In planta gene targeting

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The development of designed site-specific endonucleases boosted the establishment of gene targeting (GT) techniques in a row of different species. However, the methods described in plants require a highly efficient transformation and regeneration procedure and, therefore, can be applied to very few species. Here, we describe a highly efficient GT system that is suitable for all transformable plants regardless of transformation efficiency. Efficient *in planta* GT was achieved in *Arabidopsis thaliana* by expression of a site-specific endonuclease that not only cuts within the target but also the chromosomal transgenic donor, leading to an excised targeting vector. Progeny clonal for the targeted allele could be obtained directly by harvesting seeds. Targeted events could be identified up to approximately once per 100 seeds depending on the target donor combination. Molecular analysis demonstrated that, in almost all events, homologous recombination occurred at both ends of the break. No ectopic integration of the GT vector was found.

plant biotechnology | plant breeding | gene technology | double-strand-break repair

Since the first report on gene targeting (GT) in plants was published (1), various approaches were tested to improve the efficiency of the method (2–5), which has been summarized in recent reviews (6, 7). We were able to demonstrate that the integration of a transfer DNA (T-DNA) by homologous recombination (HR) into a specific locus could be enhanced by two orders of magnitude via double-strand-break (DSB) induction using a site-specific endonuclease (8). More recently, by the use of zinc finger nucleases (9), which, in principle, can be used to induce a DSB at any genomic site, endogenous loci have been targeted in *Arabidopsis* (10), tobacco (11), and maize (12) at high frequencies (13). Some time ago, a method for in vivo targeting was developed in *Drosophila*. A stably integrated donor precursor molecule is first circularized by the FLP recombinase and subsequently linearized via cutting a single I-SceI recognition site, generating the actual GT vector (14). However, such a technique has not been successfully transferred to plants. We have previously shown that DNA can be efficiently excised from the genome *in planta* by the use of a site-specific endonuclease (15). To test whether the combination of this approach with DSB-induced recombination might lead to an efficient GT system, that is independent of transformation, we performed a proof-of-concept (POC) experiment in *Arabidopsis* using I-SceI (16) as a site-specific nuclease.

Results

Generating Homozygous Single-Copy GT Lines. Our *in planta* GT system is based on three different constructs (two shown in Fig. 1*A*) that were transformed independently by floral dipping. The target locus contains a truncated β-glucuronidase (GUS) gene (*uidA*) that can be restored via GT. DSB induction at the two I-SceI recognition sites flanking a kanamycin-resistance gene would result in excision of the kanamycin-resistance gene and in activation of the target locus for HR. The donor locus contains a GT cassette that is also flanked by two I-SceI recognition sites, resulting in the release of a linear GT vector after I-SceI expression. Homology between the activated target site and the GT vector sequence is 942 bp on one end and 614 bp on the other. In addition, the donor construct had 599 bp of sequence homology upstream and downstream of the I-SceI sites, so that after excision of the GT vector, the resulting DSB could be repaired either by nonhomologous end joining (NHEJ) or by single-strand annealing (SSA).

Single-copy lines were identified and characterized for each construct (four for the target, three for the donor and five for the I-SceI expression), and all possible combinations of target/donor loci were crossed. Lines homozygous for both constructs were established. The I-SceI expression line Ubi::I-SceI#10 was then crossed with all 12 different target/donor lines.

Quantification of Somatic GT Events in the F1′ Generation. The F1′ generation was screened for somatic DSB-induced GT events, which can be easily visualized as blue sectors after histochemical GUS staining (see Fig. S1 for a general crossing scheme). Somatic GT events of different target/donor combinations were quantified (Fig. 2*A*). Indeed, 20–60 blue spots or sectors per plant could be detected in 14-d-old seedlings. In contrast, no blue sectors were found in controls without I-SceI expression, indicating that GT can be enhanced *in planta* by orders of magnitude via DSB induction (Fig. 3*B*). In addition, there is probably a positive effect on the recombination frequencies if the target and the donor construct are located on the same chromosome.

