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Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination

(site-specific integration/one-sided invasion/I-*Sce* I/DNA polymerase slippage/*Agrobacterium*)

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ABSTRACT **Genomic double-strand breaks (DSBs) are key intermediates in recombination reactions of living organisms. We studied the repair of genomic DSBs by homologous sequences in plants. Tobacco plants containing a site for the highly specific restriction enzyme I-*Sce* I were cotransformed with *Agrobacterium* strains carrying sequences homologous to the transgene locus and, separately, containing the gene coding for the enzyme. We show that the induction of a DSB can increase the frequency of homologous recombination at a specific locus by up to two orders of magnitude. Analysis of the recombination products demonstrates that a DSB can be repaired via homologous recombination by at least two different but related pathways. In the major pathway, homologies on both sides of the DSB are used, analogous to the conservative DSB repair model originally proposed for meiotic recombination in yeast. Homologous recombination of the minor pathway is restricted to one side of the DSB as described by the nonconservative one-sided invasion model. The sequence of the recombination partners was absolutely conserved in two cases, whereas in a third case, a deletion of 14 bp had occurred, probably due to DNA polymerase slippage during the copy process. The induction of DSB breaks to enhance homologous recombination can be applied for a variety of approaches of plant genome manipulation.**

Double-strand breaks (DSBs) play an important role in homologous recombination in eukaryotes (1–3). Recently, experimental evidence that meiotic recombination in yeast is initiated in most, if not all, cases by DSBs was supplied (4). Mitotic gene conversion in mating type switching in yeast is initiated by a DSB at the recognition site of HO endonuclease (for review, see ref. 5). Knowledge on the importance of DSBs for homologous recombination has been employed to induce recombination reactions. Cleavage of the HO nuclease recognition site by induction of the HO nuclease expression has been used to enhance intra- and intermolecular recombination in yeast cells (for review, see ref. 6). Also the yeast mitochondrial I-*Sce* I endonuclease (7), which has an 18-bp recognition site (8), was used to induce recombination reactions. In previous studies performed with I-*Sce* I, specific DSBs were induced *in vivo* in plasmid DNA transfected or injected into eukaryotic cells (9–12). Recently I-*Sce* I-mediated induction of genomic DSBs and their repair by homologous recombination were described for mouse cells (13, 14).

The role of genomic DSBs in homologous recombination in plants has not been studied yet. However, several lines of indirect evidence indicate that transient DSBs enhance homologous recombination. Factors that introduce unspecific DSBs in DNA, such as x-rays (15, 16) or methyl methanesulfonate (17), were shown to enhance intrachromosomal homologous recombination in plants. Excision of transposable elements was correlated with increased frequencies of recombination at the donor site between repeats flanking the element (18, 19) or between repeats at ectopic sites (A. Levy, personal communication).

We demonstrated that expression of the I-*Sce* I gene led to specific DSBs *in vivo* in DNA molecules transfected into plant protoplasts (10). As a consequence extrachromosomal homologous recombination was induced. This reaction proceeds mainly via single-strand annealing and thus seems to be different from recombination reactions in the chromosome (as discussed in ref. 20). In the present communication we analyzed the mechanism by which DSBs at specific sites in the plant genome are repaired with homologous sequence information, and we tested whether induction of DSBs is correlated with an increase in recombination frequencies.

MATERIALS AND METHODS

Cloning Procedures. *Construction of the I-*Sce *I expression vector.* The plasmid p35SISceI+ (10) containing an artificial I-*Sce* I open reading frame (ORF) under the control of the cauliflower 35S promoter and the cauliflower terminator was cut with *Bam*HI and the ORF containing fragment was inserted in the unique *Bam*HI site of the binary vector pCGN1589 (21) to result in the plasmid pCISceI.

Construction of the target site. Into the *Eco*RV site of the plasmid pAF100 (22), the *Xho* I–*Acc*65I-cut, blunt-ended DNA fragment of the plasmid pAH5 (23) carrying the C-terminal half of an artificial, intron-containing kanamycin resistance gene was cloned. The resultant plasmid pAFAH was cut by *Acc*65I, and the specific fragment was ligated into the *Acc*65I-digested binary vector pCH (24), resulting in the plasmid pTS.

