Corrections

PHYSICS, BIOPHYSICS AND COMPUTATIONAL BIOLOGY

The authors note that reference 19 appeared incorrectly. The updated reference appears below. This error does not affect the conclusions of the article.


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PLANT BIOLOGY

The authors wish to note that they inadvertently copied text in their introductory paragraph and in the first three sentences of their second paragraph from reference 15 [Lloyd A, Plaisier CL, Carroll D, Drews GN (2005) Targeted mutagenesis using zinc-finger nucleases in Arabidopsis. Proc Natl Acad Sci USA 102:2232–2237] without proper attribution. The authors apologize for the oversight.

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The authors note the following statement should be added to the Acknowledgments: “We also acknowledge the support of The Knut and Alice Wallenberg Foundation (Project KAW 2004.0119) and the Medical Faculty at Lund University.”

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Site-directed mutagenesis in *Arabidopsis* using custom-designed zinc finger nucleases

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Site-directed mutagenesis in higher plants remains a significant technical challenge for basic research and molecular breeding. Here, we demonstrate targeted-gene inactivation for an endogenous gene in *Arabidopsis* using zinc finger nucleases (ZFNs). Engineered ZFNs for a stress-response regulator, the *ABA-INSSENSITIVE4* (*ABI4*) gene, cleaved their recognition sequences specifically in vitro, and ZFN genes driven by a heat-shock promoter were introduced into the *Arabidopsis* genome. After heat-shock induction, gene mutations generated I cleavage sites in dsDNA breaks, increased error-prone rejoining frequency by 2.6-fold, with an increased end-degradation. These data demonstrate that an approach using ZFNs can be used for the efficient production of mutant plants for precision reverse genetics.

A major focus of plant biotechnology is genetic modification and improvement of crop plants. With this aim in mind, large-scale genome analyses have been performed for the model plant *Arabidopsis*, and also for many important crop plants species, including rice, maize, wheat, soybean, and tomato. The enormous amount of genome-sequence information now available has intensified the need for methods that can use this data to generate targeted modifications in plant genes. Site-directed mutagenesis methods could be used experimentally to investigate plant gene function or for genetic modification in plant cells. This is especially important for species that lack readily available mutant collections, including most crops.

The most widely used site-directed mutagenesis strategy is gene targeting (GT) via homologous recombination (HR). Efficient GT procedures have been available for more than 20 y in yeast (1) and mouse (2). Successful GT has also been achieved in *Arabidopsis* and rice plants (3–6). Typically, GT events occur in a fairly small proportion of treated mammalian cells (approximately 1% of the total random integration events in mouse ES cells). However, GT efficiency is extremely low in higher plant cells [0.01–0.1% of the total number of random integration events (7)]. The low GT frequencies reported in higher plants are thought to result from competition between HR and nonhomologous end joining (NHEJ) for repair of dsDNA breaks (DSBs), whereas the main pathway of DSB repair in higher plants seems to be NHEJ (8, 9). As a consequence, the ends of a donor molecule are likely to be joined by NHEJ rather than participating in HR, thus reducing GT frequency. There is extensive data indicating that DSBs repair by NHEJ in higher plants is error-prone. Often, DSBs are repaired by end-joining processes that generate insertions and/or deletions (10, 11). Taken together, these observations suggest that NHEJ-based strategies might be more effective than HR-based strategies for targeted mutagenesis in higher plants.

Indeed, expression of I-Sce I, a rare cutting restriction enzyme, has been shown to introduce mutations at I-Sce I cleavage sites in *Arabidopsis* and tobacco (12). Nevertheless, the use of restriction enzymes is limited to rarely occurring natural recognition sites or to artificial target sites. To overcome this problem, zinc finger nucleases (ZFNs) have been developed. ZFNs are chimeric proteins composed of a synthetic zinc finger–based DNA binding domain and a DNA cleavage domain. By modification of the zinc finger DNA binding domain, ZFNs can be specifically designed to cleave virtually any long stretch of dsDNA sequence (13, 14). An NHEJ-based targeted mutagenesis strategy was developed recently in several organisms by using synthetic ZFNs to generate DSBs at specific genomic sites (15–19). Subsequent repair of the DSBs by NHEJ frequently produces deletions and/or insertions at the joining site.

