Double-Strand Break-Induced Recombination Between Ectopic Homologous Sequences in Somatic Plant Cells

Holger Puchta

Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), D-06466 Gatersleben, Germany

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ABSTRACT

Homologous recombination between ectopic sites is rare in higher eukaryotes. To test whether double-strand breaks (DSBs) can induce ectopic recombination, transgenic tobacco plants harboring two unlinked, nonfunctional homologous parts of a kanamycin resistance gene were produced. To induce homologous recombination between the recipient locus (containing an I-*Sce*I site within homologous sequences) and the donor locus, the rare cutting restriction enzyme I-*Sce*I was transiently expressed via Agrobacterium in these plants. Whereas without I-*Sce*I expression no recombination events were detectable, four independent recombinants could be isolated after transient I-SceI expression, corresponding to approximately one event in 10^5 transformations. After regeneration, the F_1 generation of all recombinants showed Mendelian segregation of kanamycin resistance. Molecular analysis of the recombinants revealed that the resistance gene was indeed restored via homologous recombination. Three different kinds of reaction products could be identified. In one recombinant a classical gene conversion without exchange of flanking markers occurred. In the three other cases homologous sequences were transferred only to one end of the break. Whereas in three cases the ectopic donor sequence remained unchanged, in one case rearrangements were found in recipient and donor loci. Thus, ectopic homologous recombination, which seems to be a minor repair pathway for DSBs in plants, is described best by recombination models that postulate independent roles for the break ends during the repair process.

DOUBLE-STRAND breaks (DSBs) are critical lesions in eukaryotic genomes. Even a single genomic DSB is able to block cell division in mammalian cells (Huang *et al.* 1996). Efficient repair of DSBs is therefore important for the survival of all organisms. In principle, breaks can be repaired by two modes, illegitimate and homologous recombination. The main mode of repair in somatic plant cells seems to be illegitimate recombination (Puchta and Hohn 1996; Salomon and Puchta 1998), although studies with intrachromosomal recombination substrates indicate that breaks can also be repaired by the use of homology close to the break (Lebel *et al.* 1993; Puchta *et al.* 1995; Chiurazzi *et al.* 1996). In a recent report, occurrence of ectopic homologous recombination in tobacco could be demonstrated by the use of two unlinked transgenes, one carrying a transposable element inserted in a nonfunctional part of the β-glucuronidase (GUS) gene and the other containing the complementing GUS sequences (Shalev and Levy 1997). In this system, however, due to the destructive assay, no recombinant cells could be regenerated, and Southern analysis elucidating the molecular structure of the recombinants could not be performed. Therefore no conclusion about the mechanism of the reaction could be drawn.

Address for correspondence: Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Corrensstrasse 3, D-06466 Gatersleben, Germany. E-mail: puchta@ipk-gatersleben.de

The elucidation of recombination mechanisms in eukaryotes has been greatly helped by the establishment of techniques for inducing *in vivo* transient genomic DSBs via rare cutting restriction endonucleases (for review see Haber 1995; Jasin 1996). We recently established a system for *in vivo* induction of DSBs at specific genomic sites in transgenic plants by transient expression of the highly specific restriction endonuclease I-*Sce*I. Our previous analyses have focused on the repair of genomic DSBs with extrachromosomal homologous sequences supplied on T-DNAs. The results indicated that DSBs enhance integration of T-DNAs via homologous recombination by several orders of magnitude (Puchta *et al.* 1996) and that DSBs are predominately repaired by mechanisms involving one-sided invasion of the 3′ ends of the break (Puchta 1998). The aim of the experiments described here was to elucidate whether DSBs can also induce recombination between ectopic homologous sequences in the plant genome and, if so, what the underlying recombination mechanisms are.

MATERIALS AND METHODS

DNA constructs: The I-*Sce*I expression vector pCISceI (Puchta *et al.* 1996) contains a synthetic I-*Sce*I open reading frame (ORF) under the control of the cauliflower 35S promoter (Puchta *et al.* 1993) between T-DNA borders. The recipient site pTS (Puchta *et al.* 1996) in the tobacco line 1-12 (Figure 1) contains, next to an I-*Sce*I restriction site, the C-terminal half of an intron-containing kanamycin resistance gene (Hrouda and Paszkowski 1994). The progeny of the