Heritable GT Events in the F2′ Generation. After demonstrating that GT in plants is possible with this system, we tested whether GT events could also be transferred to the germline. The target/donor combination line T-13/D-28, in which both constructs are located on chromosome I, was selected, and its F2′ generation was screened for completely stained plants (Fig. 2*B*). Approximately 50,000 seeds in four independent experiments were screened, and >350 blue seedlings were obtained (Table 1), corresponding to a GT frequency of 6.8×10^{-3}. To avoid the standard histochemical staining procedure, which is lethal to plants, batches of seeds from different transgenic lines (F2′) were grown on agar plates and then stained 14 d postgermination for a short time by covering the agar plates with a buffer solution containing the histochemical staining substrate X-Gluc. As soon as the roots of some individual seedlings became bluish, the corresponding plants were transferred to new agar plates for further cultivation. Afterward, those plants were transferred to soil, and the primary GUS staining results were confirmed by standard histochemical staining of entire detached shoot-leaves. This procedure allowed the identification of 20 individual F2′ plants with abundant GUS activity that survived the histochemical staining protocol and that were used for further molecular analysis by PCR and Southern blotting. The GT events detected by histochemical GUS staining were confirmed molecularly by Southern blot characterization of the T-13/D-28 F3′ progeny. The Southern blot analysis of the GT lines GT-1 to GT-6 is shown in Fig. 1. To confirm that the GUS activity indeed arose because of the HR-mediated restoration of the GUS gene in the target construct, Southern blot analysis using probe A on EcoRI-

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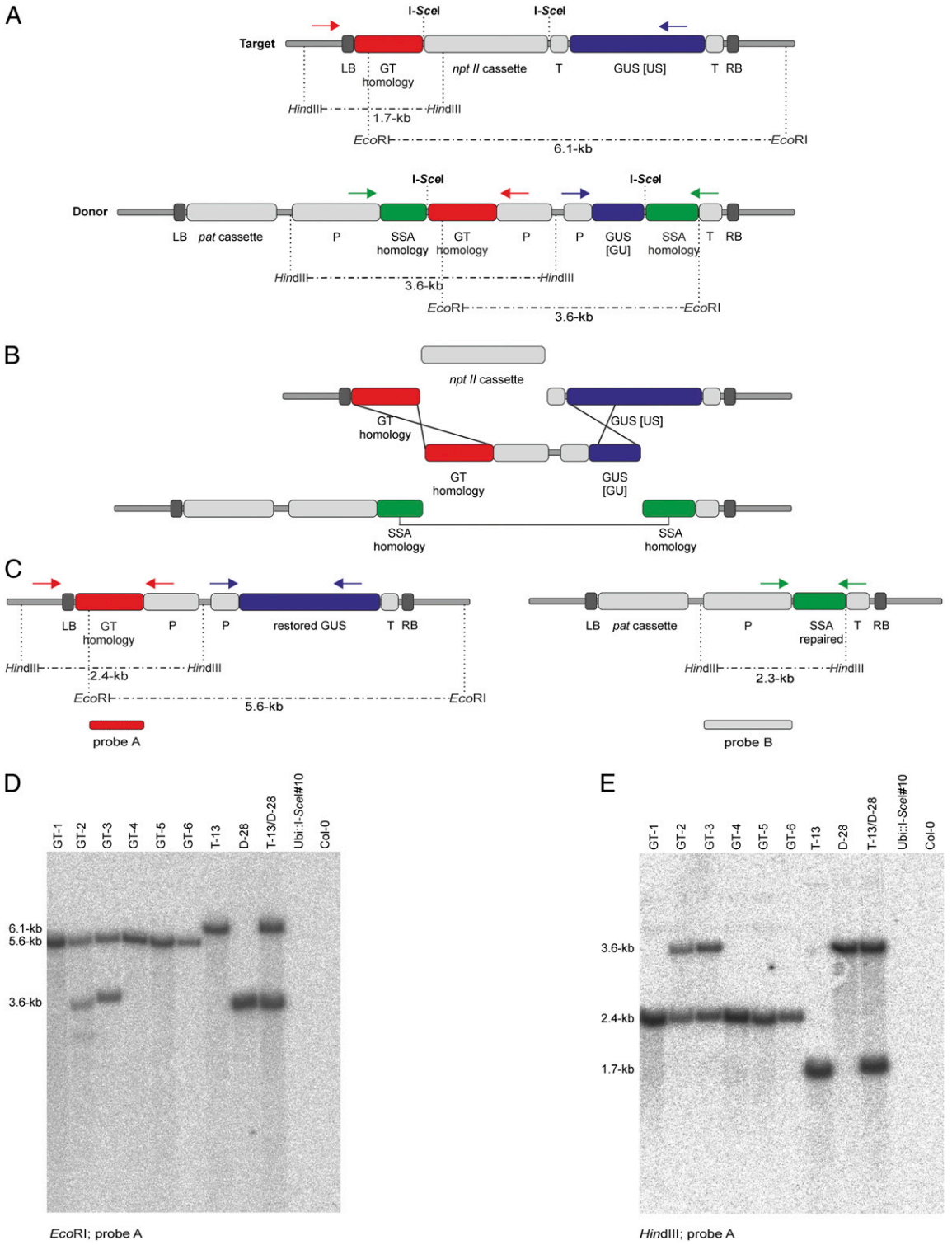