To our knowledge, two groups have successfully applied ZFNs to genetically modify genes in zebrafish embryos by using specific zinc finger motifs engineered to recognize distinct DNA sequences (16, 17). The ZFN-encoding mRNA was injected into one-cell embryos and a high percentage of animals carried the desired mutations and phenotypes. These latter studies demonstrated that ZFNs can specifically and efficiently create heritable mutant alleles at loci of interest in the germ line, and that ZFN-induced alleles can be propagated in subsequent generations.

Although precise genetic modification using ZFNs has been successfully applied to higher plants (15, 20, 21), to our knowledge, only a study of Lloyd et al. (15) presented a detailed analysis of the NHEJ-based targeted-mutation strategy with a model system using a synthetic target site for a previously reported three-finger-type ZFN_QQR (22) in the *Arabidopsis* genome. Thus, the next step in the establishment of this approach is to target endogenous gene loci in the genome of higher plants. In addition, further investigation into the precise conditions and effectors required for application of ZFNs in plants would also be invaluable.

In this report, we show that ZFNs can efficiently cleave and stimulate mutations at an endogenous target gene in *Arabidopsis*. For this demonstration, we selected the *ABA-INSSENSITIVE4* (*ABI4*) gene as a target gene. *ABI4* encodes a member of the ERF/AP2 transcription factor family and plays a role in regulating abscisic acid (ABA) (23), which controls a number of agronomically important traits, including plant responses to abiotic stress and seed development (24). We achieved targeted mutagenesis at a rate of approximately 0.26% to 2.86% in *Arabidopsis* somatic cells, and transmission of the induced mutation in the

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target gene to subsequent generations. The mutant line showed the expected mutant phenotypes. In addition, we applied ZFN-mediated targeted mutagenesis to the NHEJ-deficient Arabidopsis mutant altau80. We found that deficiency of Ku80 enhances error-prone rejoining of ZFN-cleavage ends, with increased end-degradation. These results indicate that ZFNs can form the basis of a highly efficient method for site-directed mutagenesis of higher plant genes.

Results

Custom Designed ZFNs Targeting the ABI4 Gene. To demonstrate ZFN-mediated site-directed mutagenesis in Arabidopsis using the ABI4 gene as a target gene, we first identified full consensus ZFN target sites \(5^\prime\)-NCNCCNCN\(\text{N}(\text{N}4\sim7\text{)}\)NGNGNNNN3\(\prime\) (\(N = A, C, G, \text{and } T\)) in ABI4 (Fig. 1). A combination of the three ZF arrays for \(5^\prime\)-GGAGGAGGA-3\(\prime\) (ZF\_AAA) and \(5^\prime\)-G-TGGCCGGCG-3\(\prime\) (ZF\_TCC) targeting ABI4 was designed using the zinc finger modules for 5\(\prime\)-GNN-3\(\prime\) triplets reported by Liu et al. (25) and Segal et al. (26) (SI Materials and Methods, Fig. S1, and Table S1). ZF arrays assembled in the pGP-FB vector were tested in quantitative bacterial two-hybrid (B2H) assays. According to LacZ reporter assay results, all assembled ZF arrays showed high affinity for their target, with transcription activated by more than fourfold (Fig. S2). Thus, these modular assembled ZF proteins were used in the construction of target ZFNs. We next performed in vitro DNA digestion assays with ZFN proteins synthesized using an in vitro translation system with wheat germ extracts (Fig. S3, A and B). We confirmed that the combination of ZFN\_AAA and ZFN\_TCC digested the target sequence of the ABI4 gene (Fig. S3 C and D).