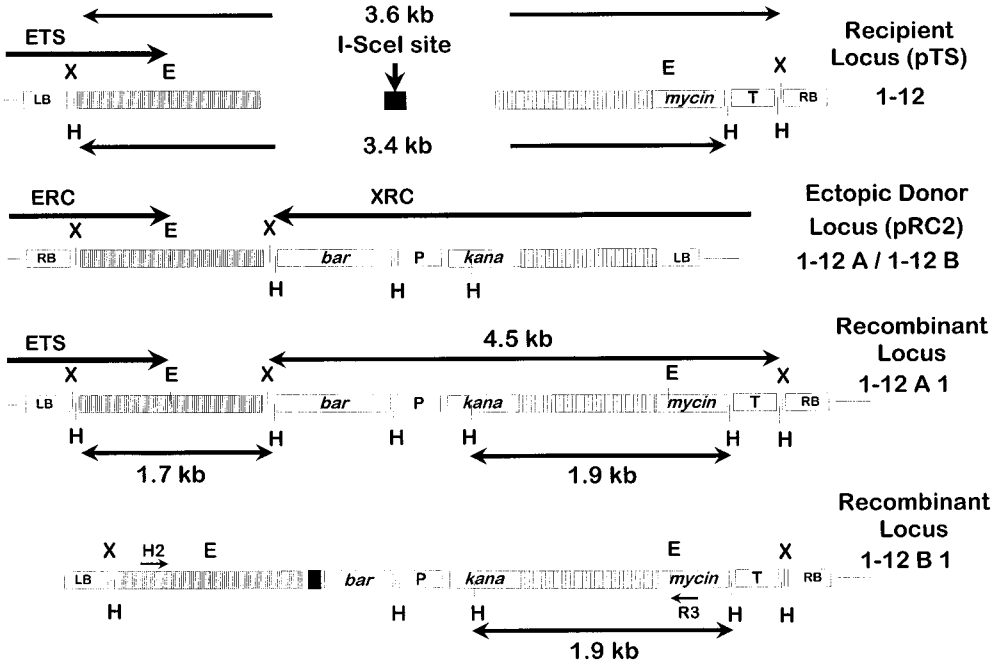

Figure 1.—Maps of the transgenes: 1-12 represents the recipient locus, pTS drawn as T-DNA integrated into the plant chromosome. Between the homologous regions (shaded box), an I-*Sce*I-site (black box) was inserted. 1-12 A / 1-12B represents the donor locus pRC2 integrated in an ectopic position into the plant chromosome. The recombinant locus 1-12 A 1 resulted from a conservative gene conversion event. The recombinant locus 1-12 B 1 resulted from a one-sided homologous DSB repair event. The homologous region within the *kanamycin* gene (gray shaded) is 1 kb in length, whereas the homology at the other end (dark shaded, *hygromycin*-specific sequences) is 1.3 kb in length. In case of homology the *kanamycin* gene is restored. After Southern blotting of *Hin*dIII (H)-digested DNA, the original recipient locus pTS should give a hybridization signal of 3.4 kb with a *kanamycin*-specific probe and homologous recombinants should show a 1.9-kb *kanamycin*-specific fragment and a 1.7-kb *hygromycin*-specific fragment. In pTS, a 3.6-kb fragment can be obtained by *Xba*I digestion, and a 4.5-kb fragment is indicative of homologous recombination. ETS, transgene-genome junction specific for the recipient locus (pTS); ERC and XRC, transgene-genome junctions specific for the donor locus (pRC2); bar, bar gene conferring resistance to glufosinate; LB, left border; RB, right border; P, cauliflower mosaic virus 35S promoter; T, cauliflower mosaic virus terminator; H2 and R3, PCR primers used for amplification and sequence analysis of the recombined locus of 1-12 B 1.

transgene line 1-12 used in this study contains a single copy of pTS at the transgene locus. Originally, the line 1-12 contained most probably two copies of pTS in tandem (Puchta *et al.* 1996), one of which was apparently lost during earlier propagation. The binary vector pRC2 (Figure 1) carries on its T-DNA next to the left border a 1-kb sequence homologous to kanamycin-specific sequences of pTS and next to its right border a 1.3-kb sequence homologous to hygromycin-specific sequences upstream of the I-*Sce*I site of pTS (Puchta 1998). The bar gene can be used as a transformation marker, resulting in glufosinate-resistant plants.