Fig. 1. Outline of the *in planta* GT system. (*A*) Schematic representation of the stably integrated T-DNA constructs used as donor and target sequences. Donor and target T-DNA each carry two I-SceI recognition sites flanking the GT cassette (donor) or a kanamycin-resistance cassette (target). (*B*) I-SceI expression led to DSB induction, resulting in an activated target locus and an excised GT vector. HR-mediated integration of the GT vector within the activated target sequence restored a truncated GUS gene that can be detected via histochemical staining. Homologous sequences were placed upstream and downstream of the GT cassette within the donor construct, so that the activated transgene could be repaired either by NHEJ or by single-strand annealing (*C*). (*D* and *E*) Southern blot analysis of six selected GT lines after selfing. (*D*) HR-mediated restoration of the GUS ORF resulted in a 5.6-kb fragment, whereas a 6.1-kb fragment was indicative of the original target sequence and a 3.6-kb fragment was indicative of the original donor sequence. (*E*) HR-mediated integration of the target vector on the other side of the DSB yielded a 2.4-kb fragment. The original target sequence resulted in a 1.7-kb fragment, whereas a 3.6-kb fragment was indicative of the original donor sequence. Additionally, the original donor construct could be detected in GT-2 and GT-3 (*D* and *E*). Primers for PCR amplification of the recombination events and sequence analysis are shown as red, blue, and green arrows.

digested DNA was performed (Fig. 1*D*). Whereas a 6.1-kb fragment is indicative of the native target locus (T-13) and a 3.6-kb fragment of the donor construct (D-28), integration of the GT vector via HR would result in a 5.6-kb fragment. In all 20 tested lines, such a 5.6-kb fragment could be detected (see GT-1 to GT-6). Moreover, in two lines (GT-2 and GT-3), the original donor construct was still present (Fig. 1*D*). The easiest explanation for this finding is that the recombined target and nonrecombined donor molecules were combined by self-pollination of nonclonal germ cells. Because targeting might occur during most of the life cycle, nonclonal progeny might arise from these plants. Thus, pollen carrying a targeting event might fertilize an egg cell that harbors a nonrecombined donor locus or vice versa. To further confirm these results, the recombined GUS fragment from all 20 lines was PCR-amplified. Sequence analysis revealed the presence of the restored GUS sequence without any mutation, demonstrating that the gene was indeed restored by HR. Previous work on DSB repair in plants showed that, in many cases, HR occurs only at one side of the DSB (17, 18). Therefore, the other GT junction, which was not selected for marker gene restoration, was analyzed by Southern blotting. A HindIII digest of the genomic DNA of plants GT-1 to GT-6 hybridized with probe A is shown in Fig. 1*E*. A 1.7-kb fragment is indicative of the original target locus (T-13), whereas a 3.6-kb fragment represents the native donor vector (D-28); a 2.4-kb fragment is expected after HR-mediated precise integration of the excised GT vector. In all six lines shown, the GT-specific fragment could be detected (Fig. 1*E*), and, moreover, the same two lines mentioned before (GT-2 and GT-3) also contained a fragment indicating the presence of the original donor construct. Altogether, the 2.4-kb fragment was detected in 19 of 20 lines. To confirm these results on the sequence level, PCR analysis of this junction was performed on the 19 positive lines. In all cases, sequence analysis verified that accurate integration by HR had occurred. Thus, in contrast to previous targeting experiments (2, 8), one-sided events occur rarely under our experimental conditions. Notably, in these Southern blot experiments, no fragments of other sizes were detected in any recombinant lines using probe A. This result indicates that the excised GT vector never integrated elsewhere in the genome by NHEJ. In previous GT studies, lines that contained the successfully targeted locus were often found to also contain one or several randomly integrated copies of the vector (19). For biotechnological applications, these transgene sequences must be out-crossed before such plants can be used in the field. To demonstrate the general applicability of the targeting system, two other target/donor combinations were also tested. These experiments resulted in totally blue plants obtained at frequencies in a comparable range as with the T-13/D-28 combination. Whereas a somewhat lower GT frequency of 1.4×10^{-3} was obtained for the T-13/D-32 combination, an even higher frequency of 8.3×10^{-3} was obtained for the T-27/D-32 combination (Table 1). Taken together, we obtained 843 GT plants out of 10 independent experiments using 3 different target/donor combinations.

