAA
CAAGTGCG
```

7 bp deletion
LB Filler GACGTTTTTAATAACAAGTGTTAAATAC
LB + 1bp deletion

```
ATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGGGGTGGTTTTTCTTTTCAC
CAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAA
```

BarP$_{nos}$-P$_{Rps5a}$GFPGUS

```
CGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAATTCAGATCGGCTGAG
TGGCTCCTTCAACGTTGCGGTTCTGTCAGTTCCAAACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGG
TCATAACGTGACTCCCTTAATTCTCCGCTCATGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATC
AG
```

7 bp + RB deletion
RB filler C

```
TGAACATGAGTGCGTATGGCATAAGAGAAGTCGACCACACCATTAATGACTTTAAATCAAGCCCAAAAGT
TCTTTCTTTTAACAGTGGAAACAAACCTCGGACCAATCTCAGCCACACAAATTCTCCTTTTTATTTTATG
TTGTTTTTTCTTGACGTTGATGAATTCTTGTTTTTGGTTATAAATTTTTTATATTACTTCCATTACTCAG
CACTTTCGTAAAATTTAATGAATTGATGATACTAGTTGAGTCTCCCTCCTCTCTAATTGTTTATGTGTTT
TTTTTCATTTGTTTCTGTGCTACTACACT
```

Figure 2 Outline of the Arabidopsis GT system. (a) The T-DNA containing the target locus containing the P$_{Rps5a}$-*gfp/gus* reporter gene including the ZFN target sequence and the P$_{nos}$-*ppt* selection marker was stably integrated. ZFNs were stably integrated in this line. Their functionality was tested by detection of footprints in their target sequence. GT was induced by introduction of a GT T-DNA construct with homology to the target locus (P$_{nos}$P$_{Rps5a}$ and GUS*)*, but missing the coding region of the *ppt* selection marker and having the *gfp* coding sequence replaced by *hpt*. GT will result in the recombined locus as shown. *Hin*dIII sites generating the DNA fragment that was detected after GT by the *hpt* probe on the Southern blots (Figure 7b) are shown. (b) Sequence of the chromosome 4 plant DNA flanking the T-DNA containing the target locus in the selected Arabidopsis line as determined by TAIL-PCR and PCR. LB, left border; RB, right border.

that contained the Rps5a constructs (4.4 ± 2.5). The constitutive 35S promoter constructs gave up to 200-fold higher expression (727 ± 365), which was comparable with its tamoxifen-induced version (874 ± 1020).

Subsequently, the activity of the ZFNs was evaluated. Although footprints could be detected in primary transformed plants, we routinely used T2 seedlings as a source for DNA isolation since these could be grown, induced (in case of the pINTAM-ZFNs) and analysed at any convenient time point. Genomic DNA isolated from a mixture of ten seedlings was pre-digested with *Eco*RI and used as template in PCR reactions (Figure 5). PCR products derived from all different types of transgenic plants, harboring ZFNs driven by the different promoters, were digested with *Eco*RI and *Eco*RI resistant fragments were cloned and sequenced (Figure 6a). The target plant line that did not

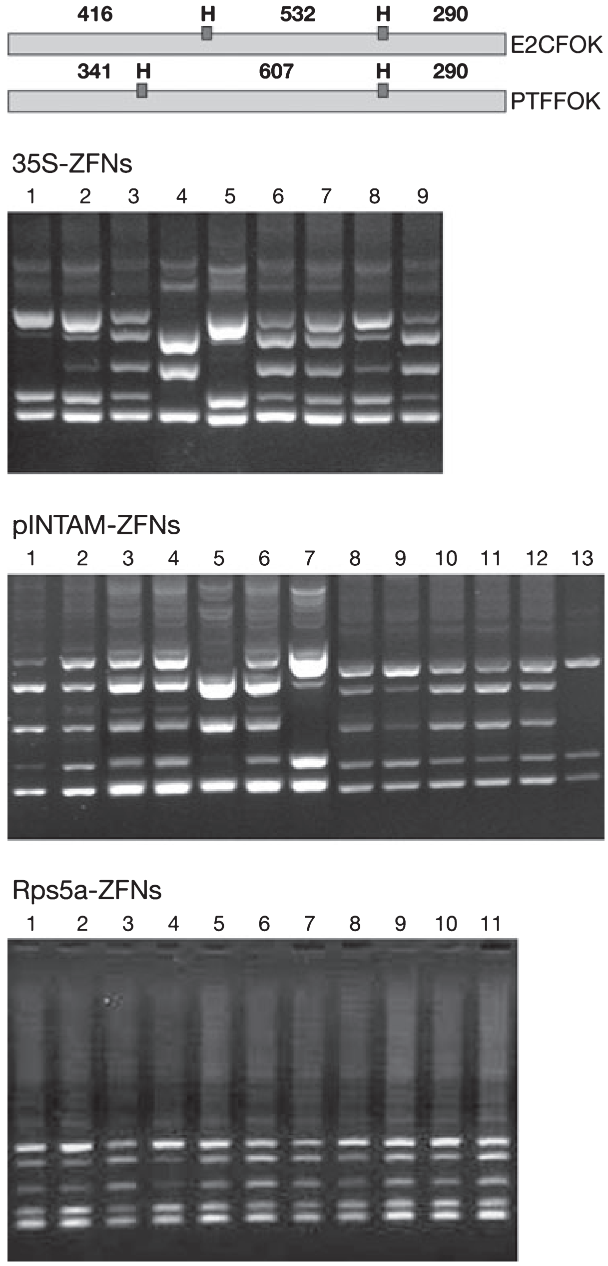


Figure 3 ZFN pairs in target lines. Arabidopsis target lines transformed with E2CFOK and PTFFOK under control of the 35S promoter (35S-ZFNs, lanes 1–9), the pINTAM promoter (pINTAM-ZFNs, lanes 1–13) or the Rps5a promoter (Rps5a-ZFNs, lanes 1–11) were analysed by PCR, using primers SP258 and SP259, amplifying both E2CFOK and PTFFOK and subsequent *Hin*dIII (H) restriction analysis for the presence of the coding regions of both ZFNs. The expected lengths of DNA fragments obtained after amplification and digestion of E2CFOK and PTFFOK are shown at the top.

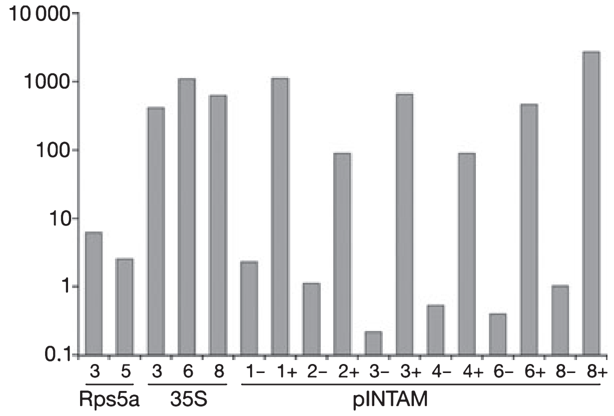


Figure 4 Relative expression levels of the two ZFNs. Several plant lines containing ZFNs under control of the Rps5a promoter, the 35S promoter or the pINTAM promoter, compared to the housekeeping gene *roc1* were analysed by RT-QPCR. For the pINTAM constructs, expression was determined in the absence (–) and presence (+) of tamoxifen.