Production of the repair construct. A specific polylinker with the sequence 5′-pGAATTCGTTA ACGAGCTCTC TAGAAGCTTC CCGGGCTCGA GTTAAC-3′ was cloned into *Eco*RI-digested pUC7 DNA. A *Hin*dIII cassette containing the *bar* gene, which codes for the phosphinothricin acetyltransferase (25), was integrated in the *Hin*dIII site. Via PCR, a fragment containing the right border and the "overdrive" sequences of the *Agrobacterium* strain C58 was cloned between the *Sac* I and *Xba* I sites. Via PCR, a fragment of the kanamycin gene from the plasmid pTZAH271 (26) fused to the left border of the *Agrobacterium* strain C58 was obtained and cloned between the *Xma* I and *Xho* I sites of the vector,

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Abbreviations: DSB, double-strand break; ORF, open reading frame; T-DNA, transferred DNA.

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resulting in the plasmid pUC1S. A PCR fragment of the hygromycin gene of pCH was cloned in parallel orientation to the intron sequence (as in pTS) into the *Xba* I-site of pUC1S. The cloned sequence was excised by digestion with *Hpa* I and ligated to a *Bgl* II-digested, blunt-ended, borderless derivative of the binary vector pBin19 (27). The resulting binary vector pRC carried in its transferred DNA (T-DNA) a sequence homologous to both sides of the I-*Sce* I site of pTS (Fig. 1). All binary plasmids were transferred to the *Agrobacterium* strain GV3101(pPM6000) (24) and used for plant transformation.

Transformation of Tobacco Seedlings with *Agrobacterium*. Transformations of tobacco seedlings were done as described (28). Three to 4 days after inoculation, the seedlings were transferred onto Murashige and Skoog (MS) plates containing hormones and 100 μg of kanamycin sulfate per ml or 20 μg of glufosinate ammonium per ml. Regeneration of plants from resistant calli was done as described (29).

Segregation of the selfed transformants was tested by putting seeds onto MS medium supplemented with 50 μg of hygromycin per ml (target lines), 500 μg of kanamycin sulfate per ml (recombinant lines), or 20 μg of glufosinate ammonium per ml.

Southern Blot Analysis. Total DNA isolation and Southern blot analysis were performed as described (30, 31). The probes [kanamycin, *Hin*dIII fragment of pAH5 (23) containing the complete intron sequence; and hygromycin, *Sac* II-fragment of pCH (24) containing the complete ORF of hygromycin phosphotransferase and parts of the nopaline promoter and terminator] were labeled with [α-^{32}P]ATP using the random primed labeling kit of Boehringer Mannheim.

Amplification and Sequence Analysis of the Genomic Recombination Joints. PCR was done as described (29) using the oligonucleotides pP1 (5′-pGCGCAAGCTT CCGACCTGTC CGGTGCCCTG-3′) and pP2 (5′-pCCGGAAGCTT CAACGTCGAC CACAGCTGCG-3′) (see Fig. 1). The PCR fragment was digested with *Hin*dIII and cloned in the polylinker of pUC18. Sequencing was performed by the Labor für DNA-Analytik (Freiburg, Germany) using an automated laser florescent sequencing device.

RESULTS

Experimental Setup. The aim of the present study was to analyze the repair of a specific DSB in the genome of somatic plant cells via homologous recombination. We cloned, in *Agrobacterium* vectors, the three components that are needed for the setup of such a system: the target sequence with the restriction site, the repair construct carrying sequence homologies to the target, and the expression cassette for the gene coding for the restriction enzyme. The binary vector pTS (target site) contained on its T-DNA the target sequence, which consists of a hygromycin resistance gene and, as parts of an artificial kanamycin resistance gene (23), an artificial intron based on intron sequences of the parsley 4-coumarate ligase and an exon that contained a part of the bacterial neomycin phosphotransferase ORF. Between the two genes, an I-*Sce* I site was placed (Fig. 1). The repair construct pRC contained on its T-DNA a hygromycin resistance gene (homologous to the target sequence), a phosphinothricin resistance gene [bar (ref. 25)], and, as the complementary part of the artificial kanamycin resistance gene, the promoter, the first exon, and the intron, which is homologous to the target sequence (Fig. 1; for details of cloning see *Materials and Methods*). The expression cassette of the I-*Sce* I gene (pCISceI) consisted of a cauliflower mosaic virus 35S promoter, an artificial I-*Sce* I ORF, and the cauliflower mosaic virus terminator (10).