Transformation of tobacco seedlings with Agrobacterium: Transformation of tobacco seedlings with Agrobacteria was done as described (Puchta 1999). After growth, bacteria were centrifuged and resuspended in 10 mm $MgSO_4$. The OD_{600} of the suspension was measured and bacteria were diluted in 10 mm $MgSO_4$ to $OD_{600} = 0.5$. Three days after inoculation the seedlings were transferred onto Murashige and Skoog (MS) medium containing phytohormones and the relevant antibiotic (100 mg/liter kanamycin sulfate or 20 mg/liter glufosinate-ammonium). Regeneration of plants from resistant calli was carried out as described (Tinland *et al.* 1995). Segregation of the selfed transformants was tested by putting seeds onto MS medium supplemented with 500 mg/liter kanamycin sulfate or 20 mg/liter glufosinate-ammonium. Statistical comparisons of the actual segregation to the expected Mendelian segregation were done by a χ^2 goodness of fit test (Simpson *et al.* 1960).

DNA analysis: Total DNA isolation (Salomon and Puchta 1998) and Southern blotting (Swoboda *et al.* 1994) were done as described. A *Hin*dIII fragment of the plasmid pAH5 (Hrouda and Paszkowski 1994) containing parts of the kanamycin gene and a *Bam*HI fragment carrying the hygromycin resistance gene of pTS were labeled with [α-^{32}P]CTP using the random primed labeling kit (Megaprime DNA labeling system RPN1607, Amersham, Little Chalfont, U.K.).

PCR and sequence analysis: Genomic DNA was analyzed via PCR as described (Salomon and Puchta 1998) using the primers H2 5′ pATGCCTGCGG GTAAATAGC 3′ and R3 5′ pAGCCGCCGCA TTGCATCAGC C 3′ (for binding sites see Figure 1) in a Gene Amp PCR-System 2400 (Perkin-Elmer, Norwalk, CT). The following program was used for amplification: 5 min denaturation at 94°, 40 cycles of 20 sec at 93°, 35 sec at 55°, and 2.5 min at 68°, and a final step of 7 min at 68°. The amplification product was cloned into the pCR 2.1-Topo vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and propagated in TOP10 One Shot *Escherichia coli* cells (Invitrogen) according to manufacturer's instructions. After subcloning, sequence analysis was performed with the automatic DNA-sequencer ALF-Express (Pharmacia, Uppsala, Sweden).

RESULTS

Production of transgenic tobacco lines harboring an ectopic donor locus: The aim of the present study was to elucidate whether a specific DSB can be repaired by ectopic homologous sequences in the plant genome and what kind of products can arise. For this purpose we used the tobacco line 1-12, which contained at a single genomic locus one copy of the recipient sequence pTS (Figure 1). pTS contains, next to a nonfunctional part of a kanamycin resistance gene, the 18-bp restriction site of I-*Sce*I. It was shown previously, by transient expression of the restriction endonuclease I-*Sce*I, that

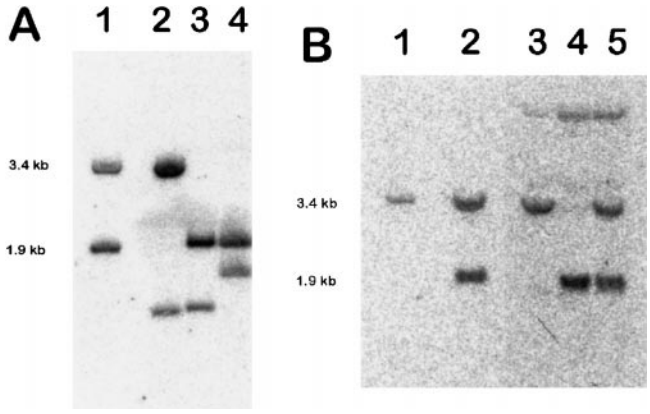

Figure 2.—Southern blot analysis of recombinant lines. *Hin*dIII-digested DNA blotted against a kanamycin-specific probe. (A) Lane 1, 1-12c; lane 2, 1-12 A; lane 3, 1-12 A 1; and lane 4, 1-12 A 2. (B) Lane 1, line 1-12; lane 2, line 1-12c; lane 3, line 1-12 B; lane 4, 1-12 B 1; and lane 5, 1-12 B 2. The 3.4-kb fragment is indicative for the recipient construct pTS and can be detected in line 1-12 (B1), in positive control line 1-12c (A1 and B2), in lines 1-12 B (B3) and 1-12A (A2), and in recombinant line 1-12 B 2 (B5). The 1.9-kb fragment, indicative for homologous recombination between the *kanamycin*-specific sequences of pTS and pRC2, can be detected in positive control 1-12c (A1, B2) and in all four recombinant lines (A3, A4, B4, and B5). The *kanamycin*-specific genome-pRC2 junction fragment is different for 1-12A (lower fragment) and 1-12 B (upper fragment). The fragment of the donor locus of the respective parental line is conserved in the recombinants 1-12 A 1 (A3), 1-12 B 1 (B4), and 1-12 B 2 (B5), but not in 1-12A 2 (A4).