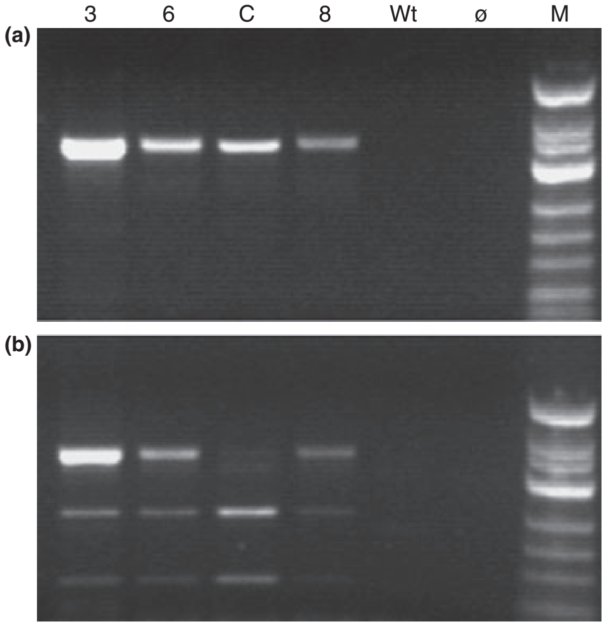


Figure 5 *Eco*RI resistant PCR fragments. Gel electrophoresis of PCR fragments of ZFN target sites from plants expressing ZFNs (35S-ZFNs plant lines 3, 6 and 8) or the control line without ZFNs (c), before (a) and after (b) digestion with *Eco*RI. M: ΦX174/ *Hin*fI molecular weight marker.

contain ZFNs did not reveal *Eco*RI resistant PCR products (Figure 5). A quantitative analysis of footprint frequency in ZFN expressing plant lines was impossible due to the cycle of *Eco*RI digestion and PCR. It was observed, however, that footprints were harder to detect when the relatively weak *Rps5A* promoter was driving ZFN expression. Most *Eco*RI resistant DNA fragments contained deletions ranging from 1 to 80 bp. Several larger deletions, up to about 200 bp, were found as well as small insertions (1–14 bp), the latter mostly accompanied by deletions. Often the repair had taken place in a region containing microho-

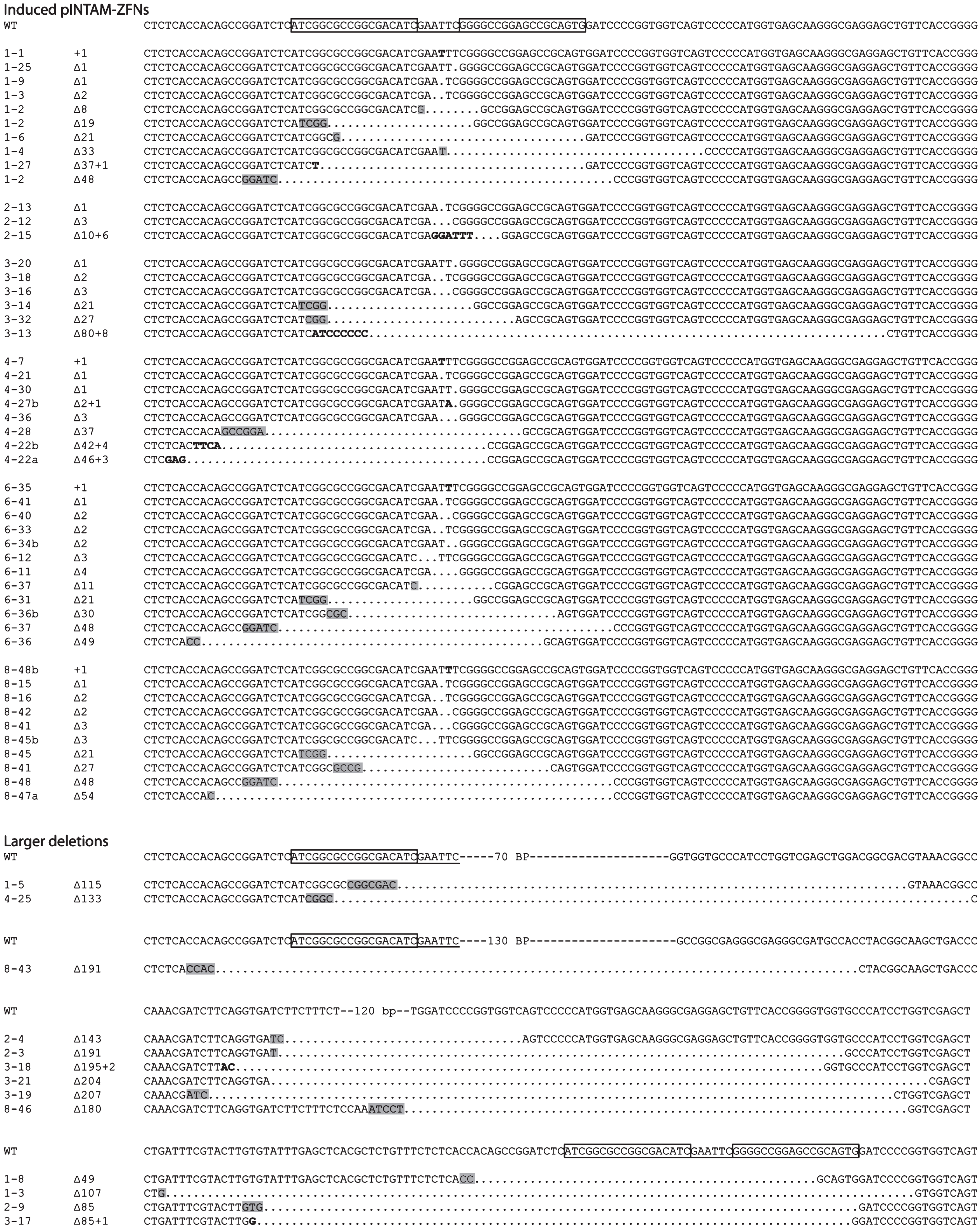


Figure 6 Sequences of *Eco*RI resistant target sites. (a) *Eco*RI digested DNA from several plant lines with different promoter-ZFN constructs was used for PCR and *Eco*RI resistant products were cloned and sequenced. ZFN binding regions are boxed and the *Eco*RI site is underlined. Deleted nucleotides are indicated by dots and inserted nucleotides in bold. Nucleotides which can be placed left or right of the deletion are shown in grey. The sizes of the deletions and insertion are indicated. (b) Sequences of *Eco*RI resistant PCR products obtained without pre-digesting the genomic DNA with *Eco*RI.