Engineered ZFNs Stimulate Mutations at Target Sequences in Arabidopsis Cells. To determine whether induction of ZFN activity could digest the Arabidopsis genome in vivo and induce mutations at the recognition sequence in Arabidopsis cells, we introduced the ZFN expression vector pPl.2gbPsZFN\_ABI4 (Fig. 2A) into the Arabidopsis genome via Agrobacterium-mediated transformation. In this study, the Arabidopsis heat shock protein HSP18.2 gene promoter (28) was used to drive the expression of ZFNs. This expression cassette allows inducible expression of ZFNs transiently upon heat shock, and thus has the benefit of avoiding cell toxicity (29). We transformed Arabidopsis plants (ecotype Col-0) with pPl.2gbPsZFN\_ABI4 and selected for transgenic lines. We refer to lines with introduced the ZFN expression vector pP1.2gfbPhsZFN\_ABI4 as zfn\_abi4-1, -2, -3, -5, -7, -9, -11, -12, and -13. To induce ZFN expression, 12-d-old plants from each transgenic line were subjected to a heat pulse at 40 °C for 90 min; genomic DNA was isolated from true leaves of these plants 48 h after heat shock. We also isolated genomic DNA from true leaves of these lines before heat induction as a control. To determine whether induction of ZFN activity could induce mutations at its recognition sequence, we developed a method of identifying mutations coupled with a mismatch-specific endonuclease: Surveyor nuclease. PCR using primers flanking the ZFN recognition sequence was performed on genomic DNA extracted from cells treated with ZFNs. PCR products were cloned into pCR-TOPO vector, and plasmid clones possessing ZFN-mediated mutations were identified using the Surveyor nuclease assay described in Materials and Methods. The DNA sequences of clones showing a positive signal in the Surveyor nuclease assay was determined to verify that these clones contained mutations within the ZFN target sequence. The mutation frequency in somatic cells was calculated as the number of clones containing mutations per total number of randomly picked clones. As summarized in Table 1, in heat-treated seedlings, 0.26% to 2.86% of clones contained ZFN-induced mutations in the ABI4 target sequence. In contrast, control seedlings, in which ZFN expression was not induced by heat pulse, gave rise to no clones containing a mutation. Of 47 clones sequenced, 33 (70%) contained substitution mutations, and 14 (30%) were simple deletions of 1 to 3 bp in the target sequence of zfn\_abi4 lines (Fig. 2B and Table S2). These data indicate that ZFNs can efficiently stimulate mutations at their recognition sequences in Arabidopsis cells.

![Fig. 1. Consensus ZFN target sites in the Arabidopsis ABA-INSENSITIVE4 (ABI4) gene. The schematic representation of the ABI4 gene is shown at the top. Asterisk indicates the position of the mutation in the abi4 mutant. AP, AP2 domain; ST, serine- and threonine-rich domain; Q, glutamine-rich domain; Acid, activation domain. Target sites of ZFN monomers are highlighted with gray bars. The putative cleavage region is shown in lowercase letters. Mutations are indicated in purple boxes. The putative site of the double-strand break is double-underlined. Predicted repaired DNA sequences at ZFN target sites (bold) are shown in Fig. S3 C and D.](image-url)
of mutations in zfn_abi4 lines. If mutations are induced in the L2 cells of the shoot apical meristem, these cells possessing mutations form a sector in the primary inflorescence that incorporates germ line cells. To detect such mutation sectors, genomic DNA extracted from several flower clusters, including the terminal flower, of each zfn_abi4 line was analyzed for the presence of mutations at the cleavage site in ABI4 with the Surveyor nuclease assay. We detected mutations in two of nine lines, zfn_abi4-1 and zfn_abi4-9 (Fig. S4). We further collected progeny seeds (approximately 300 seeds) from the primary inflorescence of the two lines, and scored progeny seedlings with mutations at the cleavage site were identified: seven plants from 96 of randomly selected plants in zfn_abi4-1 (Fig. S4). Sequence analysis revealed that all seven progeny seedlings showed a single base deletion at the same position, which produced a stop codon after Thr158 of ABI4 (Fig. 2C). It was thought that they are heterozygous mutants for the ABI4 locus, according to the Surveyor nuclease assay, because heteroduplex DNA of the ABI4 fragment was detected with this assay by using genomic DNA from only a single mutant plant. These results suggest that a single cell harboring the mutation created by ZFN-cleavage produced a sector, and the sector produced germ line cells, which exist as chimeras in reproductive tissue. In zfn_abi4-9, progeny seedlings containing mutations at the cleavage site were also identified: three plants from 96 of randomly selected plants (Fig. S4). All three were substitution mutations at the same position, which caused an amino acid change at Val159 (V159 to Ile, Fig. 2C) and correspond to mutants heterozygous for the ABI4 locus as seen in line zfn_abi4-1 according to sequencing and Surveyor nuclease analyses. For subsequent phenotypic analysis, we selected a single plant each from zfn_abi4-1 and zfn_abi4-9 progeny plants, named zfn_abi4-1 and zfn_abi4-9–1, respectively. T3 progeny of zfn_abi4-1 and zfn_abi4-9–1 were produced to select plants homozygous for these mutations. T3 seeds from T2 plants of these two lines segregated with a 1:1:2:1 ratio of homoygous mutation to heterozygous mutation to WT, following Mendelian rules (homo:hetero:WT, 9:19:12; n = 40, χ² = 0.55, P = 0.760 in zfn_abi4-1–1; homo: hetero:WT, 9:23:8, n = 40, χ² = 0.95, P = 0.622 in zfn_abi4-9–1). ABI4 is an ERF/AP2 transcription factor and plays a role in ABA and sugar signaling during seed development of Arabidopsis. Previous studies have shown that abi4 mutants are insensitive to ABA and high concentrations of sugars during seedling development (30–32). To investigate the functionality of zfn_abi4 mutations in Arabidopsis, we next asked whether zfn_abi4 mutants show a phenotype similar to that of the abi4 mutant. zfn_abi4-1–1 and zfn_abi4-9–1 mutant plants exhibited no morphological alterations under normal growth conditions; however, the germination rates of the zfn_abi4-1–1 plants and abi4, but not those of zfn_abi4-9–1 plants, were higher than those of WT plants on ABA-containing medium (Fig. 3A and C). The zfn_abi4-1–1 mutant plants were also able to grow on a high concentration of glucose (Fig. 3B and Fig. S5A), suggesting that this mutant showed ABA and glucose insensitivity. Germination of zfn_abi4-1–1 was also tolerant to NaCl and mannitol as seen in abi4 (Fig. S5 B and C).