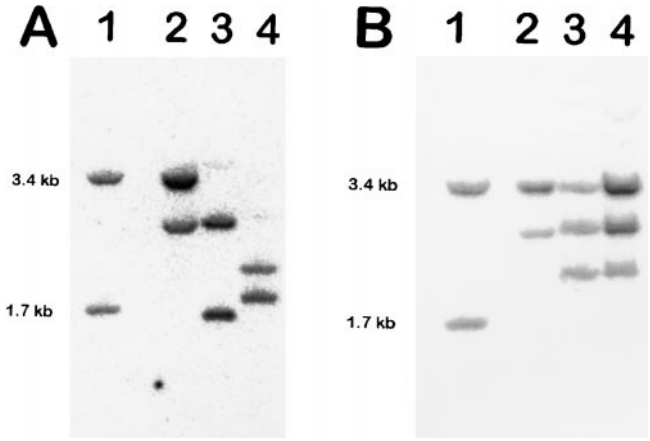

Figure 3.—Southern blot analysis of recombinant lines, *Hin*dIII-digested DNA blotted against a hygromycin-specific probe. (A) Lane 1, 1-12c; lane 2, 1-12 A; lane 3, 1-12 A 1; and lane 4, 1-12 A 2. (B) Lane 1, line 1-12c; lane 2, line 1-12 B; lane 3, 1-12 B 1; and lane 4, 1-12 B 2. The 3.4-kb fragment is indicative for the recipient construct pTS and can be detected in positive control line 1-12c (A1 and B1), in lines 1-12 A (A2) and 1-12 B (B2), in recombinant line 1-12 B 2 (A5), and apparently also in line 1-12 B 1 (A4). However as no 3.4-kb *kanamycin*-specific fragment is present in line 1-12 B 1 (see Figure 2, B4) and the intensity of the *hygromycin*-specific fragment is about half of that for 1-12 B and 1-12 B2, it seems that there is a second 3.4-kb *hygromycin*-specific fragment present in all three 1-12 B lines, together with the 3.4-kb hygro- and *kanamycin*-specific fragment of pRC2 in lines 1-12 B and 1-12 B 2. The 1.7-kb fragment indicative for homologous recombination between the hygromycin-specific sequences of pTS and pRC2 is detectable in positive control line 1-12c (A1, B1) and in recombinant line 1-12 A 1 (A3). The *hygromycin*-specific genome-pRC2 junction fragments are different for the original lines 1-12A (one fragment below the 3.4-kb fragment) and 1-12 B (two fragments, one of 3.4 kb and one smaller). The fragment(s) of the respective parental line are (is) conserved in the recombinants 1-12 A 1 (A3), 1-12 B 1 (B3), and 1-12 B 2 (B4), but not in 1-12A 2 (A4). New fragments arose due to nonhomologous recombination in lines 1-12 A2 (A4), 1-12 B 1, and B 2 (B3, B4, smallest fragment, each).

DSBs can be induced *in vivo* in plant cells (Puchta *et al.* 1993) and that after induction of a genomic DSB a T-DNA carrying homologies of 1.0 and 1.3 kb to the broken ends (pRC2) can integrate into the recipient locus of the line 1-12 (Puchta *et al.* 1996; Puchta 1998). Next, the line 1-12 was stably transformed with pRC2 to obtain plants that harbor the homologous sequence in ectopic positions of the genome. Primary transformants were regenerated and transgenic lines in which glufosinate resistance was segregating in a 3/1 fashion were identified indicating the presence of a single transgene locus. Furthermore, a second segregation analysis was performed on the progeny of transgenic plants that were hemizygous for pTS (hygromycin resistance) and pRC2 (glufosinate resistance) to find out whether both loci were genetically linked. Two lines, 1-12 A and 1-12 B, were chosen for further analysis. The transgene loci in these lines were not linked genetically (rejection $P = 0.05$) as selection markers were transferred independently to the progeny ($\chi^2 = 0.85$ for 1-12 A; $\chi^2 = 2.74$ for 1-12 B).