35S-ZFNs

WT		CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAATTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-6	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA.TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-5a	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA.TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-11a	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAATT.GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-34	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA..TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-11	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA..CGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-7	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCG..TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-9a	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAAT..GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-24	Δ2 +1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA**G**.CGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-39	Δ3	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATC...TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-15	Δ3	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCG...TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-38	Δ4	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATC....TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-5	Δ4	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA....GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-4	Δ6	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATC......GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-29	Δ8	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGA........TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-17	Δ9	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGAC.........GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-33	Δ9	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACAT.........GGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-1a	Δ12	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA............GCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-8a	Δ12	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCG............GGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-6a	Δ12 +1	CTCTCACCACAGCCGGATCTCATCGGCGCCG**A**...........TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-8	Δ21	CTCTCACCACAGCCGGATCTCATCGG.....................GGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-31	Δ27	CTCTCACCACAGCCGGATCTCATCGG...........................AGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-20	Δ32 +5	CTCTCACCACAGCCG**TATC**...........................GGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-21	Δ41 +14	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATC**CACTGCGGCTCCGG**...........................CCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
3-2a	Δ48	CTCTCACCACAGCCGGATC..CCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-64	+1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA**A**TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
6-70	+1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA**T**TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
6-50	+2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA**AA**TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGG
6-21a	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA.TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-53	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA.TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-77	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATC.AATTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-54	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAATT.GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-45	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA..CGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-67	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCG..TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-79	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA..TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-16a	Δ3	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA...CGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-71	Δ6	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA......GCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-20a	Δ8	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACAT........GGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-14a	Δ21	CTCTCACCACAGCCGGATCTCATCGG.....................GGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-41	Δ48	CTCTCACCACAGCCGGATC..CCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
6-74	Δ58	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA..GAGGAGCTGTTCACCGGGG
6-73	Δ68 +10	CTCTCACCACAGCCGGATCTCATCGGCGCCGG**GGACATGGAT**..GAGGAGCTGTTCACCGGGG
6-18a	Δ63	CTCTCACCA...TGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-27a	+1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA**A**TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
8-107	Δ1 +1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA**A**TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-28a	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA.TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-25a	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA.TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-83	Δ1	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAATT.GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-93	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCG..TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-85	Δ2	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACAT..AATTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-29a	Δ3	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATC...TTCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-84	Δ3	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA...CGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-96	Δ3	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA...GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-95	Δ5	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACAT.....TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-29a	Δ10 +6	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGA**GGATTT**....GGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-109	Δ20	CTCTCACCACAGCCGGATCTCAT....................TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-32a	Δ21	CTCTCACCACAGCCGGATCTCATCGG.....................GGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-26a	Δ30 +12	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCG**CAGTGGTTCCCA**..................GGTGGTCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-33a	Δ33	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAAT.................................CAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
8-31a	Δ59	CTCTCACCACAGCCGG...TCAGTCCCCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG

Rps5a-ZFNs (pSDM 3837 lines)

5	Δ5	CTCTCACCACAGCCGGATCTCATCGGCGCCGGCGACATC.....CGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGTCC
3	Δ48	CTCTCACCACAGCCGGATC..CCCGGTGGTCAGTCC

B

No EcoRI predigestion

3-52a	Δ1	CACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA.TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGT
3-104b	Δ1	CACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA.TCGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGT
3-116b	Δ1	CACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAATT.GGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGT
3-113b	Δ2	CACCACAGCCGGATCTCATCGGCGCCGGCGACATCGAA..CGGGGCCGGAGCCGCAGTGGATCCCCGGTGGTCAGT
3-42b	Δ48	CACCACAGCCGGATC..CCCGGTGGTCAGT

Figure 6 (Continued)

mology (Figure 6, grey boxes), suggesting that in these cases microhomology mediated end-joining (MMEJ; McVey and Lee, 2008) had been used for repair.

In order to get more reliable insight in the frequency of mutations within the target sequence, PCR products amplified using undigested DNA templates from a mixture of T2 seedlings expressing 35S-ZFNs (plant line 3) were cloned and sequenced directly, thus without any selection by means of *Eco*RI digestion. Out of 240 cloned PCR fragments, 5 were missing the *Eco*RI site. The sequences of these five clones showed deletions of 1–48 bp (Figure 6b). This means that in about 2% of the cells the target sequence contained footprints, which were likely formed after ZFN-induced DSB formation followed by incorrect repair via NHEJ. The majority of the target sites was unaltered and was therefore still potential substrates for ZFN cleavage. Remarkably, no aberrant phenotypes were observed in any of the plant lines expressing the ZFNs, indicating the absence of ZFN toxicity.

ZFN-induced gene-targeting in Arabidopsis plants

Since the ZFNs were shown to create DSBs in their target sequence, the next step was to perform gene-targeting experiments at this locus. Due to the fact that the majority of potential ZFN target sites was still intact in cells of T2 seedlings, even when the ZFN pair was under control of the constitutive 35S promoter, we decided to investigate whether it was possible to achieve GT by introducing a homology carrying construct with the commonly used floral dip procedure. With a potentially active ZFN pair expressed in flower buds and undamaged target sites still present in most of the cells, it should be sufficient to employ an *Agrobacterium* strain carrying no more than a single T-DNA for which HR-mediated integration at the target locus can be demonstrated. The experimental design of the GT assay is shown in Figure 2a. A GT T-DNA is introduced by floral dip transformation into a plant line already containing the target locus as well as the genes encoding the ZFNs, the latter located on one T-DNA with the kanamycin selection marker. We chose to use plants with ZFN expression driven by the *Rps5A* promoter in order to avoid unwanted constitutive activity of the ZFN in differentiated tissues. The fact that this promoter is predominantly active in meristematic cells, such as present during flower and gamete formation as well as early embryos (Weijers *et al.*, 2001), ensures that ZFN expression will be at hand during the process of floral dip transformation. The GT T-DNA was designed to be similar to the sequence at the target locus, but the *gfp* coding region was replaced by the *hpt* coding region and the *ppt* coding region, present in the target locus, was omitted. Upon integration via HR the *hpt* gene will replace the *gfp* coding region and will be linked to *ppt* and to the chromosome 4 plant DNA flanking the target locus. Transgenic seeds from the floral dip transformation were selected on hygromycin. It should be noted that random integration of the GT construct carrying the *Rps5A*-driven *hpt* gene also results in hygromycin resistant seedlings. Therefore, PCR was used to detect GT events. Pools of 20 hyg^R plants were analysed with primers in *hpt* and chromosome 4. Of the 152 pools (3040 plants) that were analysed, three pools contained one plant each that produced a PCR product of the correct size with primers in the *hpt* gene and the downstream flanking region of chromosome 4 (Figure 7a), indicating that the *hpt* coding region was integrated via homologous recombination. In two of the three plants (GT1 and GT3) the correct band was also detected with primers in the upstream flanking region of chromosome 4 and the *hpt* coding region. These two plants represent so-called true gene targeting events (TGT). In GT2, homologous recombination in the region upstream of the *hpt* gene linking the *ppt* gene and *hpt* gene, could not be detected, indicating that integration at this end did not occur via HR. Longer extension times also did not result in PCR products with *ppt* and *hpt* specific primers. Therefore it seems that integration of the T-DNA at this end also did not occur via NHEJ. This plant represents an ectopic gene targeting (ETG) event. In all three plants the original target locus was still present, indicating that the plants were heterozygous. No GT events were detected in 2860 hyg^R plants that did not contain the ZFNs.

To confirm the GT events, Southern blot analysis was performed. Genomic DNA of the plants GT1, GT2, and GT3 was digested with *Hin*dIII and probed with *hpt* (Figure 7b), detecting GT T-DNA fragments with one recognition site in the T-DNA and one recognition site in the flanking plant DNA (Figure 2a). A 4.6 kb hybridizing fragment expected for a GT event was detected in DNA from all three plants. In the lanes with DNA from both GT1 and GT3 many additional hybridizing bands were visible. These bands probably represent randomly integrated T-DNAs. The additional, small band (1 kb) in the lanes with GT2 DNA probably represents a partial T-DNA.