Elimination of the Excised Kanamycin-Resistance Gene. To determine whether the excised kanamycin-resistance gene originating from the target locus was still present somewhere in the genome, a membrane carrying MfeI-digested genomic DNA was hybridized with a kanamycin-specific probe. A signal with the targeting vector was only detected in T-13 control plants, suggesting that the excised resistance cassette did not reintegrate into the genome by NHEJ in any of the plants analyzed (Fig. S2). This observation is consistent with the fact that randomly integrated copies of the excised GT vector were also not detected in any recombinant line.

DSB Repair Within the Donor Locus. To test whether the break in the donor construct was repaired by NHEJ or SSA following excision of the GT vector, the fragment was PCR-amplified from those lines that still harbored the repaired donor locus for sequence analysis. In all six lines tested, the break was repaired by the use of homology, thus by SSA. To confirm these PCR-based results, the membrane carrying the HindIII-digested DNA was hybridized with probe B after stripping. A 2.3-kb fragment is indicative of a SSA-mediated DSB repair, which was reproved in all six lines (Fig. S3).

Mendelian Inheritance of the Restored GUS Reporter Gene. To show that the restored GUS reporter gene was inherited in a Mendelian fashion, segregation of blue staining was examined in the F3′ generation of fully blue F2′ plants. In all cases tested, the Mendelian segregation pattern of the GUS gene was confirmed, being equivalent to 100 or 75% blue plants, depending on homozygous or heterozygous F2′ plants regarding the GT event (Table S1). The proper segregation of the modified locus in all lines can be taken as evidence of the absence of large-scale rearrangements like translocations, associated with the targeted events.

Discussion

Our results clearly demonstrated the feasibility of an *in planta* GT technique that does not rely on efficient transformation and regeneration procedures. The strength of the established technique is also demonstrated by the fact that, in contrast to previous targeting experiments in plants (20, 21), we were able to obtain hundreds of targeting events. We obtain seeds with a perfectly targeted locus at a frequency comparable to *Arabidopsis* T-DNA transformation (22).

Whereas conventional GT approaches rely on the generation of a very large number of transformation events, our GT method does not require a minimum efficiency. Upon expression of a DSB-inducing endonuclease, the GT vector is set free during the lifecycle of a plant in a large number of cells. Thus, it is the organism itself, not the experimenter, that supplies the necessary number of events to obtain successful targeting. Moreover, because only one or two copies of the target vector are set free per cell, the number of unwanted random integration events is minimized, in contrast to conventional GT approaches in which often multiple copies of a vector are transferred into a single cell. Taking our data into consideration, one might speculate about an enhancement of GT frequencies if the target and the donor locus are located on the same chromosome. However, only further experiments will tell, whether physical proximity has any influence on the GT reaction. Although a marker gene was used for the POC experiment, this technique could be applied to any endogenous locus using synthetic nucleases. It should make no difference whether the nuclease cuts once or twice within the target locus. The setup used in this study demonstrates a further application of GT: it can be efficiently used for the simultaneous elimination of a genomic fragment out of the target locus. In none of the recombinants analyzed did we find this fragment integrated elsewhere in the genome. In a typical endogenous *in planta* GT approach, only one cleavage site is present within the target locus. In previous work, we were able to show that T-DNA integrates into a specific locus that has been activated within a single cleavage site by HR (8). It has been reported previously that T-DNA can also integrate via NHEJ into a site activated by a DSB (23–25). Indeed, with such a setup, GT should also become possible in plants without the use of homology.