Functional Test of the Recombination Components. Before experiments on homologous integration were performed, the function of each component of the system was tested. The plant expression cassette for the I-*Sce* I gene was shown previously

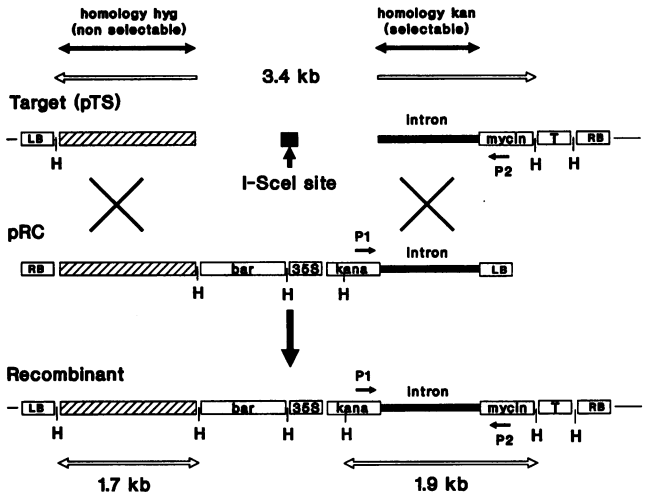

FIG. 1. Design of the experiment. pTS represents the target locus, drawn as T-DNA integrated into the plant chromosome. Between the hygromycin resistance gene (hatched box) and a nonfunctional 3′ half of the kanamycin resistance gene containing an intron, an I-*Sce* I site was cloned. pRC is the repair construct; between the two homologous regions, namely, the hygromycin resistance and the 5′ end of the kanamycin resistance gene, a phosphinothricin resistance gene (bar) was placed. After homologous recombination induced by a DSB at the I-*Sce* I site, recombinants are expected to have the structure depicted in the lower part of the figure. Whereas after Southern blot analysis of *Hin*dIII (H)-digested DNA, the original target locus should give a hybridization signal of 3.4 kb, both with a kanamycin- and a hygromycin-specific DNA probe, a recombinant arising by homologous recombination of the left arm of the vector should show a 1.9-kb kanamycin-specific band, and a recombination of the right arm of the vector should show a 1.7-kb hygromycin-specific band. Oligonucleotides P1 and P2 were designed to amplify the recombined intron sequences in the plant genome for sequence analysis. LB, left border; RB, right border; 35S, cauliflower mosaic virus 35S promoter; and T, cauliflower mosaic virus terminator.

to be active in creating specific DSBs in plasmids carrying the restriction site (10). To assure that the constructs used as partners for the recombination reactions were functional we coinoculated tobacco seedlings with an *Agrobacterium tumefaciens* strain that contained the target locus on its T-DNA (pTS) and with bacteria that contained the repair construct (pRC). We obtained ≈1% of kanamycin-resistant calli in all transformation events indicating that extrachromosomal homologous recombination between T-DNA molecules had taken place at a similar frequency as described (24, 32). Southern hybridization of callus DNA revealed the diagnostic kanamycin resistance gene-specific 1.9-kb fragment (Fig. 1), indicating that the gene was restored accurately via homologous recombination (data not shown).

By inoculation of tobacco with *Agrobacterium* containing the plasmid pTS, hygromycin-resistant transgenic plants were obtained that contained the I-*Sce* I site within the target sequence in the plant genome. Transgenic lines were identified that contained transgenes in a single locus as indicated by Mendelian segregation. For further experiments, plant lines 1-7, 1-12, and 1-16 were chosen. Southern blot analysis of these lines revealed that each of them contained several intact unities of the target locus (a 3.4-kb *Hin*dIII band hybridizing with either hygromycin or kanamycin specific probes; Fig. 2 *A* and *B*, lanes 3 and 12; Fig. 3 *A* and *B*, lanes 6). Besides the intact copy in the case of line 1-7, one (Fig. 3*B*, lane 6) and in the case of line 1–16, two smaller kanamycin-specific *Hin*dIII fragments were detected in blots (Fig. 3*B*, lane 8), indicating that these lines carry additional kanamycin-specific sequences at the target locus. The intactness of the genomic I-*Sce* I sites was demonstrated by a combined *Hin*dIII/I-*Sce* I restriction di-