Progeny of the GT plants was analysed by PCR for the segregation of the *gfp* and *hpt* genes (Figure 7c). In GT2, segregation was only observed for the *hpt* locus. Selection on hygromycin of GT2 progeny showed that GT2 contained one active *hpt* gene (180 hyg^R : 53 hyg^S; 3 : 1). All hyg^R progeny plants still contained the *gfp* gene, indicating that HR had taken place between the target locus and the GT T-DNA through one-end invasion and gene conversion. Apparently, this was followed by release of the T-DNA, completed with the newly acquired sequence of chromosome 4, and integration elsewhere in the genome. However, segregation of *gfp* and *hpt* and thus of the target locus and the recombined GT locus was observed in GT1 and GT3. Three classes of GT1 and GT3 progeny

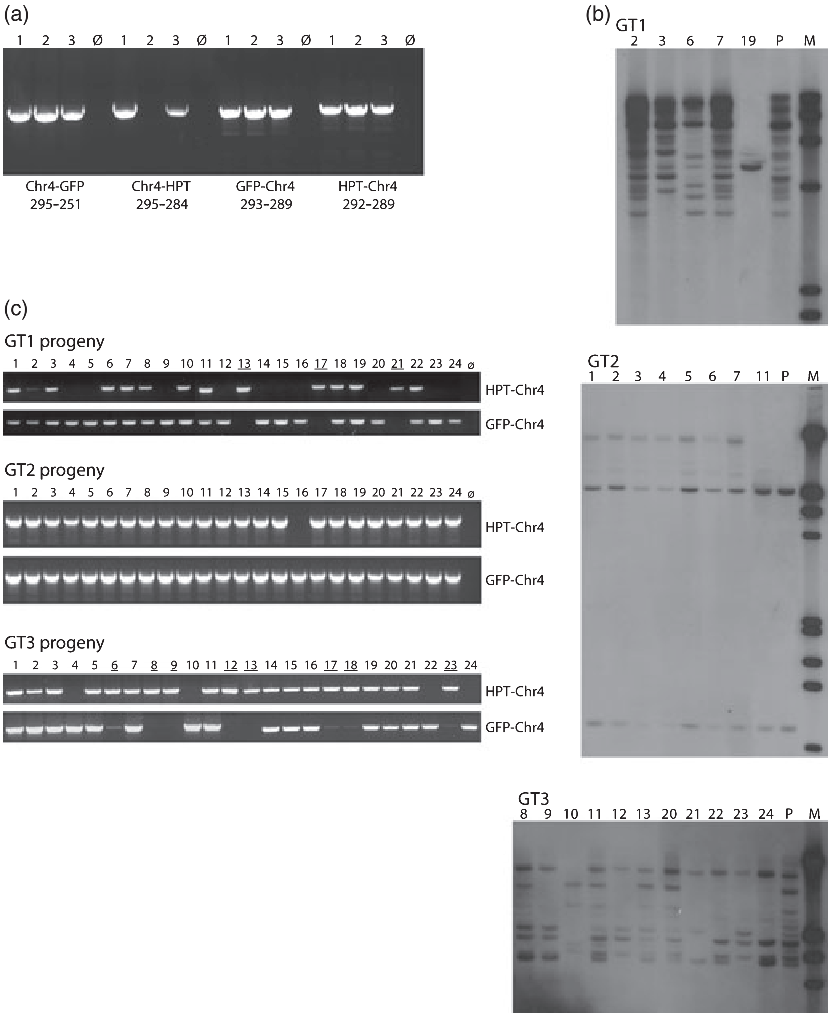


Figure 7 GT analysis in Arabidopsis. (a) PCR on primary GT plants with primers in chromosome 4 (SP295 or SP289) flanking the target T-DNA combined with primers in the *hpt* gene (SP284 or SP292) or *gfp* gene (SP251 or SP293) to detect homologous recombination. (b) Southern blot analysis of DNA from primary transformants (P) and progeny GT plants (numbers). Genomic DNA was digested with *Hin*dIII and blots were probed with the *hpt* gene. Molecular weight markers (M) are Lambda (*Hin*dIII) (top panel) or Lambda (*Eco*RI/*Hin*dIII). The lanes in panel GT1 are derived from several different blots and band intensities cannot be compared. (c) PCR on progeny of GT plants using a primer in chromosome 4 flanking the target locus (SP289) combined with primers in the *hpt* gene (SP292) or *gfp* gene (SP293) to detect segregation. Plants homozygous for the GT locus are underlined. For GT2 23 hyg^R progeny plants and one hyg^S progeny plant (16) were used for PCR.

plants were obtained; homozygous *gfp*, homozygous *hpt* and heterozygous plants. Southern blot analysis of the progeny of GT1 and GT3 showed that some of the additional bands representing randomly integrated T-DNAs were not present anymore (Figure 7b). In one GT1 progeny plant all of the extra bands had been lost (Figure 7b; GT1 plant 19). This means that the extra copies were not linked to the GT locus and that they could be lost by selfing. PCR analysis of the ZFN genes showed that in some GT1 progeny plants these genes were lost through segregation (results not shown). One of these is the heterozygous GT1 plant 19, which also did not contain randomly integrated T-DNAs. By selfing this plant we obtained homozygous plants in which both target loci had been replaced by the GT T-DNA and in which the randomly integrated T-DNAs as well as the ZFNs were not present anymore (results not shown). In all hyg^R GT2 progeny plants, the band of expected size (4.6 kb) is present as well as the small band (Figure 7b), which is probably linked to the ectopic GT locus.

Finally, the full-length T-DNA inserts comprising the target locus and the recombined GT locus in GT1 and GT3 were amplified with primers in chromosome 4 and cloned. Since the plants that were used were heterozygous for the target locus and the recombined GT locus, two different T-DNAs were cloned. Recombined GT clones from both GT1 and GT3 were sequenced from the N-terminal part of the *ppt* coding region to the N-terminal of the *hpt* coding region and from the C-terminal part of the *hpt* coding region to chromosome 4. The sequences were identical to those expected from precise homologous recombination between the target locus and the GT T-DNA (results not shown). Clones of the original target locus from both the GT1 and GT3 lines were sequenced and both still contained intact ZFN recognition sequences, indicating that either this chromosome had not been broken by the ZFNs or that repair had occurred here via precise NHEJ. Taken together, these results show that GT occurred in plants with ZFN activity, whereas no GT was detected in a comparable number of plants without ZFNs.

Discussion

The ZFNs that were generated at the onset of this study contained 6-fingered (6ZF) PZF domains. Hence, in theory, any dimeric ZFN that can be composed within a cell expressing one or both of these ZFN should preferably interact with a 2 × 18 bp DNA sequence, given that the spacing in between the 18 bp sequence allows the dimer to do so. Any particular need for such long ZFN recognition sites has thus far not been demonstrated. In fact, most other studies targeted 2 × 9 or 2 × 12 bp half-sites, corresponding with two PZFs each consisting of 3 or 4 ZFs (Lloyd *et al.*, 2005; Urnov *et al.*, 2005; Wright *et al.*, 2005; Beumer *et al.*, 2006; Doyon *et al.*, 2007; Lombardo *et al.*, 2007; Moehle *et al.*, 2007; Cai *et al.*, 2008; Maeder *et al.*, 2008; Tovkach *et al.*, 2009; Townsend *et al.*, 2009). Thus far only one study made use of ZFNs with longer DNA binding domains containing 5 or 6 ZF (Shukla *et al.*, 2009). Our choice was inspired by our earlier findings that 6ZF PZFs were superior regarding their *in vivo* interaction with chromosomal DNA in yeast cells (Neuteboom *et al.*, 2006). Moreover, in the same study the binding characteristics of 6ZF PZF domains PTF and E2C were carefully analysed. For the purpose of investigating ZFN-induced mutagenesis and GT in Arabidopsis, the performance of the PTFFOK and E2CFOK ZFN pair was also first evaluated in a yeast assay to ensure that they were active on chromatin-embedded chromosomal DNA.

The linker between the PZF and the *Fok*I nuclease domain was 21 amino acids, similar to the length of the linker used by Smith *et al.* (1999). The typical footprints resulting from imperfect DSB repair within and around a unique 2 × 18 bp target site were only detected with the 6 bp spacer between the ZFN recognition half sites and not with the 16 bp spacer. Recently, it was reported that ZFNs containing linkers of up to 20 amino acids bind also to target sequences with longer spacers (Händel *et al.*, 2009). We did not observe this, indicating that probably the amino acid sequence of the linker also determines the affinity for the target site.