It did not escape our attention that in contrast to previous targeting experiments (18, 19), in almost all cases, integration of both ends of the vector into the target locus, as well as the repair of the DSB at the vector excision site in the donor locus, was attributable to HR. This might be taken as indication that in contrast to in vitro cell culture, during plant development at specific time points, some cells might be especially prone to HR. Therefore, it will be of interest to test, by the use of specific promoters, whether certain cell types are especially prone for GT.

With the knowledge obtained in our study, achieving multiple targeting events with a single transformation event, which subsequently enters the germline and contributes to the genotype and phenotype of the offspring generations, should be possible. The most obvious application of such an approach is the use of artificially constructed modular endonucleases, such as meganucleases (26), zinc finger nucleases (27), or transcription

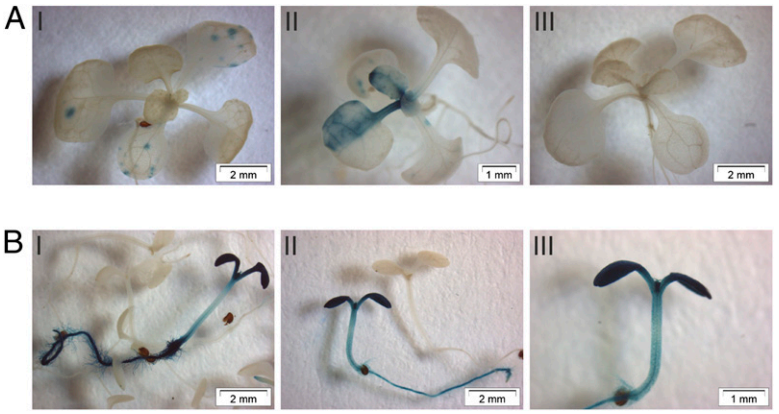

Fig. 2. Somatic and heritable GT events. (*A*) Selected subset of 14-d-old F1′ generation seedlings showing blue spots (*I*) or blue sectors (*II*) after histochemical staining. *III* represents a negative control without I-SceI expression. (*B*) Selected subset of 7-d-old F2′ generation seedlings in which the GT event entered the germline, resulting in completely blue plants after histochemical staining.

activator-like effector nucleases (28), that are designed to cut at a specific site in the gene of interest. Recent studies demonstrated that the aforementioned nucleases can be applied in the same way as I-SceI used in our study for different DSB-induced recombination reactions in plants (8, 15, 19, 29). For commercial application, the donor vector might contain a GT cassette flanked by two recognition sites of a site-specific endonuclease that would also cut within the target locus (Fig. 4). Moreover, the donor vector might also contain the ORF of this required nuclease under the control of an inducible or organ-specific promoter that is active late in development and not during the transformation process. An ideal expression pattern for the nuclease would be in the reproductive tissue, to ensure that targeted events will be present among the progeny. The high frequency of targeted seeds obtained in our study indicates that identification can be performed by simple PCR analysis without the need for positive/negative selection. Because the donor locus can be segregated from the targeted integration, the final plant that will go to the field will carry only the designed change in the target without any additional transgene sequences. To accelerate commercial GT, the process could be preformed with heterozygous lines (target and/or donor) instead of homozygous ones, such as the lines used in the POC experiment described in this study. Because our GT approach does not rely on high transformation/regeneration efficiencies, it should be applicable to all transformable plant species, even if transformation efficiency is extremely low.