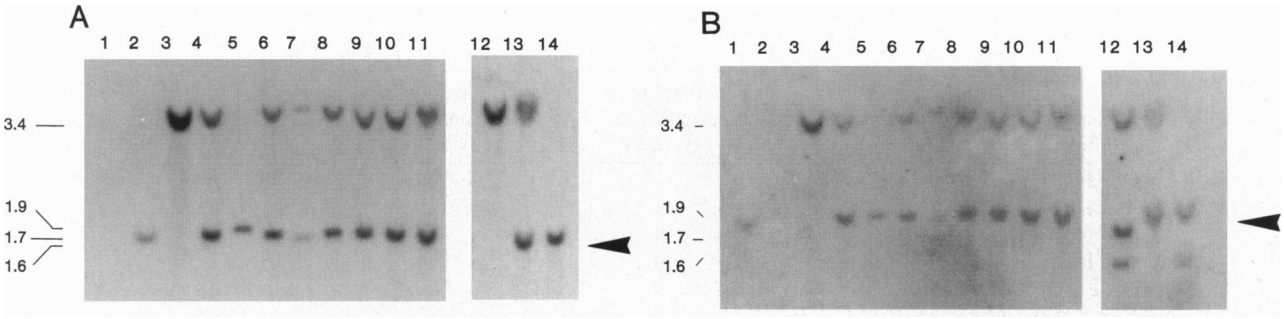

FIG. 2. Southern blot analysis of recombinant lines in which both ends of the targeting vector were integrated by homologous recombination. (*A*) *Hin*dIII-digested plant DNA probed with a hygromycin-specific probe. (*B*) The identical blot after reprobing with a kanamycin-intron specific probe. Lane 1, wild-type tobacco DNA mixed with 1.9-kb band cut out of pAH5, specific for the functional kanamycin resistance-intron gene; lane 2, wild-type tobacco DNA mixed with 1.6-kb specific hygromycin resistance band resulting from a digestion of pTS with *Sac* II; lane 3, target line 1-12; lanes 4–11, recombinants A, B, C, D, E, F, G, and H of target line 1-12; lane 12, target line 1-16; lanes 13 and 14, recombinants A and B of target line 1-16.

gestion. In all lines upon I-*Sce* I digestion the 3.4-kb *Hin*dIII band was replaced by two kanamycin- or hygromycin-specific bands of about 1.7 kb each (data not shown). Taken together, these assays demonstrated that all components of the site-specific recombination experiments were functional.

Induction of Homologous Integration by I-*Sce* I Expression. Seedlings at the cotyledon stage of the selfed primary transformants 1–7, 1–12, and 1–16 were inoculated with two different *Agrobacterium* strains, one carrying the I-*Sce* I expression cassette (pCISceI), the other the repair construct (pRC), in a ratio 4:1. The transformation efficiency was determined as described by diluting the *Agrobacterium* strain carrying the binary pRC with the transfer deficient strain 6000K (29) and subsequent counting of the phosphinothricin (PPT)-resistant calli. To identify putative homologous recombinant events, kanamycin selection was applied and plants were regenerated from kanamycin-resistant calli. Table 1 shows the number of calli obtained in which the kanamycin resistance gene was restored by homologous recombination as demonstrated by Southern blot analysis (see below).

Our experiments indicated a strong recombination-enhancing effect of induction of I-*Sce* I-specific DSBs, to $>10^{-3}$, in comparison to gene targeting experiments ($\approx 10^{-5}$). To strengthen this correlation, we coinoculated seedlings of the transgenic line 1–12 with different ratios of the two *Agrobacterium* strains. As shown in the lower part of Table 1, there seems to be some correlation between the frequencies of homologous recombination and the concentration of the I-*Sce* I gene in the inoculum. Whereas we could not detect any recombinants ($<6 \times 10^{-4}$) when the copy numbers of the I-*Sce* I genes were low in comparison to the repair construct, the highest recombination frequency (2%) was found when the *Agrobacterium* strain containing the I-*Sce* I gene was used in a 9-fold excess over the repair strain.

Molecular and Genetic Analysis of the Obtained Recombinants. In Table 1, only those kanamycin-resistant calli that proved to be the result of homologous recombination events are listed (see below). However, we also obtained calli (one for lines 1–7 and 1–12 each and nine for line 1–16) in which the kanamycin resistance was either due to illegitimate recombination events (molecular data not shown) or "escapes." Illegitimate recombination leading to kanamycin resistance may be explained by unexpected splicing of the intron sequence, whereas escapes may have arisen due to the relatively weak kanamycin selection, which had to be applied due to the relatively low level of resistance expressed by the artificial kanamycin construct (23).

Taking all three lines together, 17 kanamycin-resistant calli (see Table 1) were identified in which a new 1.9-kb kanamycin gene specific *Hin*dIII fragment that is diagnostic for homologous recombination between the incoming T-DNA and the chromosomal target locus could be detected.