Once a single target locus was introduced into Arabidopsis and homozygous lines were obtained, the strategy that was used to finally obtain GT in those plants encompassed a two-step procedure: after raising plants harbouring ZFNs, these very plants were subsequently transformed with a GT donor construct. So far, ZFN-induced GT in eukaryotic cells has almost exclusively been accomplished by the simultaneous introduction of DNA molecules encoding ZFNs and an incoming donor GT construct with homology to the site of interest where the DSB should occur. As far as plant cells are concerned, the target cells used for transformation aimed at ZFN-induced GT thus far were protoplasts (Wright *et al.*, 2005; Townsend *et al.*, 2009) or cultured cells (Cai *et al.*, 2008; Shukla *et al.*, 2009). Although this does not at all affect any of the conclusions regarding successful ZFN-mediated GT events, the very processes of protoplasts isolation or tissue culture steps needed to support growth or regenerations of cells and tissues might have a detrimental effect on the normal state of DNA metabolism, the state as it is maintained in unstressed cells or tissues. These tissue culture approaches may lead to somaclonal variations and chromosomal rearrangements (Kaeppler *et al.*, 2000; Mohan Jain, 2001; An *et al.*, 2005). Floral dip transformation, as it is routinely performed with intact Arabidopsis plants, does not necessitate any kind of regeneration of deliberately prepared cells or tissues. This method in fact prevents the tissue culture steps that may induce unwanted changes in the genome. Although the method is mainly used for Arabidopsis, efforts have resulted in protocols for floral dip transformation of other species, like wheat (Zale *et al.*, 2009). Furthermore, the female gametes that are transformed via floral dip transformation are in a developmental phase just before or after meiosis (ovule primordia, megasporocytes or megagametocytes) (Desfeux *et al.*, 2000) and might therefore be equipped with highly active HR DNA repair systems, more so than somatic cells or cells experiencing stress by artificial manipulations.

As mentioned above, the expression of ZFNs can potentially be accompanied by toxic side-effects, possibly caused by high expression or off-target binding of the ZFNs (Cornu *et al.*, 2008). However, it cannot be excluded that in cases where detrimental effects are observed, these are at least partly the consequence of *in vitro* manipulations of target cells rather than a harmful effect of ZFNs. In this respect, our data that footprints were observed in primary transformants expressing ZFNs as well as in seedlings derived from these plants without noticeable negative effects upon plant growth and development that could be attributed to ZFN expression are very much encouraging regarding the feasibility of using ZFNs in plants. To our knowledge, these are the first data providing evidence that multicellular organisms can very well tolerate expression of active ZFNs. For all ZFN expressing plant lines maintained in our laboratory, the offspring is of perfectly normal wild type appearance.

It can be argued that the absence of any detrimental effects of ZFN expression also has to be attributed to the fact that only a small percentage (2%) of the cells from plants expressing the ZFNs contains footprints, even when the ZFNs were expressed using the 35S promoter. This may imply that the ZFNs were unstable or not very active. Although ZFN protein was easily detectable in yeast, we could not detect ZFN protein in plants on Western blots using anti-FLAG antibodies. In addition, full-length ZFN mRNA levels were very low in plants. This could mean that plant specific RNases or proteases degrade our ZFN mRNAs or proteins. Recently, it was shown that by modulating ZFN protein levels, ZFN toxicity can be reduced (Pruett-Miller *et al.*, 2009). Thus, low protein levels may also be a reason for the absence of toxic effects. Another possibility, which might very much be the consequence of using unstressed plant material as also mentioned above, is that most DSBs that were formed were rapidly repaired without leaving any footprint due to the fact that the repair system remains very active during the normal processes of embryo-, seedling- and plant development. In this respect, it is of particular interest to note that even in mammalian cell extracts, the majority of DSBs are repaired without leaving any footprint (Kuhfittig-Kulle *et al.*, 2007). The footprints that were detected in our study were all rather typical mutations as they are created via NHEJ or MMEJ repair of DSBs, thus resulting in deletions and insertions. The size of the deletions observed was limited. However, these results greatly depend on the size of the PCR fragments that were isolated and cloned. It cannot be excluded that also larger deletions had been created upon DNA repair.

With healthy plant lines expressing ZFNs, but with the large majority of target sites still unaltered, it was possible to perform another round of floral dip transformation aimed at GT by means of a HR event at a newly generated DSB. For the GT experiments, plants with Rps5a-driven ZFNs, with by estimation much less than 2% of footprints within the ZFN target sites, were used for floral dip. As already mentioned above, the Rps5A promoter is rather active in dividing cells, so also in developing flowers, gametes, zygotes and early embryos (Weijers *et al.*, 2001). Therefore, during floral dip of these plants a significant number of the cells that are transformed with the GT construct may have a ZFN-induced DSB.

We observed a GT frequency of 10^{-3} in our GT assay in the presence of functional ZFNs. Considering the estimates of GT frequency in several different plant species ranging from 10^{-4} to 10^{-6} (Paszkowski *et al.*, 1988; Lee *et al.*, 1990; Offringa *et al.*, 1990; Halfter *et al.*, 1992; Hrouda and Paszkowski, 1994; Miao and Lam, 1995; Risseeuw *et al.*, 1995; Hanin *et al.*, 2001), our data suggest a 10- to 1000-fold increase in GT frequency due to ZFN expression. Since we did not find any GT event in plants without ZFNs, it is currently not possible to know the exact increase of GT frequency by ZFNs in our test system. In literature, much higher ZFN-induced GT frequencies were reported (Wright *et al.*, 2005; Cai *et al.*, 2008; Shukla *et al.*, 2009; Townsend *et al.*, 2009). However, it should be realized that a comparison cannot be made as the methods that were used differ very much from ours, involving different plant species, *in vitro* cultured plant material, different methods of DNA delivery and different genomic loci. In future experiments, direct selection of GT events will enable us to screen larger numbers of transformants, with and without ZFNs. This should allow us to determine with precision the fold increase in GT by ZFN-induced DSBs by means of the floral dip method. The results presented here corroborate recent findings in very different systems that ZFN-induced mutagenesis and ZFN-induced GT are very promising technologies for making precise changes in the plant genome at any given locus. Of particular importance in this respect is the finding that even prolonged exposure of the cells of multicellular organisms to ZFNs with sufficient specificity seems to present no particular fundamental problem, not even for their offspring.

Experimental procedures

Construction of yeast strains with the zinc-finger nuclease target site and vectors for the expression of ZFNs

The plasmid pINT1 (Meijer *et al.*, 1999) was digested with *Bam*HI and annealed primers for target sites with a 6 bp spacer (GATCTC-

ATCGGCGCCGGCGACATCGAATTCGGGGCCGGAGCCGCAGTG and GATCCACTGCGGCTCCGGCCCCGAATTCGATGTCGCCGGCGCCGATGA) or a 16 bp spacer (GATCTCATCGGCGCCGGCGACATCAGCTGAATTCACTAGTGGGGCCGGAGCCGCAGTG and GATCCACTGCGGCTCCGGCCCCACTAGTGAATTCAGCTGATGTCGCCGGCGCCGATGA) were ligated and confirmed by sequencing. Plasmids containing the binding site in the correct orientation were linearized with *Sac*I and *Nco*I, isolated from agarose gel and transformed to the yeast strain 21R⊿mel1 (Melcher *et al.*, 2000) by a PEG/LiAc transformation method (Gietz *et al.*, 1992). Yeast colonies were checked by PCR for correct insertion at the *PDC6* locus.

Plasmid pET-15b:ΔQNK-F_N (Smith *et al.*, 1999) (kind gift of Dr Chandrasegaran, John Hopkins University, Baltimore, MD, USA) was digested with *Bam*HI and *Spe*I and the ~0.6 kb band containing the coding region of the *Fok*I endonuclease domain was cloned into *Bgl*II and *Spe*I-digested pSKN-SgrAI, pSKN-SgrAI-PTF1 and pSKN-SgrAI-E2C1 (Neuteboom *et al.*, 2006). These PZF will be further referred to as PTF and E2C, respectively. *Escherichia coli* DH5α strains containing the pSKN-SgrAI plasmids with a *FokI* domain were always grown in the presence of 20 mM glucose to suppress protein expression.