Analyzing the molecular structure of pRC2 integrated into the genome, it was found that in the case of line 1-12 A, a single, complete copy of the T-DNA was inserted in the genome. This is shown by the occurrence of only one specific genome-T-DNA junction fragment for both ends of the T-DNA (the *kanamycin*-specific fragment, Figure 2A, lane 2 [*Hin*dIII-digest], Figure 4, lane 3 [*Xba*I-digest], and the *hygromycin*-specific fragment, Figure 3A, lane 2 [*Hin*dIII-digest]). In line 1-12 B two copies of pRC2 were inserted at a single locus, apparently with some genomic sequences between them. This is documented by the occurrence of two specific fragments for both genome-T-DNA junctions (*kanamycin*-specific fragments in Figure 4, lane 6 [*Xba*I-digest], and *hygromycin*-specific fragments in Figure 3B, lane 2 [*Hin*dIII-digest]; one fragment is comigrating with the internal 3.4-kb fragment of the recipient locus—see below). The integrity of the homologous regions of pRC2 was also tested via Southern blotting: Because the homologous sequence within the *hygromycin* gene is flanked by *Xba*I restriction sites (Figure 1), hybridization with a *hygromycin*-specific probe should reveal a 1.3-kb *Xba*I-specific fragment. In both transgenic lines 1-12 A and 1-12 B

TABLE 1

DSB-induced ectopic homologous recombination

Transgene line	I-*Sce*I	HR *Kan*^{r} [a]	Transformed cells (T)[b] $\times 10^5$ (seedlings)	Frequency H/T
1-12A	−	0	2.0 (2007)	$< 5.0 \times 10^{-6}$
(5 experiments)	+	2	1.9 (1905)	10.5×10^{-6}
1-12B	−	0	4.0 (3984)	$< 2.5 \times 10^{-6}$
(11 experiments)	+	2	3.9 (3940)	5.1×10^{-6}

HR, homologous recombinants; *T*, transformants.

[a] Kanamycin-resistant calli in which the kanamycin gene was restored by homologous recombination as shown by Southern hybridization. The calli were obtained from independent experiments.

[b] Based on the estimation of 100 transformation events per seedling as demonstrated in previous experiments (Tinland *et al.* 1995; Puchta 1998).

this fragment was clearly visible (data not shown). Furthermore, via *Hae*III digestion the integrity of a 0.8-kb fragment covering most of the 1.0-kb homologous sequence of the *kanamycin* gene of pRC2 can be checked. Molecular hybridization revealed the presence of such a fragment in lines 1-12 A and 1-12 B (data not shown), demonstrating that the T-DNA sequences of pRC2 were not rearranged in both lines.

Induction of recombination: To induce ectopic recombination, F_1 seedlings of the lines 1-12 A and 1-12 B were inoculated with Agrobacteria carrying the I-*Sce*I open reading frame under the control of plant expression signals on their T-DNA to achieve transient expression of the enzyme (Puchta *et al.* 1996). In parallel experiments an equivalent number of seedlings were inoculated with the same Agrobacterium strain devoid of the binary vector. Altogether 5 transformations were done with line 1-12 A and 11 with 1-12 B (see Table 1). In total, 4 kanamycin-resistant calli were obtained, 2 for each line. The calli originated from independent experiments. In previous experiments with pRC2 supplied as T-DNA ~10–20 kanamycin-resistant calli were obtained per transformation experiment, using half the number of seedlings (Puchta 1998). This indicates that DSBs can be repaired by pRC2 as extrachromosomal T-DNA much more efficiently than by pRC2 as ectopic genomic sequence.

TABLE 2

Segregation for kanamycin resistance in the selfed progeny (F_1) of the tobacco lines, with a kanamycin gene restored by homologous recombination.

Line	Kanamycin *Kan*^{r}	Kanamycin *kan*^{s}	r/s	χ^2
1-12 A	Sensitive			
1-12 A 1	163	57	2.9/1	0.05
1-12 A 2	107	40	2.7/1	0.16
1-12 B	Sensitive			
1-12 B 1	185	72	2.6/1	0.64
1-12 B 2	128	48	2.7/1	0.24

The χ^2 values indicate that the ratios do not deviate significantly from Mendelian segregation (rejection $P < 0.05$).

Kan^{r}, resistant; *kan*^{s}, sensitive; r/s, ratio of kanamycin resistant to sensitive seedlings.

Genetic and molecular characterization of the recombinants: Tobacco plants were regenerated from the kanamycin-resistant calli and selfed. The progeny were grown on selection medium to verify that kanamycin resistance was restored and transferred to the next generation. All four lines showed a clear-cut Mendelian segregation in the F_1 generation (Table 2). The recombinants were then analyzed at the molecular level to further characterize the recombination products.

