Breaking news: Plants mutate right on target

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For millennia, early human civilizations observed phenotypic changes in animals and plants and used these for domestication (1). In recent decades, scientists around the world induced random mutations, mainly in crop plants, to widen the mutation spectra to be used for extensive screening for varieties useful for agriculture and science (2). As mutagens, ethyl methanesulfonate, radiation, *Agrobacterium tumefaciens*-mediated T-DNA transformation, and transposon mutagenesis have been used. Distinction between WT and mutant was dependent on the phenotype, on sequence specificity of the mutagenizing DNA (in case of transposons or T-DNA), or could be accomplished with tilling. Alternatively, gene expression could be suppressed by use of small interfering RNAs. Targeted mutagenesis in plants, however, was only recently developed, and examples of Zinc finger nuclease (ZFN)-mediated targeting of natural genes by homologous recombination have been published recently (3–6). Now, an even more straightforward technique for mutation, the site-specific breaking and error-prone repair of endogenous genes in *Arabidopsis thaliana* by the plant machinery, is the topic of two reports presented in PNAS (7, 8). A similar approach using a custom-made meganuclease was also reported for maize (9).

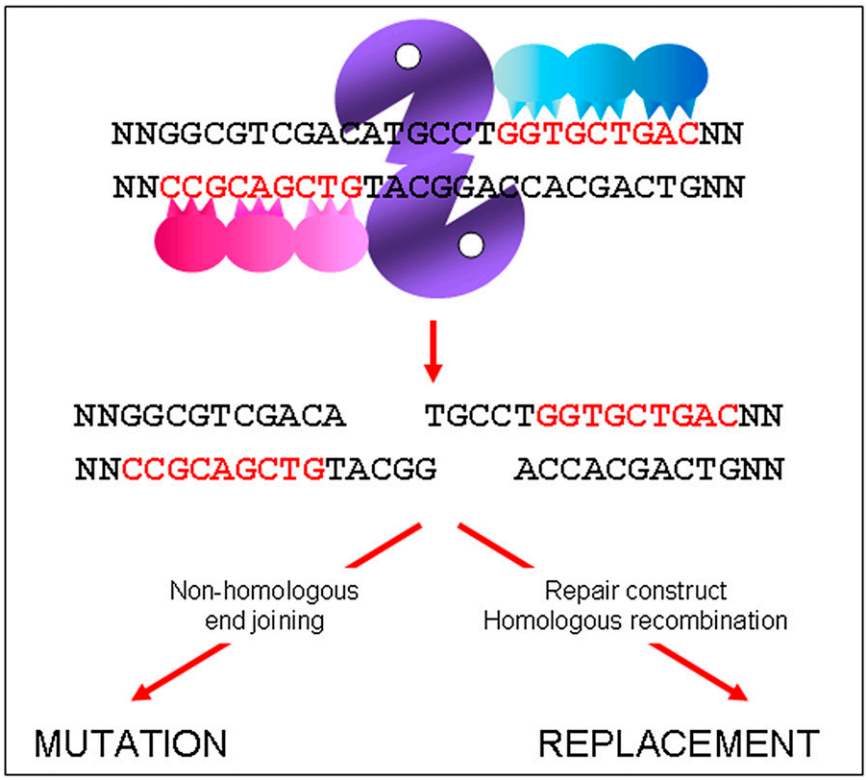

Fig. 1. Target for ZFN, with the two different subunits of the enzyme drawn below and above the target sequence. The sequence in between is being cut, upon dimerization of the enzyme, yielding a 4-bp-long 5′ overhang. This can be repaired by the host-specific NHEJ activity, usually leaving small deletions or insertions behind. Alternatively, a homologous rescue construct, supplied at the time of enzyme activation, is used by the host's homologous recombination activity, to replace the endogenous sequence.

In recent years, the development and use of ZFNs or meganucleases, especially for animal systems, increased like an explosion. Meganucleases are reengineered homing endonucleases mostly based on I-CreI that is found in chloroplasts of *Chlamydomonas reinhardtii* (10). ZFNs rely on the combination of a nuclease domain supplied by the enzyme FokI and sequence-specific Zinc-finger domains designed using specialized programs or assays (11). Proof of concept for the successful activity has been provided earlier (12), and the utility of site-specific induction of double-stranded breaks (DSBs) and their repair by nonhomologous joining of the ends or their repair using homologous rescue sequences has been demonstrated in animal and plant systems (Fig. 1).

Whereas the pathway using homologous sequence is not as efficient, both in plants and in animals (except for ES cell lines), direct error-prone rejoining of the broken ends by endogenous pathways turned out to yield mutated versions of the targeted gene in relatively high proportions in animal systems (11). This reflects the relatively higher efficiency of the error-prone nonhomologous repair pathway compared with that dependent on homology, both in higher animal and higher plant systems.