We further analyzed the molecular structure of the recombinants to learn more about the mechanism of homologous recombination in plant cells. If the recombination reaction occurs in a conservative way, homologous recombination should proceed simultaneously at both ends of the break in the double-stranded genomic DNA, resulting also in a new 1.7-kb

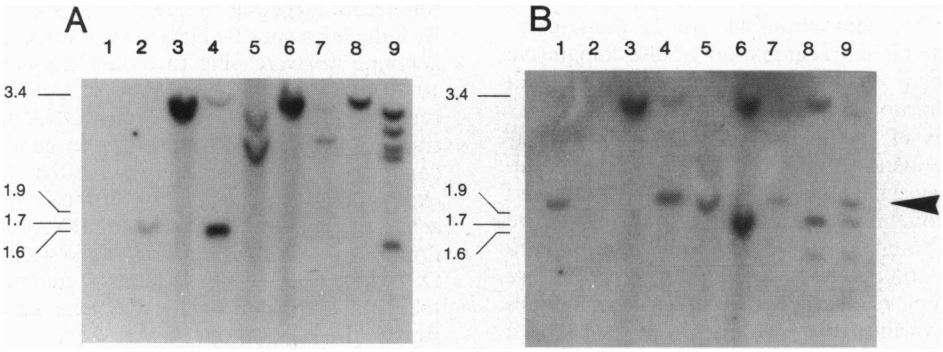

FIG. 3. Southern blot analysis of recombinant lines in which only the intron-carrying end of the targeting vector was integrated by homologous recombination. (*A*) *Hin*dIII-digested plant DNA probed with a hygromycin-specific probe. (*B*) The identical blot after reprobing with a kanamycin-intron specific probe. Lane 1, wild-type tobacco DNA mixed with a 1.9-kb DNA fragment cut out of pAH5, specific for the functional kanamycin resistance-intron gene; lane 2, wild-type tobacco DNA mixed with a 1.6-kb DNA fragment resulting from a digestion of pTS with *Sac* II specific for hygromycin resistance gene; lane 3, target line 1-12; lanes 4 and 5, recombinants J and K of the target line 1-12 [note that the hygromycin-specific band of 1-12 J (lane 4) is 1.5 kb smaller than the expected homologous joint of 1.7 kb and also the 1.6-kb control fragment in lane 2]; lane 6, target line 1-7; lane 7, recombinant B of the target line 1-7; lane 8, target line 1-16; lane 9, recombinant C of the target line 1-16.

Table 1. Homologous integration of the repair construct in the target site after expression of I-*Sce* I

Transgene line	Ratio, repair construct/ I-*Sce* I gene	No. of transformants produced by: Homologous recombination (H), Kan^r*	Illegitimate recombination (I), bar^r × 10^2	Frequency, H/I × 10^{-3}
1-7	0.2/0.8	2	9.3	2.2
1-12	0.2/0.8	1	1.9	5.2
1-16	0.2/0.8	4	6.0	6.7
1-12	0.8/0.2	0	16.4	<0.6
	0.5/0.5	4	14.0	2.8
	0.1/0.9	6	3.2	18.8

To exclude the multiple propagation of clonal material, only one callus per inoculated seedling was taken into account and regenerated.
*Kanamycin resistant calli in which the kanamycin gene was restored by homologous recombination as analyzed by Southern blot analysis.

hygromycin-specific *Hin*dIII DNA fragment (Fig. 1). Hybridization of the blots with the hygromycin-specific probe indeed revealed such a fragment in 12 cases (1 case in line 1-7, 1 case in line 1-16, and 10 cases in line 1-12). In these lines no other "new" hygromycin-specific bands could be detected (e.g., Fig. 2*A*) indicating that, apart from the homologous integration event, no other copies of pRC were integrated in the genomes. Interestingly, in two cases (Fig. 2*A*, lanes 5 and 14) the 3.4-kb fragment representing the complete copy of the target site was lost, indicating that in these cases the other copies of the target site (pTS) in the chromosomal locus were lost. This may have occurred upon cleavage at several I-*Sce* I sites of the tandemly repeated transgene with concomitant removal of sequences in between, followed by homologous recombination with the repair construct.

In the remaining five cases (one event in line 1-7, one event in line 1-16, and three events in line 1-12) the 1.7-kb hygromycin-specific band was replaced by one or several hygromycin-specific DNA bands of different sizes (Fig. 3*A*, lane 4, a 1.5-kb band; lanes 5, 7, and 9, several bands). In two lines the original 3.4-kb band specific for the target locus was also lost (Fig. 3*A*, lanes 5 and 7). Thus in these five cases homologous recombination occurred only at one end (containing the kanamycin sequences) of the repair construct. A different recombination mechanism has to be postulated for these events in comparison to the 12 events described above (for details, see *Discussion*).