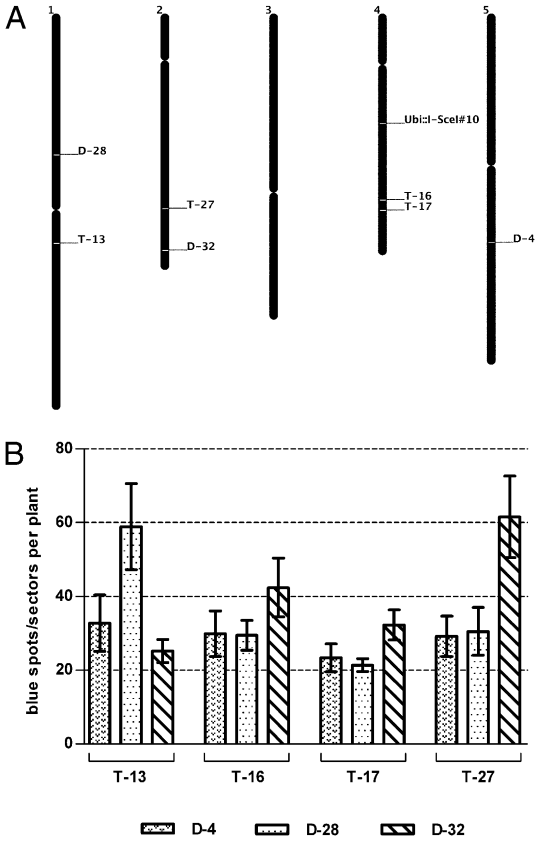

Fig. 3. Chromosome map and quantified GT events in F1′ generation. (*A*) The chromosome map depicts the integration loci of all single-copy target, donor, and I-SceI expression lines used in this study. After crossing the target and donor lines, we obtained 12 different combinations that were crossed with the I-SceI expression line Ubi::I-SceI#10. We quantified recombination in 14-d old F1′ seedlings after histochemical staining. The results are shown in *B* as blue sectors per plant for all different combinations. Two of the target/donor combinations that have donor and target constructs located on the same chromosome show an enhanced rate of HR-mediated GUS restoration via GT.

Materials and Methods

Strains. All lines are in a Columbia-0 background. Seeds were sown on agar plates containing GM or on substrate containing 1:1 Floraton 3 (Floragard) and Vermiculit (Deutsche Vermiculite Dämmstoff).

T-DNA Constructs. The target construct, VC-SBT359-6qcz, contains the following elements from LB to RB: 35SpA[AS] (polyadenylation signal of the CaMV 35S gene in the antisense orientation); DsRed2[AS] [cds (coding sequence) of the DsRed2 fluorescent protein in the antisense orientation]; bidirectional promoter [OAS/FD promoter: genomic DNA sequence located between the *Arabidopsis* ferredoxin and *O*-acetyl-serine genes, able to drive expression of both flanking ORFs (30); in this construct, it is driving the expression of the DsRed and the Npt2 genes]; Npt2 (kanamycin-resistance gene); CatpA (polyadenylation signal of the *Solanum tuberosum* cathepsin D gene); US [truncated, nonfunctional fragment of the GUS gene (*uidA*) lacking 88 bp at the 5′ end of the cds]; and NOS terminator (terminator of the *Agrobacterium tumefaciens* nopaline synthase gene). The different elements were cloned into a pSUN3 binary vector backbone (31) using standard molecular biology techniques and validated by sequencing.

The donor construct, VC-SBT366-12qcz, contains a phosphinothricin-resistance expression cassette next to the LB, followed by a targeting cassette. The phosphinothricin expression cassette consists of the NOS promoter (promoter of the *A. tumefaciens* nopaline synthase gene), the pat cds (phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes*), and the NOS terminator (terminator of the *A. tumefaciens* nopaline synthase gene). The targeting cassette contains the following elements: SP (32); ZsGree (truncated, nonfunctional fragment of the *Zoanthus sp.* green fluorescent protein gene missing 94 bp at the 3′ end of the gene); 35SpA[AS] (polyadenylation signal of the CaMV 35S gene in the antisense orientation); DsRed2 [AS] (cds of the DsRed2 fluorescent protein in the antisense orientation); pLEB4[AS] (seed-specific promoter of the *Vicia faba* legumin B4 gene); 35S promoter (promoter of the of the CaMV 35S gene); GU (truncated, nonfunctional fragment of the GUS gene missing 1,191 bp at the 3′ end of the cds); een (truncated, nonfunctional fragment of the *Zoanthus sp.* green fluorescent protein gene missing 9 bp at the 5′ end of the gene); and LB3UT (terminator of the *Vicia faba* legumin 1B3 gene).