Lack of Ku80 Reduces the Fidelity but not the Efficiency of NHEJ. As described earlier, after ZFN-ABI4 cleavage, the majority of ZFN-mediated mutations resulted in substitution-type mutations without large deletion or insertions (Table 1, Table S2, and Fig. 2B). The Arabidopsis protein AtKu80 plays a role in end-protection of DSBs. We hypothesized that a lack of Ku protein activity would result in drastic modifications such as large deletion and/or insertion at DSB ends. To test this hypothesis, we explored the type and frequency of mutations in the NHEJ-deficient Arabidopsis mutant plant, atku80 (ecotype WS background, ref. 33). Heterozygous Atku80/atku80 plants were transformed with pPi.2gfbPhsZFN-ABI4, and three T1 transgenic lines of heterozygous Atku80/atku80 plants were recovered by PCR genotyping. Segregated WT and homozygous atku80/atku80 plants were recovered from three lines (lines 80–1, 80–2, and 80–3). These lines showed a 3:1 ratio of segregation for blasticidin-SR to blasticidin-SR when T2 seeds were plated on selection medium. After obtaining T3 seeds, we recovered plant lines segregating for WT (Atku80/Atku80) and homozygous for blasticidin-SR, and lines homozygous for atku80/atku80 and blasticidin-SR from 80–1, 80–2, and 80–3, respectively. These lines were used to test for ZFN-mediated mutagenesis. Fig. 4 shows a comparison of the mutation frequency and spectrum in the segregating WT and atku80 plants. As seen in Fig. 4A, the mutation frequency of ecotype WS is similar to that seen earlier with ecotype Col-0 (Table 1). Furthermore, we found that the mutation frequency in atku80 was comparable to that in WT (Fig. 4A). It is interesting to note that the fraction of

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**Table 1. Frequency of ZFN-induced mutations in the ABI4 gene**

<table>
<thead>
<tr>
<th>Treatment/line</th>
<th>Frequency of mutations, % (no. of clones tested)</th>
<th>Deletion mutations/total number of mutations (percentage of deletion mutations)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat induced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>zfn-abi4-1</td>
<td>2.86 (384)</td>
<td>4/11 (36.3%)</td>
</tr>
<tr>
<td>zfn-abi4-2</td>
<td>1.30 (384)</td>
<td>1/5 (20.0%)</td>
</tr>
<tr>
<td>zfn-abi4-3</td>
<td>1.82 (384)</td>
<td>2/7 (28.6%)</td>
</tr>
<tr>
<td>zfn-abi4-5</td>
<td>0.78 (384)</td>
<td>1/3 (33.3%)</td>
</tr>
<tr>
<td>zfn-abi4-7</td>
<td>0.52 (384)</td>
<td>0/2 (0%)</td>
</tr>
<tr>
<td>zfn-abi4-9</td>
<td>2.34 (384)</td>
<td>2/9 (22.2%)</td>
</tr>
<tr>
<td>zfn-abi4-11</td>
<td>1.82 (384)</td>
<td>3/7 (42.9%)</td>
</tr>
<tr>
<td>zfn-abi4-12</td>
<td>0.52 (384)</td>
<td>1/2 (50%)</td>
</tr>
<tr>
<td>zfn-abi4-13</td>
<td>0.26 (384)</td>
<td>0/1 (0%)</td>
</tr>
<tr>
<td>Not induced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>zfn-abi4-1</td>
<td>0 (768)</td>
<td>–</td>
</tr>
<tr>
<td>zfn-abi4-9</td>
<td>0 (768)</td>
<td>–</td>
</tr>
<tr>
<td>zfn-abi4-11</td>
<td>0 (768)</td>
<td>–</td>
</tr>
</tbody>
</table>