For expression in yeast, the different ZFN constructs were cloned as *Not*I fragments into similarly digested p425ADHl or p426ADHl vectors. p426ADHl, which has been described previously (Neuteboom *et al.*, 2006), allows selection on medium lacking uracil. The vector p425ADHl, which allows selection on medium lacking leucine, was constructed from the vector p425ADH (Mumberg *et al.*, 1995) by replacing the original linker sequence with the linker sequence of p426ADHl.

Yeast nuclease assay

p425ADHl and p426ADHl plasmids, either without insert or containing ZFN fusions, were transformed separately as well as simultaneously to yeast strains containing the artificial target site and colonies were selected on minimal medium supplemented with the appropriate amino acids. For the nuclease assay, yeast cells were grown in selective liquid medium to an $\text{O.D.}_{600\text{nm}}$ of 0.8–1.0. Cells from 1 mL of culture were washed twice with 1 mL TE buffer. Following the last centrifugation step, excess liquid was aspirated and the cells were resuspended and disrupted by freeze–thawing. Subsequently, samples were heated at 94 °C for 5 min with occasional shaking. Cell debris was removed by centrifugation for 5 min and 2 μL of the denatured genomic DNA sample was used for PCR amplification. PCR was performed with REDTaq™ polymerase (Sigma-Aldrich, St. Louis, MO, USA) in 25 μL using 15 pmol each of TargetFW primer and TargetREV primer (Table 1). The presence of the *Eco*RI site was evaluated by restriction analysis.

Construction of the ZFN target site, the GT repair construct, ZFN expression vectors and plant transformation

The ZFN target sequence was inserted in the *Bam*HI site of pGPTV-BAR containing the *Rps5a-gfp/gus* reporter (Weijers *et al.*, 2001) resulting in pSDM3832 (target T-DNA). The *gfp* coding sequence (*Bam*HI/*Sca*I fragment) was replaced by *hpt* (*Bgl*II/*Bam*HI fragment)

Table 1 PCR primers

Primer	Sequence	Characteristics
targetFW	ATTCGTTTGAGTACACTACTAATGGC	ZFN target sequence (yeast) sense
targetREV	AACATTATACTGAAAACCTTG	ZFN target sequence (yeast) antisense
SP258	CGACTACAAGGACGACGACG	N-terminus ZFN sense
SP259	CCTCTAAGGTTAATGTGCC	C-terminus ZFN antisense
SP272	CCCTAATGAATGGTGGAAAG	Fok domain sense
SP273	GTTGGTGACGGACGAGATTAC	PTF antisense
SP274	GCTACGAGAAAAGGACTTCC	E2C antisense
SP250	CTCTGCCGTCTCTCTCATTCG	ZFN target sequence (Arabidopsis) sense
SP251	CTTGAAGAAGTCGTGCTGCTT	ZFN target sequence (Arabidopsis) antisense
SP283	GTAGTGGTTGACGATGGTGC	ppt sense
SP284	CACGAGATTCTTCGCCCTCC	hpt antisense
SP289	CTGAGATTGGTCCGAGGTTTG	Chr4 right flank sense
SP292	CCGAGGGCAAAGAAATAGAG	hpt sense
SP293	GTCCGCCCTGAGCAAAGACC	gfp sense
SP295	GACACGGATATACATACACGAGTTTTGTGG	Chr4 left flank antisense
ROC3.3	CCACAGGCTTCGTCGGCTTTC	ROC1 sense
ROC5.2	GAACGGAACAGGCGGTGAGTC	ROC1 antisense
MC141	CGATTCCGGAAGTGCTTGAC	Hpt sense
MC142	GGTCGGCATCTACTCTATTC	Hpt antisense
XB1200FW	AATTCGATATCCACCATG	Linker pCambia1200del
XB1200RV	GTGGATATCGAATT	Linker pCambia1200del
NOS 1	GATTGAATCCTGTTGCCGGTCTT	Tail PCR
NOS 2	GCATGACGTTATTTATGAGATGG	Tail PCR
NOS 3	CGCAAACTAGGATAAATTATCGC	Tail PCR
AD3	(A/T)GTGNAG(A/T)ANCANAGA	Tail PCR

from pGPTV-HPT (Becker *et al.*, 1992), resulting in pSDM3833 (positive control). The GT construct was created by cloning the *Nhe*I/*Pme*I fragment of pSDM3833 in pCambia1200del (*Xba*I/*Eco*RV), creating pSDM3834. pCambia1200del is a derivative of pCambia1200 with the *Xmn*I–*Bst*XI fragment containing *35S-hpt* replaced for a linker (XB 1200FW/RV) containing an *Eco*RV site. PTFFOK and E2CFOK were cloned as *Not*I fragments in pGPTV derivatives (Becker *et al.*, 1992; *kan* or *hpt* selection markers), in which the ZFNs are driven by the *Rps5a* promoter (pSDM3835, pSDM3836), the 35S promoter (pSDM3838, pSDM3839) or the tamoxifen-inducible 35S promoter (pSDM3840, pSDM3941) (Friml *et al.*, 2004). An *Xba*I linker was cloned in the *Pme*I site in pSDM3836 and the *Rps5a*-PTFFOK gene from pSDM3836 was cloned as an *Xba*I fragment in pSDM3835 containing *Rps5a*-E2CFOK, resulting in pSDM3837. The two ZFN genes in pSDM3837 were placed in inverted orientation. Plasmids are listed in Table 2.

Plant vectors were introduced in *Agrobacterium tumefaciens* AGL1 (Lazo *et al.*, 1991) by electroporation.

Arabidopsis plants (ecotype Col-0) were transformed via the floral dip method (Clough and Bent, 1998) and primary transformants were selected on MA solid medium without sucrose supplemented with nystatin (100 μg/mL), timentin (100 μg/mL) and the appropriated antibiotics (ppt 15 μg/mL; hpt 15 μg/mL; km 30 μg/mL) for selection of transformed plants.

DNA isolation and PCR analysis

Seedlings, leaves or flowers were disrupted to a powder under liquid N_2 in a TissueLyser (Retch, Haan, Germany). The powder was mixed with 500 μL CTAB extraction buffer (2% CTAB (*N*-cetyl-*NNN*-trimethyl ammonium bromide), 1.4 M NaCL, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0) supplemented with RNase (0.2 mg/mL) and incubated for 15 min at 37 °C. Subsequently, samples were incubated 15 min with 100 μL chloroform at 65 °C and twice extracted with phenol/chloroform (1/1) and once with chloroform. DNA was precipitated with 10 μL 3 M NaAC (pH 5.2) and 1 mL ethanol, washed, dried and dissolved in 50–200 μL water. One μL (usually 0.1 μg DNA) was used for PCR reactions in a final volume of 25 μL with either REDTaq™ (Sigma-Aldrich, St. Louis, MO, USA) or Phusion (Finnzymes, Espoo, Finland) polymerase. PCR primers are shown in Table 1. For amplification of ZFN coding regions SP258 and SP259 were used. For detection of GT events SP251, SP283, SP284, SP289, SP292, SP293 and SP295 were used in various combinations. For analysis of the T-DNA-plant DNA border sequence, TAIL-PCR was performed according to Liu *et al.* (1995), with specific primers NOS1-3 and random primer AD3. For PCR and cloning of the complete target locus and GT locus SP295 and SP289 were used.