Table 1. Gene targeting frequencies in three different target/donor combination lines

Experiment	Total no. of seedlings	Blue seedlings	Blue seedlings/total no. of seedlings
T-13/D-28			
1	1.3×10^4	114	1:112
2	1.3×10^4	93	1:136
3	1.2×10^4	67	1:184
4	1.3×10^4	80	1:157
Total	5.1×10^4	354	1:147
T-13/D-32			
1	1.8×10^4	34	1:540
2	1.5×10^4	18	1:838
3	1.5×10^4	20	1:766
Total	4.8×10^4	72	1:715
T-27/D-32			
1	1.8×10^4	189	1:77
2	1.5×10^4	86	1:175
3	1.5×10^4	142	1:107
Total	4.8×10^4	417	1:120

According to the quantification of blue spots/sectors in the F1′ generation the target/donor combination lines T-13/D-28, T-13/D-32, and T-27/D-32 were chosen for detection of completely blue plants in the F2′ generation.

The different elements were cloned into a pSUN3 backbone (31) using standard molecular biology techniques and validated by sequencing. The codon-optimized I-SceI-ORF (33) driven by the *Petroselinum crispum* Ubiquitin4-2 promoter was cloned into the binary vector pPZP221 (34). The three resulting binary vectors were electroporated into *Agrobacterium* strain *GV3101*.