*The percentage of deletion mutations was calculated as follows: percentage of deletion mutations = no. of deletion mutations/total no. of mutations × 100.
The mutation frequency achieved here clearly could enable an efficient site-directed mutagenesis procedure in higher plants, in contrast to conventional GT procedures without the help of ZFNs, which have much lower mutation frequencies; typically fewer than 10^{-7} GT events per cell or 10^{-8} to 10^{-9} GT events per integration event (15). The dramatically higher mutation frequencies with the ZFN-mediated procedure can be compared with those obtained by HR-mediated procedures, supporting the view that the error-prone end-joining pathway for DSBs repair is highly active in Arabidopsis somatic cells. The frequency of mutagenesis identified here (Table 1) is comparable to, albeit slightly lower than, that identified for similar experiments with ZFNs reported by others. Heat-inducible expression of ZFN-QQR (22) in Arabidopsis produced approximately 7% mutated progeny seedlings with an artificial target site (15). Maeder et al. (34), by using induced ZFN SR2163, found a mutation frequency of 5% at the tobacco SuIR4 locus. These differences might be a result of differences in protein stability and/or activity of ZFNs. In addition, accessibility of ZFNs to chromatinized DNA might depend on ZFN design and each gene locus. These, and perhaps other yet-uncharacterized, features could affect mutation frequency.

We found many substitution mutations at the edge of the putative DNA cleavage sequence (Fig. 2B and Tables S2 and S3). One possible mechanism that might explain this observation is the single-base insertion coupled with single-base deletion caused by the MRE11/RAD50 complex. This complex has exonuclease activity, but can delete a few bases, and functions to process DSB ends to make blunt ends (8). It is interesting to note that the mutation profile obtained in this study is slightly different from that found in previous studies with I-Sce I (12) and ZFNQQR (15) for target cleavage sites at the synthetic and exogenous integrated gene loci of Arabidopsis. These studies showed that the majority of mutations were highly varied, with longer deletions and/or insertions of 1 to 20 bp, and sometimes much longer (12, 15). One possible explanation for these differences is that binding activity to chromatinized target DNA of ZFNs could differ in each study, thus triggering different types of repair pathways or factors. Thus, longer end-degradation at the cleavage sites may have occurred in other studies compared with ours. We thought it possible that there might be higher activity of end-protection by DNA-PK/Ku at the cleavage sites under our conditions in a target gene locus–dependent manner, and depending on the cleavage activity of the specific ZFNs used.

We compared ZFN-mediated mutations in WT and Ku80-deficient mutant plants. As shown in Fig. 4 B and C, our data clearly indicate that deficiency of AtKu80 increases deletion size at the cleavage site of ABI4 as expected. We also found that a high level of NHEJ mutagenesis was maintained in the absence of Ku80, suggesting that a secondary, inaccurate repair pathway(s) function in Arabidopsis cells (Fig. 4A). Schulte-Uentrop et al. (35) reported similar results with Ku80-deficient mouse cells; efficient end-joining activity was observed in both WT and Ku80-deficient mouse cells after introducing DSBs enzymatically. However, deletions at the break site in Ku80-deficient mouse cells were significantly longer than in WT mouse cells.