1-12 A 1: The recombinant 1-12 A 1 arose due to homologous recombination of both ends of the DSB with the genomic donor sequence. Southern Blotting with *Hin*dIII-digested DNA revealed the presence of a 1.9-kb *kanamycin* specific fragment [compare Figure 2A, lane 1, the positive control line 1-12c, in which homologous integration of pRC2 as T-DNA in the recipient locus was achieved by DSB-induction (Puchta *et al.* 1996) with 1-12 A 1, lane 3] and a 1.7-kb *hygromycin*-specific fragment (compare Figure 3A, the positive control line 1-12c in lane 1 with 1-12 A 1, lane 3), indicative of homologous recombination at both ends of the break (see also Figure 1 for constructs). The two junctions replace the original pTS-specific fragment of the recipient locus, which was lost during the recombination reaction. A Southern blot with *Xba*I-restricted DNA confirmed these findings: a new 4.5-kb *kanamycin*-specific fragment of same size as in the positive control line 1-12c is detectable in line 1-12 A 1 (Figure 4, compare lanes 2 and 4), whereas the 3.6-kb pTS-specific fragment of the recipient locus is gone. Comparison of the pRC2-specific fragments of the line 1-12 A with the recombinant 1-12 A 1 indicates that during the recombination reaction no change of the ectopic donor locus pRC2 occurred (Figure 3A, identical fragment in lanes 2 and 3 and Figure 4, identical fragment in lanes 3 and 4). In accordance with the molecular data, the segregation of

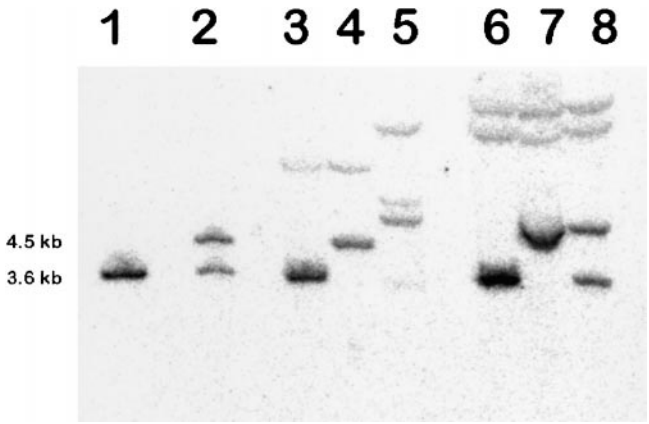

Figure 4.—Southern blot analysis of recombinant lines, *Xba*I-digested DNA blotted against a *kanamycin*-specific probe. Lane 1, line 1-12; lane 2, line 1-12c; lane 3, 1-12 A; lane 4, 1-12 A 1; lane 5, 1-12 A 2; lane 6, 1-12 B; lane 7, 1-12 B 1; and lane 8, 1-12 B 2. The 3.6-kb fragment, indicative of pTS2, is present in 1-12, 1-12c, 1-12 A, 1-12 B, and 1-12 B 2. The 4.5-kb fragment, indicative of homologous recombination of pTS with pRC2, is present in positive control line 1-12c and recombinant line 1-12 A 1. In lines 1-12 B 1 and 1-12 B 2 a recombinant fragment of larger size (in 1-12 B 2 slightly bigger than in 1-12 B 1) can be detected. The *kanamycin*-specific genome-pRC2 fragments are different for the original lines 1-12A (one fragment above the 4.5-kb fragment) and 1-12 B (two fragments at the top of the blot). As before (see Figures 2 and 3) the fragment(s) of the donor locus of the respective parental line are (is) conserved in the recombinants 1-12 A 1 (A3), 1-12 B 1 (B3), and 1-12 B 2 (B4), but not in 1-12A 2 (A4).

glufosinate resistance (bar gene) in the F_1 generation was close to 15/1 ($\chi^2 = 0.03$), demonstrating that the resistance gene was copied in its functional form into the break, resulting in two independently segregating entities of the gene.

In principle—at least according to the DSB repair (DSBR) model of recombination (Szostak *et al.* 1983)—gene conversions can be accompanied by crossover of flanking sequences. It was therefore checked whether in this case the conversion was accompanied by a crossover of flanking sequences. For this purpose plants of the selfed F_1 progeny of the regenerated mother plant were analyzed via Southern blotting. As in the recombinant 1-12 A 1, the donor locus pRC2 is hemizygous; the recombined recipient locus is expected to be present in absence of pRC2 in 3 out of 16 F_1 plants. Indeed 2 plants that carried the recombined recipient locus but lacked pRC2 were found in 11 kanamycin-resistant tested. Molecular hybridization with probes specific for the T-DNA transgene junctions flanking the homology (kanamycin, the 4.5-kb fragment and XRC; hygromycin, ERC and ETS, see Figure 1) were used to test whether a crossover had occurred. Both flanking fragments of the recombined recipient locus (the 4.5-kb fragment and the ETS junction; compare Figure 5, A and B) were detected, whereas both flanking fragments of the donor locus pRC2 (XRC and ERC) were absent (in Figure 5, A and B, compare lane 3 with lanes 4 and 5) in these plants. Thus, the gene conversion was not accompanied by crossover.