Table 2 Plasmids

Plasmid number	Main feature	Plant selection marker
pSDM3832	Target locus T-DNA	ppt
pSDM3833	Positive control	ppt, hpt
pSDM3834	GT T-DNA	hpt
pSDM3835	Rps5aE2CFOK	km
pSDM3836	Rps5aPTFFOK	hpt
pSDM3837	Rps5aE2CFOK, Rps5aPTFFOK	km
pSDM3838	35SE2CFOK	km
pSDM3839	35SPTFFOK	hpt
pSDM3840	pINTAME2CFOK	km
pSDM3841	pINTAMPTFFOK	hpt

RT-PCR

For expression analysis of ZFN constructs, pools of ten days old seedlings were frozen in liquid N_2. For expression of pINTAM-ZFNs, seedlings were incubated for 48 h in liquid ½MS alone or ½MS supplemented with 1 μM tamoxifen for induction of the promoter. Tissue was disrupted to a powder under liquid N_2 in a TissueLyser (Retch, Haan, Germany). RNA was isolated using RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA). Residual DNA was removed with DNA-*free*™ (Ambion, Austin, TX, USA). cDNA was produced on 0.5 μg RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and PCR was performed on 0.25 μL cDNA using the iScript SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a final volume of 25 μL with 0.4 μm of each primer using a DNA Engine Thermal Cycler (MJ Research, Ramsay, MI, USA) equipped with a Chromo4 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Expression of ZFNs was analysed with primers SP258 and SP274 for the E2C PZF domain, with primers SP258 and SP273 for the PTF PZF domain and with primers SP272 and SP259 for the *Fok*I domain. Normalization of relative gene expression was based on expression of the housekeeping gene *roc1* (primers ROC3.3, ROC5.2).

Nuclease assay in Arabidopsis

Genomic DNA was digested with *Eco*RI and target sites were amplified with Phusion polymerase (Finnzymes, Espoo, Finland) using primers SP250 and SP251. PCR products were digested with *Eco*RI and analysed by gel electrophoresis. *Eco*RI resistant fragments were isolated from 5% polyacrylamide gels (in some cases amplified by PCR to obtain enough material) and cloned in pJet1.2 (Fermentas, Burlington, Ontario, Canada). DNA sequences were determined by ServiceXS (Leiden, the Netherlands).

Southern blot analysis

Plant DNA (5 μg) was digested with *Hin*dIII and separated in 0.7% agarose gels, blotted onto Hybond-N and hybridized in DIG easy hyb (Roche Diagnostics, Mannheim, Germany) supplemented with 50 μg/ml herring sperm DNA with a *hpt* probe, labelled in a PCR reaction with primers MC141 and MC142 using DIG-labelling mix (Roche Diagnostics, Mannheim, Germany). After 16–20 h, blots were washed twice with 2× SSC; 0.1% SDS at room temperature and three times with 0.2×SSC; 0.1% SDS at 65 °C. Detection was performed using the DIG wash and block buffer set and CDP-star (Roche Diagnostics, Mannheim, Germany) according the manufacturers protocol.

Acknowledgements

We want to thank Dr S. Chandrasegaran for the plasmid containing the *Fok*I coding sequence and Martin Brittijn for preparation of the figures. This work was financially supported by a grant from the Dutch ministry of economical affairs (GenTap IS054064) and a grant from the EU (Recbreed KBBE-2008-227190).

References

An, G., Lee, S., Kim, S.H. and Kim, S.R. (2005) Molecular genetics using T-DNA in rice. *Plant Cell Physiol.*, **46**, 14–22.

Becker, D., Kemper, E., Schell, J. and Masterson, R. (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.*, **20**, 1195–1197.

Beumer, K., Bhattacharyya, G., Bibikova, M., Trautman, J.K. and Carroll, D. (2006) Efficient gene targeting in Drosophila with zinc finger nucleases. *Genetics*, **172**, 2391–2403.

Bibikova, M., Golic, M., Golic, F. and Carroll, D. (2002) Targeted chromosomal cleavage and mutagenesis in Drosophila using Zinc-finger nucleases. *Genetics*, **161**, 1169–1175.

Bitinaite, J., Wah, D.A., Aggarwal, A.K. and Schildkraut, I. (1998) Fok1 dimerization is required for DNA cleavage. *Proc. Natl Acad. Sci. USA*, **95**, 10570–10575.

Cai, C.Q., Doyon, Y., Ainley, W.M., Miller, J.C., DeKelver, R.C., Moehle, E.A., Rock, J.M., Lee, Y.-L., Garrison, R., Schulenberg, L., Blue, R., Worden, A., Baker, L., Faraji, F., Zhang, L., Holmes, M.C., Rebar, E.J., Collingwood, T.N., Rubin-Wilson, B., Gregory, P.D., Urnov, F.D. and Petolino, J.F. (2008) Targeted transgene integration in plant cells using designed zinc finger nucleases. *Plant Mol. Biol.*, **69**, 699–709.

Camenisch, T.D., Brilliant, M.H. and Segal, D.J. (2008) Critical parameters for genome editing using zinc finger nucleases. *Med. Chem.*, **8**, 669–676.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735–743.

Cornu, T.I., Thidodeau-Beganny, E., Alwin, S., Eichtinger, M., Joung, J.K. and Cathomen, T. (2008) DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol. Ther.*, **16**, 352–358.

Desfeux, C., Clough, S.J. and Bent, A.F. (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the Arabidopsis floral-dip method. *Plant Phys.*, **123**, 895–904.

Doyon, Y., McCammon, J.M., Miller, J.C., Faraji, F., Ngo, C., Katibah, G.E., Amora, R., Hocking, T.D., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D. and Amacher, S.L. (2007) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.*, **26**, 702–708.

Dreier, B., Beerli, R.R., Segal, D.J., Flippin, J.D. and Barbas, 3rd C.F. (2001) Development of zinc finger domains for recognition of the 5′-ANN-3′ family of DNA sequences and their use in the construction of artificial transcription factors. *J. Biol. Chem.*, **276**, 29466–29478.

Dreier, B., Fuller, R.P., Segal, D.J., Lund, C.V., Blancafort, P. and Huber, A. (2005) Development of zinc finger domains for recognition of the 5′-CNN-3′ family DNA sequences and their use in the construction of artificial transcription factors. *J. Biol. Chem.*, **280**, 35588–35597.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B.F., Ljung, K., Sandberg, G., Hooykaas, P.J.J., Palme, K. and Offringa, R. (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science*, **306**, 862–865.

Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, G.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucl. Acids Res.*, **20**, 1425.

Halfter, U., Morris, P.C. and Willmitzer, L. (1992) Gene targeting in *Arabidopsis thaliana*. *Mol. Gen. Genet.*, **231**, 186–193.

Händel, E.-M., Alwin, S. and Cathomen, T. (2009) Expanding or restricting the target site repertoire of zinc-finger nucleases: the inter-domain linker as a major determinant of target site selectivity. *Mol. Ther.*, **17**, 104–111.

Hanin, M., Volrath, S., Bogucki, A., Ward, E. and Paszkowski, J. (2001) Gene targeting in *Arabidopsis*. *Plant J.*, **28**, 671–677.

Hrouda, M. and Paszkowski, J. (1994) High fidelity extrachromosomal recombination and gene targeting in plants. *Mol. Gen. Genet.*, **243**, 106–111.

Kaeppler, S.M., Kaeppler, H.F. and Rhee, Y. (2000) Epigenetic aspects of somaclonal variation in plants. *Plant Mol. Biol.*, **43**, 179–188.

Kuhfittig-Kulle, S., Feldmann, E., Odersky, A., Kuliczkowska, A., Goedecke, W., Eggert, A. and Pfeiffer, P. (2007) The mutagenic potential of non-homologous end-joining in the absence of the NHEJ core factors Ku70/80, DNA PKcs and XRCC4-LigIV. *Mutagenesis*, **22**, 217–233.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology*, **9**, 963–967.

Lee, K.Y., Lund, P., Lowe, K. and Dunsmuir, P. (1990) Homologous recombination in plant cells after Agrobacterium-mediated transformation. *Plant Cell*, **2**, 415–425.