A recent study suggested that the DNA-PK–dependent pathway involving Ku80 competes with the DNA repair pathway involving PARP-1/XRCC1/DNA ligase II (LigIII) in human cells (36). Counterpart genes for PARP1 and XRCC1 genes have been identified in the Arabidopsis genome, but a counterpart gene for the LIGIII gene is lacking. Recently, Waterworth et al. (37) reported that Arabidopsis DNA ligase I plays an important role in DSB repair as well as in the repair pathway of single-stranded DNA breaks. Thus, it is highly possible that ligase I participates in the alternative end-joining repair pathway instead of LigIII, and that this pathway acts as a backup pathway for NHEJ in Ku80-deficient Arabidopsis cells. In addition, the microhomology-mediated end joining pathway involving PARP-1/XRCC1/LigIII

#### Error-Prone End-Joining Pathway (s) repair events increased by approximately 2.6 times in atku80 cells (Fig. 4 B and C and Table S3). In particular, deletions more than 4 bp in length increased in atku80 drastically in as many as 70% of the mutants (Fig. 4B). Thus, we conclude that AtKu80 plays a role in end-protection of enzymatically induced DSBs, although other end-joining pathway(s) repair DSB ends efficiently in Arabidopsis cells.

### Discussion

Gene knockout/inactivation is the most powerful tool for determining gene function or to permanently modify phenotype for molecular breeding in higher plants. Currently available methods for gene disruption in higher plants are limited by their efficiency, time to completion, and the potential for confounding off-target effects.

This study demonstrates the feasibility of a ZFN-based approach to site-directed mutagenesis in an endogenous gene of Arabidopsis using ABI4 (23) as a target gene. The mutation frequency achieved at the ZFN target sites in the ABI4 gene was sufficient to allow easy identification of mutants following heat-induced ZFN expression (Table 1). We further confirmed transmission of mutations to the next generation. Two transgenic lines (zfn_abi4-1 and zfn_abi4-9) from a total of nine lines gave rise to transmitted mutations (Fig. 2C and Fig. S4), and mutations in these lines were further transmitted to their progeny following Mendelian rules. The physiological phenotypes of mutant lines produced via ZNF-mediated cleavage in terms of stress and ABA responses were confirmed as being similar to those of mutants previously obtained by using the other method. zfn_abi4-1–1 plants clearly showed insensitivity to ABA and glucose (Fig. 3 and Fig. S5).

![Fig. 4. Efficiency and fidelity of NHEJ repaired DNA after ZFN cleavage in the ABI4 gene in atku80 cells. (A) The frequency of end-joining after induction of DSBs by ZFNs. The relative error-prone end-joining frequency was derived from the number of DNA clones positive for the Surveyor nuclease assay per number of DNA clones tested (n = 480 for each experiment). Experiments were performed with three independent lines, and data are presented as mean ± SD. (B) Distribution of length of deletions at individual junctions. Deletions are defined as the sum of base pairs lost at both sides of the DSB. (C) Examples of repaired DNA sequences obtained from genomic DNA of WT and atku80 plants after ZFN cleavage. ZFN recognition sites are depicted in bold. Putative cleavage regions are shown in lowercase characters. Mutations are shown in magenta-colored characters and deletion are shown as hyphens.](image-url)
(38) might also function as an error-free repair pathway. ZFNs produce 5'-cohesive ends with perfectly 4b-matched sequences, and these fragments could be employed for microhomology-mediated end joining without any modification following ZFNs cleavage. Furthermore, in the case of Ku80-deficient cells, exonuclease 1 could function efficiently compared with WT cells (39). Thus, the 3'-single-strand tail produced by exonuclease 1 might also lead to the HR repair pathway for restriction enzyme-mediated cleavage, although this pathway is restricted to the S-to-G2 phase of the cell cycle (40).

In conclusion, we have established a method for ZFN-mediated site-directed mutagenesis in Arabidopsis. By using this system, we compared profiles of mutations between WT and Ku80-deficient mutant cells. We found that a deficiency in Ku80 increased the frequency of mutations showing longer deletions of bases at the repair sites. The data indicate the existence of an alternative NHEJ pathway in Arabidopsis, and that this pathway is highly active under conditions of deficiency of the Ku80-dependent NHEJ pathway. Our data further suggest that coupling of ZFNs and Ku80-deficient cell lines could be a powerful tool with which to create many types of mutations, including substitutions, and short and long deletions of bases, at the gene of interest. In addition, this method can be further applied to crop plants such as rice, maize, wheat, soybean, and tomato, as sequence information and transformation systems are available in these species. The ZFN technology can be easily applied to establish mutation lines of genes of interest for molecular breeding.