Osakabe et al. (7) targeted the *A. thaliana* gene *ABI4*, as mutations in this gene are expected to show a strong phenotype. The activities of the respective designed ZFNs were assayed in bacterial two-hybrid systems, in vitro and in transgenic plants. The expression of the ZFNs was activated by a temperature-inducible system and candidates for mutated *abi4* were screened for, using the Surveyor nuclease assay (this assay makes use of mismatches between WT and mutated sequences using a mismatch-specific endonuclease). Frequencies as high as 3% were obtained in somatic tissue and transmission to the offspring exhibiting the expected phenotype could be demonstrated. Use of *ku80* mutant plants as targets for the ZFNs led to mutation frequencies in the same range as with WT plants, but the extent of sequence degradation at the junction sites was increased.

Zhang et al. (8) targeted two Arabidopsis genes, one coding for the *ADH 1* and the other for the *TT4*, both of which, in the homozygous mutated version, exhibit strong phenotypes. Activity and specificity of the chosen ZFNs were tested in yeast and Arabidopsis protoplasts. For induction of the ZFN proteins in the transgenic plants, an estrogen-inducible promoter was used. Somatically mutated plants could be recovered at frequencies of 7% and 16%. The efficiency in this publication may be higher than that in the

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study of Osakabe et al. (7) because induction of ZFN activity was started at the time of selection for transgenic plants. Mutations were faithfully transmitted to the next generation and exhibited the expected phenotypes.

Key to success in ZFN-dependent targeted genomic changes in plants is a combination of optimizations. These include design and test of the nucleases, the choice of the plant target tissue, the mode of introduction or induction of the enzyme activities, the absence of toxicities of the nucleases, the detection of the mutated plant individuals and absence of off-target-mutagenesis. For the design of the ZFNs, either a combination of individual Zinc fingers, as was done by Osakabe et al. (7), or a program such as OPEN, an open source for engineering active Zinc-finger arrays (4), can be used, as used by Zhang et al. (8). However, preliminary tests to assay the in vivo function of these enzymes is essential because chromatinized or possibly methylated DNA, as it exists at the target locus, will exhibit a conformation different from that of a purified molecule. Even in different tissues, this conformation may change. Activity and specificity tests have been designed in organisms such as bacteria, yeast, and plant protoplasts. In the cases published in PNAS, these preliminary tests were successful, but they probably could not guarantee target specific activity in plants.

In *A. thaliana* there is no convenient choice of target tissue other than a transgenic plant. Tobacco can also be used as protoplast (4) because these easily regenerate. Microinjection of zebrafish or *Drosophila melanogaster* embryos or mammalian cell lines or embryos with DNA or mRNA coding for the ZFNs of course constitute beautiful and efficient examples (11) that cannot be matched by plants—not yet. Inducible ZFN-gene expression in plants, just as transient expression in animal cells, circumvents/avoids enzyme toxicity. The ZFN transgene in the resulting plant mutants will of course have to be crossed out.

Identification of mutated alleles remains a challenge, at least as long as frequencies are low. However, with 7% and 16% somatic mutation frequency (8), PCR-based screening methods allow rapid identification of mutants that then can be sequenced.Thus, mutations with unknown phenotypes can be recovered.

DSB repair might contribute to the wide variation of genome sizes in plants.

Analysis of the repaired sequences allows the conclusion that the nonhomologous end joining (NHEJ) machinery was involved (7, 8). As also found in animal systems, frequently both alleles of the target locus have been repaired, which was shown to constitute two independent repair events. This finding can be taken as an indication that cutting was efficient. It also confirms that DSB-induced allelic gene conversion is an extremely rare event in somatic plant cells (13). DSBs are repaired by NHEJ with or without use of microhomologies at the break site (14), indicating two different pathways being responsible for the different patterns (15). The heterodimer of Ku80 and Ku70 is involved in the canonical pathway of NHEJ in eukaryotes by binding to broken DNA ends and enhancing ligation by ligase IV. As shown by Osakabe et al. (7), lack of the Ku heterodimer is correlated with loss of end-protection in Arabidopsis and its absence leads to enhanced degradation of the DNA ends. Repair seems to occur in this case exclusively by the alternative pathway of NHEJ, which rejoins breaks by the use of microhomologies, resulting in most cases in deletions at the break site. It is interesting to note that differences in the efficiency of protection of broken DNA ends and/or differences in the use of the canonical and alternative NHEJ pathway might have tremendous consequences in light of genome evolution. Bioinformatic analysis indicates that DSB repair might be a significant source of sequence loss during genome evolution in Arabidopsis (16). Indeed, differences between Arabidopsis and tobacco resulting from repair of a nuclease-induced DSB have been found (17). Such differences might be caused by difference in the efficiency of canonical to alternative NHEJ pathways between species. Thus, DSB repair might contribute to the wide variation of genome sizes in plants and represent a counter force to the genome enlargement by spread of retroelements (18). As we expect that ZFN-mediated mutation induction will become routine in many plant species, it will be interesting to see whether differences in quantity and quality of deletions between different plant species can be found and whether there is any correlation with the respective genome size.

The new results (7, 8) justify the hope that ZFN-mediated genomic changes in plants also become a generally used possibility and that this technology will be extended smoothly to a row of crop plants. Apart from the creation of targeted mutations useful for academia and agriculture, we hope that this technique will help to make molecular changes in plants more acceptable to the general public as the resulting mutant plants, after backcrossing to the respective WT, are devoid of transgene sequences.

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