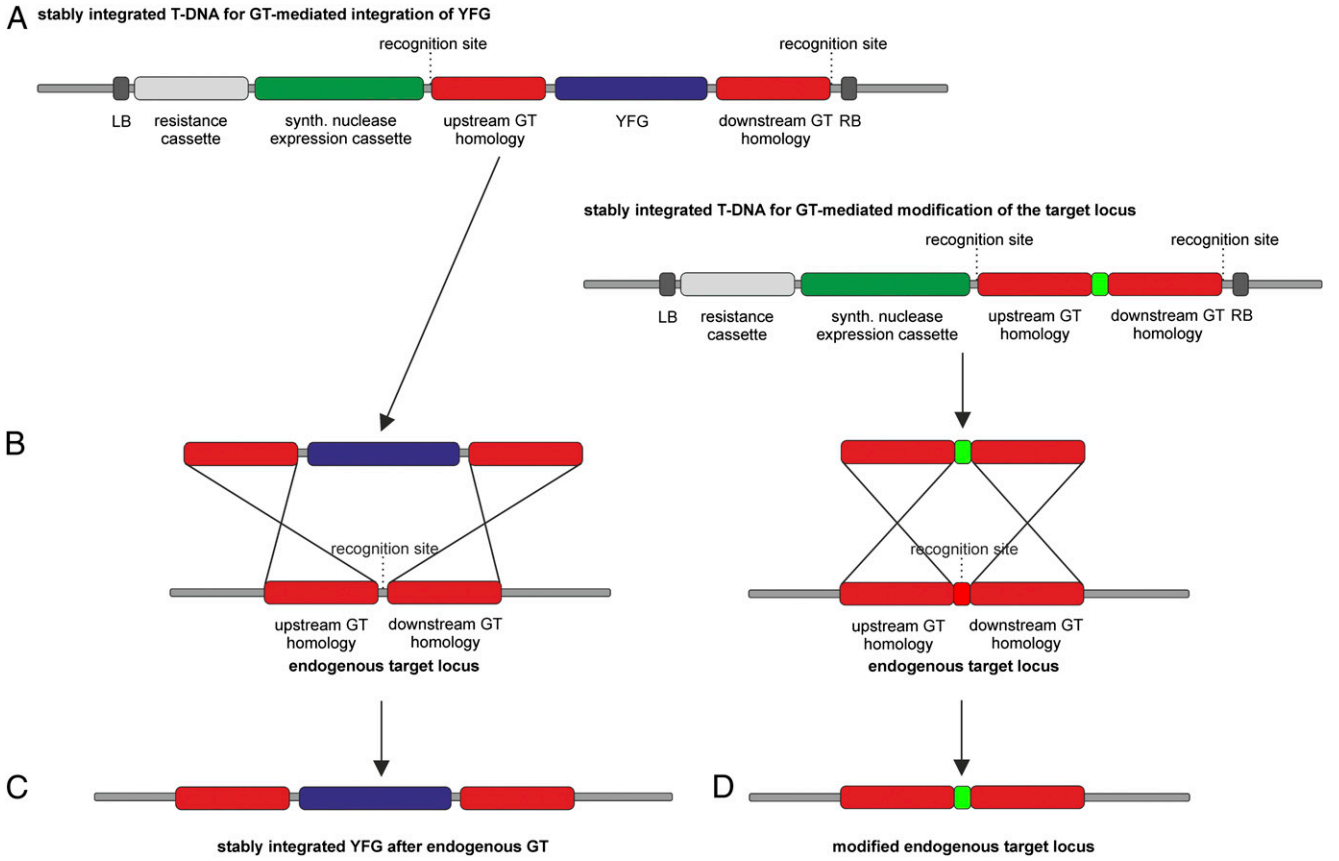

Fig. 4. Application of *in planta* GT for modifying endogenous loci. After construction of an artificial site-specific endonuclease that is able to induce a unique DSB in the endogenous locus, a single T-DNA construct is produced that harbors, besides the nuclease under the control of an inducible promoter, the targeting vector as cassette flanked by two restriction sites of the enzymes. The targeting vector might contain between homologous regions a selection marker, shown here as YFG (Your Favorite Gene), or a defined modification (*A*). After random integration in plant genome, expression of the endonuclease is induced. The GT vector is excised (*B*), and, simultaneously, the target locus is activated. The vector integrates via HR in the target leading to a stably modified gene locus (*C* and *D*).

Plant Transformation. *Agrobacterium*-mediated transformation of *Arabidopsis* plants was performed as described (22). Plants (target: kanamycin; donor: phosphinothricin; Ubi::I-SceI: gentamycin) were checked for a 3:1 segregation in the next generation to obtain lines in which the transgene was inserted at a single locus. Single-copy plant lines were identified by Southern blot analysis. Insertion junctions were determined using SiteFinder (35), inverse PCR (36, 37), or adapter ligation-mediated PCR protocol (38).

GUS Assay. GUS assays were performed as described (39). Determination of GT events that entered the germline was performed with 10-d-old seedlings (F2′ generation), cultured on sand as described (40). For nondestructive staining, the seedlings were covered after 14 d with a staining solution as described (39) but without sodium azide and incubated overnight at room temperature. Plants with bluish roots were first transferred to agar plates and, then some days later, to soil. The identification of recombinants for Southern blotting and PCR analysis was performed with plants cultivated on GM medium (F3′ generation).

Plant DNA Extraction and Southern Analysis. DNA was extracted from batches of 60 F3′ siblings representing the progeny of a recombinant F2′ plant. The extraction was performed as described (24). Southern blotting of EcoRI-, HindIII-, or MfeI-digested genomic DNA (F3′ generation) using the membrane "Hybond N+" (GE Healthcare) was performed as described (24). The DNA probes were labeled as described (41). Probe A was PCR-amplified from VC-SBT359-6qcz using the oligonucleotides 5′-GTTGTGGGAGGTGATGTC-3′ and 5′-CCGAGAACGTCATCACCG-3′; probe B from VC-SBT366-12qcz using the oligonucleotides 5′-CTTGGATTGAACAAGATGGATTGC-3′ and 5′-CAGAAGAACTCGTCAAGAAGGCG-3′. The kanamycin-specific probe was PCR-amplified from VC-SBT366-12qcz using the oligonucleotides 5′-CTAGATTCGACGGTATCGATAAGC-3′ and 5′-GATTGGTTATGAAATTCAGATGC-3′.

PCR and Sequence Analysis. Genomic DNA (F3′ generation) was analyzed by PCR using the oligonucleotides 5′-GACCACTTCGTACAACACTAG-3′ and 5′-CTACTAATCATCATCTATCTGTG-3′, which were used for detection of the upstream HR-mediated integration of the GT cassette and subsequent sequencing. Oligonucleotides 5′-GTTCATTTCATTTGGAGAGG-3′ and 5′-GACGACCAAAGCCAGTAAAG-3′ were used to amplify the restored GUS gene and for subsequent sequencing. Oligonucleotides 5′-CACTAGTCTAGAGTCGATCGAC-3′ and 5′-GGGCAATGCAGATCCGGATGC-3′ were used to amplify the restored donor region and for subsequent sequencing. PCRs and sequencing were performed as described (42).

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