Apart from the molecular analysis, we also obtained genetic evidence that the kanamycin resistance gene was restored by homologous recombination. A set of recombinant plant lines was selfed and segregation analysis for kanamycin resistance was performed on the seeds obtained. All 11 lines tested exhibited a clear Mendelian segregation of the kanamycin resistance (in all cases $\chi^2 < 0.275, P > 0.6$) demonstrating that all recombined chromosomal loci are meiotically stable.

Sequence Analysis of the Recombined Gene. Our assay system relied on the identification of recombinants by kanamycin selection. Because the homologous sequence was placed within an intron region, most sequence changes that may have arisen during the recombination reaction should be conserved due to reduced selection pressure (26). To test this, we chose to sequence the genomic recombination joints of the plant lines 1-12 G and 1-16 B in which recombination occurred on both sides of the DSB, and of the line 1-12 K in which homologous recombination occurred only on the side of the kanamycin resistance gene. The genomic junctions were amplified via PCR using the primers P1 and P2 (Fig. 1). To exclude mutations occurring during the PCR amplification step, the appropriate DNA fragments of two independent PCRs were cloned for each target line and sequenced. The two sequences of each recombination joint were compared with each other and only nucleotide changes that were found in both PCR products were considered original.

The result of this analysis indicated that in the case of the lines 1-12 K and 1-16 B, perfect homologous recombination took place—i.e., no sequence changes could be detected within the 1-kb recombinant intron. However, sequence analysis of the line 1-12 G revealed a 14-bp deletion roughly in the center of the recombinant intron. A closer look revealed that the deleted base pairs are flanked by a 3-bp repeat (TTA) and that the internal 11 bases could theoretically form a secondary structure (see also Fig. 5 and *Discussion*).

DISCUSSION

We demonstrated that artificially induced site-specific genomic DSBs in plant cells were repaired by homologous recombination with exogenously supplied DNA using two different pathways. Induction of the DSB led to drastically enhanced frequencies of homologous integration into a specific locus of the plant chromosome. Our findings might be the basis for different kinds of controlled sequence rearrangements of plant genomes.

***Agrobacterium* and Gene Targeting in Plants.** *Agrobacterium* transfers a specific segment of bacterial DNA (T-DNA) into the plant genome (for review, see ref. 33). T-DNA travels as a single strand to the plant nucleus (24). Integration is occurring via illegitimate recombination and most likely involves single-stranded T-DNA (29). In *Agrobacterium*-mediated gene targeting experiments frequencies of 8.4×10^{-5} (34), 3.3×10^{-5} (32), and $2.2–6.3 \times 10^{-6}$ (35) were reported for tobacco cells. Recently Miao and Lam (36) reported a targeting frequency of 3.9×10^{-4} for *Arabidopsis*. By inducing a specific DSB at the target sequence in the plant genome, we were able to obtain frequencies of $2.2–18.3 \times 10^{-3}$, indicating that T-DNA can be used as template for the repair of a genomic DSB. Because DNA uptake by direct gene transfer yielded low frequencies in gene targeting in plants comparable with *Agrobacterium*-mediated transfer (30, 37), we expect that a similar increase in the frequency could also be achieved via DSB induction using DNA-mediated transformation techniques. Hrouda and Paszkowski (26) found without DSB induction a gene targeting frequency of 3.8×10^{-6} for tobacco protoplasts, using the same kanamycin intron constructs also used in our study.

Genomic DSBs in Plants Can Be Repaired by Different Pathways. The analysis of recombinant plant lines allows conclusions on the mechanism of DSB repair in plant cells by homologous recombination. In 12 cases, both ends were used and in 5 cases only one end of the repair construct was used for the repair of the DSB by homologous recombination. One possible interpretation of this finding is that under our conditions recombination reactions are not occurring simulta-

neously at both sides of a DSB but that the interaction of the partners is started at one end. Similar conclusions were drawn from a kinetic analysis of recombination reactions in the mating type switching system in yeast, a special case of DSB induced homologous recombination. There, after induction of a DSB by HO nuclease the 3′ end of broken DNA distal (i.e., right of the HO site on the conventional map) to the cut is invading the intact donor duplex. Only later interactions between the proximal (left of the HO site) end of the cut and the homologous donor sequence occur to complete gene conversion (5). Gene targeting of repeated sequences in mammalian cells was also suggested to start at one end, which would explain the existence, apart from double-homologous recombinants, of a new class of recombinants in which only one of the two homologous regions underwent homologous recombination (38). This led to the proposal of the one-sided invasion model (ref. 3; see also refs. 39 and 40).