Lindhout, B.I., Pinas, J.E., Hooykaas, P.J.J. and van der Zaal, B.J. (2006) Employing libraries of zinc finger artificial transcription factors to screen for homologous recombination mutants in Arabidopsis. *Plant J.*, **48**, 475–483.

Lindhout, B.I., Fransz, P., Tessadori, F., Meckel, T., Hooykaas, P.J.J. and van der Zaal, B.J. (2007) Live cell imaging of repetitive DNA sequences via GFP-tagged polydactyl zinc finger proteins. *Nucl. Acids Res.*, **35**, e107. doi:10.1093/nar/gkm618.

Liu, Y.-G., Mitsukawa, N., Oosumi, T. and Whitter, R. F. (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* **8**, 457–463.

Liu, Q., Xia, Z., Zhong, X. and Case, C.C. (2002) Validated zinc finger protein designs for all 16 GNN DNA triplet targets. *J. Biol. Chem.*, **277**, 3850–3856.

Lloyd, A., Plaisier, C.L., Carroll, D. and Drews, G. (2005) Targeted mutagenesis using zinc-finger nucleases in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **102**, 2232–2237.

Lombardo, A., Genovese, P., Beausejour, C.M., Colleoni, S., Lee, Y.-L., Kim, K.A., Ando, D., Urnov, F.D., Galli, C., Gregory, P.D., Holmes, M.C. and Naldini, L. (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat. Biotechnol.*, **25**, 1298–1306.

Maeder, M.L., Thibodeau-Beganny, S., Osiak, A., Wright, D.A., Anthony, R.M., Eichtinger, M., Jiang, T., Foley, J.E., Winfrey, R.J., Townsend, J.A., Unger-Wallace, E., Sander, J.D., Müller-Lerch, F., Fu, F., Pearlberg, J., Göbel, C., Dassie, J.P., Pruett-Miller, S.M., Porteus, M.H., Sgroi, D.C., Lafrate, A.J., Dobbs, D., McCray Jr, P.B., Cathomen, T., Voytas, D.F. and Joung, J.K. (2008) Rapid ''open-source'' engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol. Cell*, **31**, 294–301.

Mani, M., Smith, J., Kandavelou, K., Berg, J.M. and Chandrasegaran, S. (2005) Binding of two zinc finger nuclease monomers to two specific sites is required for effective double-strand DNA cleavage. *Biochem. Biophys. Res. Commun.*, **334**, 1191–1197.

McVey, M. and Lee, S.E. (2008) MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet.*, **24**, 529–538.

Meijer, A.H., Ouwerkerk, P.B.F. and Hoge, J.H.C. (1999) Vectors for transcription factor cloning and target site identification by means of genetic selection in yeast. *Yeast*, **14**, 1407–1416.

Melcher, K., Sharma, B., Ding, W.V. and Nolden, M. (2000) Zero background reporter plasmids. *Gene*, **247**, 53–61.

Miao, Z.H. and Lam, E. (1995) Targeted disruption of the TGA3 locus in *Arabidopsis thaliana*. *Plant J.*, **7**, 359–365.

Moehle, E.A., Rock, J.M., Lee, Y.-L., Jouvenot, Y., DeKelver, R.C., Gregory, P.D., Urnov, F.D. and Holmes, M.C. (2007) Targeted gene addition into a specific location in the human genome using designed zinc finger nucleases. *Proc. Natl Acad. Sci. USA*, **104**, 3055–3060.

Mohan Jain, S. (2001) Tissue culture-derived variation in crop improvement. *Euphytica*, **118**, 153–166.

Mumberg, D., Müller, R. and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*, **156**, 119–122.

Neuteboom, L.W., Lindhout, B.I., Saman, I.L., Hooykaas, P.J.J. and van der Zaal, B.J. (2006) Effects of different zinc finger transcription factors on genomic targets. *Biochem. Biophys. Res. Commun.*, **339**, 263–270.

Offringa, R., de Groot, M.J., Haagsman, H.J., Does, M.P., van den Elzen, P.J. and Hooykaas, P.J.J. (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after Agrobacterium-mediated transformation. *EMBO J.*, **9**, 3077–3084.

Paszkowski, J., Baur, M., Bogucki, A. and Potrykus, I. (1988) Gene targeting in plants. *EMBO J.*, **7**, 4021–4026.

Porteus, M.H. and Caroll, D. (2005) Gene targetting using zinc finger nucleases. *Nat. Biotechnol.*, **23**, 967–973.

Pruett-Miller, S.M., Reading, D.W., Porter, S.N. and Porteus, M.H. (2009) Attenuation of zinc finger nuclease toxicity by small-molecule regulation of protein levels. *PLoS Genet.*, **5**(2), e1000376. doi:10.1371/journal.pgen.1000376.

Puchta, H., Dujon, B. and Hohn, B. (1996) Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. *Proc. Natl Acad. Sci. USA*, **93**, 5055–5060.

Risseeuw, E., Offringa, R., Franke-van Dijk, M.E.I. and Hooykaas, P.J.J. (1995) Targeted recombination in plants using Agrobacterium coincides with additional rearrangements at the target locus. *Plant J.*, **7**, 109–119.

Sega, D.J., Dreier, B., Beerli, R.R. and Barbas, 3rd C.F. (1999) Towards controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5′-GNN-3 ′ DNA target sequences. *Proc. Natl Acad. Sci. USA*, **96**, 2758–2778.

Shukla, V.K., Doyon, Y., Miller, J., DeKelver, R.C., Moehle, E.A., Worden, S.E., Mitchell, J.C., Arnold, N.L., Gopalan, S., Meng, X., Choi, V.M., Rock, J.M., Wu, Y.-Y., Katabah, G.E., Zhifang, G., McCaskill, D., Simpson, M.A., Blakeslee, B., Greenwalt, S.A., Butler, H.J., Hinkley, S.J., Zhang, L., Rebar, E.J., Gregory, P.D. and Urnov, F.D. (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature*, doi:10.1038/nature07992.

Smith, J., Berg, J.M. and Chandrasegaran, S. (1999) A detailed study of the substrate specificity of a chimeric restriction enzyme. *Nucl. Acids Res.*, **27**, 674–681.

Smith, J., Bibikova, M., Withby, F.G., Reddy, A.R., Chandrasegaran, S. and Carroll, D. (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucl. Acids Res.*, **28**, 3361–3369.

Tovkach, A., Zeevi, V. and Tzfira, T. (2009) A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. *Plant J.*, **57**, 747–757.

Townsend, J.A., Wright, D.A., Winfrey, R., Fu, F., Maeder, M.L., Joung, J.K. and Voytas, D.F. (2009) High-frequency modification of plant genes using zinc-finger nucleases. *Nature*, **459**, 442–445.

Urnov, F.D., Millar, J.C., Lee, Y.-L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D. and Holmes, M.C. (2005) Highly efficient endogenous human gene correction using zinc-finger nucleases. *Nature*, **435**, 646–651.

Weijers, D., Franke-van Dijk, M., Vencken, R.-J., Quint, A., Hooykaas, P. and Offringa, R. (2001) An *Arabidopsis* Minute-like phenotype caused by a semi-dominant mutation in a RIBOSOMAL PROTEIN S5 gene. *Development*, **128**, 4289–4299.

Wright, D.A., Townsend, J.A., Winfrey Jr, R.J., Irwin, P.A., Rajagopal, J., Lonosky, P.M., Hall, B.D., Jondle, M.D. and Voytas, D.F. (2005) High-frequency homologous recombination in plants mediated by zinc-finger nucleases. *Plant J.*, **44**, 693–705.

Zale, J.M., Agarwal, S., Loar, S. and Steber, C.M. (2009) Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens*. *Plant Cell Rep.*, **28**, 903–913.