1-12 B 1: The recombinant 1-12 B1 revealed a restored kanamycin gene as demonstrated by the occurrence of the 1.9-kb kanamycin-specific *Hin*dIII fragment in Figure 2B, lane 4. However, the other end of the break was not repaired by homologous recombination as no 1.7-kb hygromycin-specific *Hin*dIII fragment is present in the respective blot (Figure 3B, lane 3). The recombination reaction led to a rearrangement of the recipient locus; the kanamycin-specific *Xba*I fragment of the recipient locus seems to be a bit bigger than in 1-12c (Figure 4, compare lanes 6 and 7). No rearrangement of the donor locus took place (Figure 3B, identical fragments in lanes 2 and 3 and Figure 4, identical fragment in lanes 6 and 7).

To characterize a one-sided homologous DSB repair event as a paradigmatic case at the sequence level, PCR was used. Oligonucleotides H2 (with its binding site within the hygromycin gene) and R3 (with its binding site at the 3′-end of the kanamycin gene) were used for amplification (see Figure 1). Because in this recombinant the original recipient locus (pTS) was no longer present, a unique 3.6-kb fragment containing both the homologous and the illegitimate junction of donor and recipient sequences could be isolated, cloned, and sequenced. Comparison of the obtained sequence with pTS and pRC2 confirmed the finding of the Southern blot data (Figure 2A) that the kanamycin resistance gene was restored by homologous recombination. The other end of the break was indeed—as already indicated by Southern blotting (Figure 3B)—repaired by illegitimate recombination. The sequence of the recipient locus (pTS) was joined to that of the donor locus (pRC2) within a stretch of five homologous nucleotides (Figure 6), a pattern well known for the repair of genomic DSBs in plants (Salomon and Puchta 1998). The free 3′ end of the break was nearly completely retained, as only one out of four nucleotides within the 3′ overhang of the I-*Sce*I cut is missing in the newly formed junction (Figure 6). Most of the bar gene of the donor locus was not transferred to the recipient in the reaction—in accordance with the 3/1 segregation ($\chi^2 = 0.68$) of glufosinate resistance in the progeny of 1-12 B 1 (which is similar to the parental line 1-12 B). Thus, in 1.12 B 1 one end of the break was repaired via homologous recombination and the other end via illegitimate recombination using sequences from the donor locus pRC2.

1-12 B 2: As with line 1-12 B 1, line 1-12 B 2 resulted from one-sided invasion of homologous sequences into the DSB, leading to the restoration of the kanamycin resistance gene. This is indicated by the presence of the 1.9-kb kanamycin-specific fragment and the absence of the 1.7-kb hygromycin-specific fragment after Southern hybridization with *Hin*dIII-restricted DNA (see Figure 2B, lane 5 and Figure 3B, lane 4). In contrast to 1-12