Materials and Methods

Plant Material and Growth. Arabidopsis thaliana ecotypes Columbia (Col-0) and Wassilevskija (Ws) were used in this study. Plants were grown on a soil mixture of equal parts of vermiculite and commercial soil (Super Mic; Sakata Seed) in a growth chamber at 22 °C. Seeds were germinated and incubated on plates containing half-strength Murashige and Skoog (MS) medium solidified with 0.25% Gelrite (MS Gelrite plate; Wako) in a growth chamber at 22 °C. Seeds of the abi4 mutant were obtained from the Arabidopsis Biological Resource Center. The T-DNA insertion mutant line for AtKu80B was kindly provided by C. M. Bray (University of Manchester, Manchester, UK; ref. 33). The PCR genotyping of atku80 plants was performed according to West et al. (33).

Construction of ZFNs. ZFNs were constructed using the modular assembly method previously reported by Wright et al. (41) with slight modifications (for details, see SI Materials and Methods and Fig. S1). Amino acid sequences of helix motif in ZF are used as follows: QRAHLER for finger 1, QSGLHQLR for finger 2, and QRHALER for finger 3 in ZF_AAA; and RSDALTR for finger 1, RSDDLR for finger 2, RSDDLQ for finger 3 in ZF_TCC. The coding sequences of ZF proteins were cloned between the XbaI and BamHI sites of the pGFB8-vector (41).

Quantitative B2H Assay. B2H assays were performed according to the protocol of Wright et al. (41). Plasmid DNA and bacterial strains for B2H were obtained from Addgene.

In Vitro DNA Digestion Assay. ZFN proteins were synthesized using in vitro translation system with wheat germ extracts. Details of protein synthesis and purification in vitro digestion assay are provided in SI Materials and Methods.

Expression of ZFNs in Arabidopsis Cells. The plasmid pH2pFpsZFN_ABI4 was transformed into Agrobacterium tumefaciens strain GV3101 by electroporation and introduced into Arabidopsis by the floral dipping method (42). Transgenic plants were selected on medium containing 1.5 μg/mL lincomycin and 5 μg/mL of kanamycin, and by the appearance of GFP fluorescence (43). Seedlings were grown on plates at 22 °C for 12 d. The plates then were wrapped in plastic wrap and immersed in water at 40 °C for 90 min. Seedlings were grown for an additional 48 h at 22 °C before DNA extraction.

Analysis of Genome Editing at ZFN Target Sites. To analyze mutations ZFN-induced mutations, the 685-bp region surrounding the ZFN_AAA/ZFN_TCC pair site for ABI4 was amplified by PCR with the high fidelity DNA polymerase KOD-Plus (Toyobo). PCR products were analyzed with the Surveyor Mutation Detection Kit (Transgenicom). The detail for PCR and the nuclease assay conditions were described in SI Materials and Methods. To determine sequences of ZFN-induced mutations, the initial PCR products were cloned into the plasmid pCR-TOPO vector (Invitrogen) and Escherichia coli colonies containing ABI4 gene fragments were randomly picked. These colonies were replicated, transformed into E. coli, and these fragments could be employed for microhomology-mediated break repair. Typical results are shown in Fig. S6. The batches containing the mutations according to the Surveyor nuclease assay were reanalyzed with each single colony. Finally, the plasmid from the single colony showing a mutation signal according to the Surveyor nuclease assay was isolated and its sequence determined. To determine transmission of ZFN-induced mutations, progeny seeds from lines zfn_abi4-1 and -9 were plated on MS medium. Genomic DNA was extracted individually from true leaves of 96 randomly picked plants. Twelve DNA samples were batched and used for the Surveyor nuclease assay. Batches containing the mutations according to the Surveyor nuclease assay were reanalyzed with each single DNA sample.

Seed Germination and Root Growth Assays. Germination assays were performed as previously described (44, 45). Seeds were surface-sterilized, suspended in sterile 0.1% agar, and placed on MS plates containing 1% sucrose, 0.5 mM ABA, 6% glucose, 150 mM NaCl, or 300 mM mannitol and 0.8% agar and grown in a 16:8 h light/dark cycle at 22 °C. Germination (i.e., radical emergence) was scored at various times. Root growth assays were performed as follows. Seeds were surface-sterilized as described earlier and germinated on MS plates containing 1% sucrose and 0.8% agar at 22 °C. After 5 d, seedlings were transferred to plates containing various concentrations of NaCl, turned 90°, and incubated for additional 5 d. Root length was measured at different times after seed plating.

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gene demonstrates an open-source encode an APETALA 2 domain protein. 