Because in our targeting experiments both processes have been detected, we tried to relate the underlying mechanisms to each other. We therefore suggest that DSB repair proceeds in plant cells as depicted in Fig. 4. After induction of the DSB (Fig. 4*a*) and the production of 3′ single-stranded tails at the genomic break due to exonucleolytic degradation of the 5′ ends (Fig. 4*b*), the 3′ single-stranded tail of the DSB and the homologous 3′ end of the single-stranded repair construct anneal (Fig. 4*c*). As T-DNA is used as template for repair, it formally does not matter whether it is present in its single-stranded or double-stranded form. [Targeting of single-stranded and double-stranded vectors was shown to occur with similar frequencies and identical recombination patterns in mammalian cells (41)]. Following elongation of the distal genomic 3′ end, using T-DNA as template, the recombination reaction may proceed with or without the use of homology supplied proximal (i.e., Fig. 4 *Left*) of the DSB. If a second homologous pairing step can occur (in Fig. 4*d1*) the reaction mimics the classical double-strand break repair model. The resulting recombinant thus possesses homologous sequences at both sides of the original DSB (Fig. 4*e1*). If, on the other hand, this interaction is not possible because proximal homologies are either not present (e.g., due to an enlargement of the gap by nucleases) or not recognized, the DSB is repaired by illegitimate recombination in a nonconservative way (Fig. 4*d2*) as described by the one-sided invasion model. The resulting recombinant has thus a homologous (at the distal side of the DSB) and a nonhomologous (at the proximal side of the DSB) joint between repair construct sequences and genomic sequences (see Fig. 4*e2*).

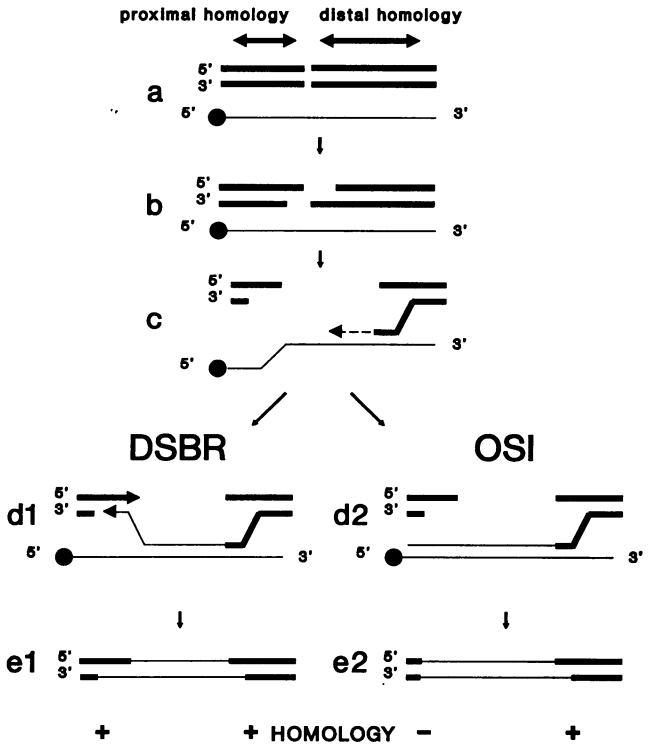

FIG. 4. Models for the repair of DSBs in the plant genome: the double-strand break repair model (DSBR) and the one-sided invasion model (OSI) as adapted to homologous integration of a single-stranded T-DNA molecule (solid circle, VirD2 molecule attached to the 5′ end of the T-strand). After the induction of the DSB at the specific site in the plant genome (*a*) and the production of free single-stranded 3′ tails by a 5′-3′ endonuclease (*b*), the T-strand can be used as repair template by one 3′ end of the broken duplex (*c*). Depending on whether the repaired 3′ end can interact with homologous sequences at the opposite side of the break the recombination reaction will be concluded by homologous recombination resulting in a conservative recombination product (DSBR) (*d1, e1*) or by illegitimate recombination resulting in a nonconservative recombination product (OSI) (*d2, e2*). For further explanations see *Discussion.*

Analysis of three joints in the homologous intron region revealed perfect sequence conservation in two cases, one double-strand break repair-like and one one-sided invasion-like event. In a third case, a double-strand break repair-like event, however, a 14-bp long deletion was detected within the homologous region. The formation of this deletion may have occurred upon copying of a single-stranded T-DNA (Fig. 4*c*) which transiently formed a small stem loop structure across which the replicating DNA polymerase slipped (Fig. 5). Both sides of the putative hairpin contain a TTA trinucleotide, enabling the DNA polymerase to switch the template, resulting in the loss of bases in the genomic DNA sequence. Our finding is especially notable in the light of a recent report, demonstrating that DSB repair in yeast is accompanied by high mutation rates, part of which might be due to secondary structure-mediated polymerase slippage (42).

It is interesting to compare our data to results obtained in gene targeting of tobacco in which no specific DSBs were induced. Out of five targeting events reported by Risseeuw *et al.* (35), only one transformant contained two homologous joints, whereas in four other cases, only one homologous joint was found (associated with deletions of the target locus). One is tempted to speculate that in all these cases a "naturally" occurring DSB within or close to the proximal side of the target

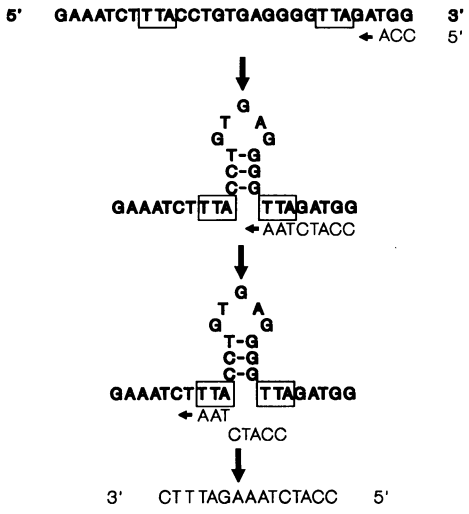

FIG. 5. Model for the deletion of 14 bp in the recombinant intron of the targeted plant line 1-12 F. (Boldface letters, T-strand; thin letters, 3′ strand of the distal end of the genomic break). As shown in Fig. 4, the T-strand is used as template for the repair of the genomic gap. For further explanations see *Discussion.*

locus was inducing the recombination reaction. In two independent cases, however, it was found that the incoming T-DNA was converted to the target locus in a nonreciprocal way and was subsequently integrated elsewhere (35, 43). In our experiments this is less likely to happen since the target sequence is activated by the DSB to act as acceptor of genetic information.

Homologous Recombination via DSB Induction—A System for the Site-Specific Integration of Foreign DNA. Homologous recombination ("gene targeting") is an attractive technique for integrating foreign DNA at a specific site in the mouse genome (44). Unfortunately, no attractive technique for targeting of genes of higher plants could yet be developed (45). As the induction of DSBs by I-*Sce* I seems to be rate-limiting in our system (Table 1), the frequency of integration, which was in the best case one homologous event out of ≈50 illegitimate ones, might be further improved by, for example, supplying more enzyme directly (46, 47).

The irreversible integration of foreign DNA into specific I-*Sce* I sites in the plant genome, which can be mapped by I-*Sce* I digestions (48), might become a powerful technique and thus may represent a viable alternative to the recently developed Cre-*lox*-mediated site-specific integration into the plant genome (49). Genomic position effects can be excluded by applying the technique in, for example, plant promoter studies. Further transgenes may be accumulated at a specific locus by introducing other target sites as *lox* sites that can be used for the integration of foreign DNA or the controlled manipulation of the transgene locus.

Induction of DSBs—A Way Out of the Gene Targeting Dilemma in Plants? The induction of a DSB might in general be one of the rate-limiting steps in gene targeting in plants. Thus a technique based on the induction of restriction-mediated DSBs might be established to target natural genes. In the current study we used a highly specific restriction endonuclease with a 18-mer recognition sequence. Although some additional sequences with minor changes in the recognition site are also recognized (8), I-*Sce* I is the most specific commercially available restriction enzyme. It is tempting to speculate that the carefully controlled use of conventional restriction enzymes (recognizing 6- or 8-bp sites) that cut in natural genes might improve gene targeting frequencies of these genes drastically.

Induction of DSBs—A Way for Controlled Change of the Plant Genome? Induction of a site-specific genomic DSB in plants should also be applicable for the induction of recombination events between two linked (cis) or unlinked (trans) genomic loci. From our present results we expect that a double-strand break repair-like recombination event should also occur between two double-stranded genomic loci, with recombination intermediates being resolved as gene conversions as well as crossovers. However, a crossover will lead to an exchange of genetic linkage. If the transgenes are located on the same chromosome, a deletion or inversion of the genetic information between the homologies would occur, depending on the orientation of the homologous sequence information (50, 51). If the transgenes are placed on different chromosomes, an exchange of chromosome arms could occur (52).

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