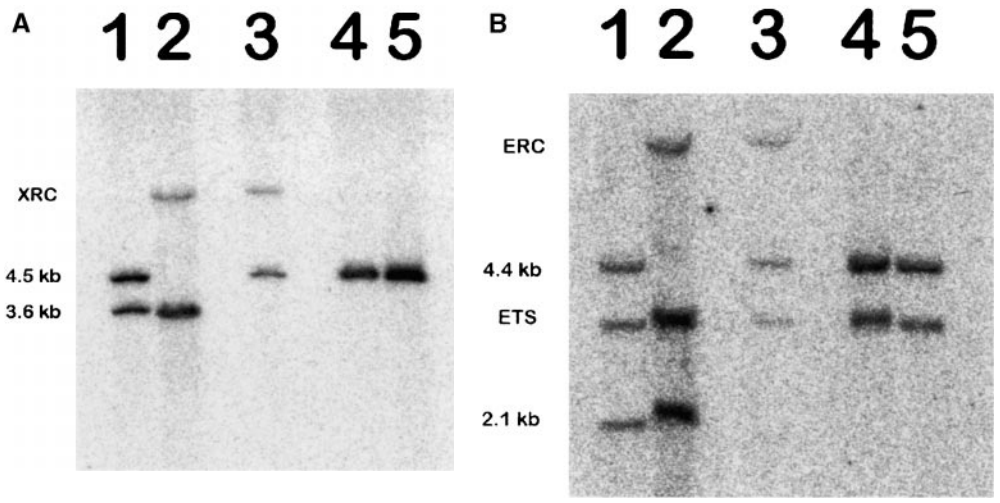

Figure 5.—Southern blot analysis of indiviual F_1 plants of the recombined line 1-12A 1. (A) *Xba*I-digested DNA blotted against a *kanamycin*-specific probe. Lane 1, positive control line 1-12 c; lane 2, line 1-12 A; lane 3, line 1-12 A 1 (mother plant); lanes 4 and 5, progeny plants of 1-12 A 1 carrying only the recombined transgene locus. (B) *Eco*RI-digested DNA blotted against a *hygromycin*-specific probe. Lane 1, positive control line 1-12 c; lane 2, line 1-12 A; lane 3, line 1-12 A 1 (mother plant); lanes 4 and 5, progeny plants of 1-12 A 1 carrying only the recombined transgene locus. The 2.1- and 3.6-kb fragments are specific for the recipient locus pTS and the 4.4- and 4.5-kb fragments for the recombinant locus (see Figure 1). The blot demonstrates that the recombination event resulting in 12 A 1 was due to a gene conversion not associated by a crossover. For a crossover, a linkage of the transgene border junction ERC and the fragments indicative for recombination (4.5 kb in A and 4.4 kb in B) is expected. This is clearly not the case, as the fragments cosegregate with ETS. Both donor locus-genome-specific junctions ERC and XRC are absent.

B 1, the unchanged recipient locus pTS is present on the homologue in the recombinant 1-12 B 2 (compare Figure 2B, lanes 4 and 5 and Figure 4, lanes 7 and 8). As in the case of 1-12 B 1, no change in the donor locus could be detected in the various blots. Thus, as in the case of 1-12 B 1, the recombination product of 1-12 B 2 is in accordance with the one-sided invasion model of recombination.

1-12 A 2: In the line 1-12 A 2, as in the other recombinants, the occurrence of the kanamycin resistance is linked to the restoration of a 1.9-kb kanamycin-specific fragment in *Hin*dIII-restricted plant DNA (Figure 2A, lane 4). The absence of the hygromycin-specific 1.7-kb fragment is indicative of a one-sided homologous recombination reaction (Figure 3A, lane 4). Surprisingly the fragment pattern of the recombinant is drastically changed in comparison to the parental line, indicating that not only the recipient locus in which the DSB was induced but also the donor locus was rearranged during the recombination reaction (compare Figures 2A and 3A, lanes 2 and 4). The presence of three different *kanamycin*-specific fragments in the *Xba*I digest (Figure 4, lane 5) also argues for a major rearrangement of this line. Due to its complex nature, the recombinant was not analyzed further.

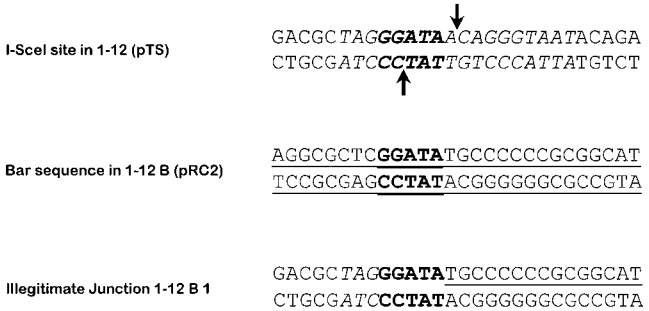

Figure 6.—Structure of the illegitimate recombination junction of pTS- and pRC2-specific sequences in 1-12 B 1. In the top the original I-*Sce*I-site (italic) within pTS is shown. The staggered cut of I-*Sce*I is indicated. Below the sequence of the recombination partner (bar gene of pRC2) is given. The two sequences were joined within a patch of 5 homologous nucleotides (boldface). Apparently the 3′ end of the upper strand of the break induced by I-*Sce*I was directly involved in the joining reaction.

DISCUSSION

In this article it is demonstrated that DSBs can induce homologous recombination between ectopic sequences in a higher eukaryote. In a recent study it was reported that the presence of the transposable element *Ac* can induce ectopic homologous recombination in somatic plant cells (Shalev and Levy 1997). The authors postulated that this was due to transiently induced breaks during transposition. The data presented here now fully sustain this hypothesis. Recently, however, it was also reported that *Ac* does not induce meiotic recombination (Dooner and Martinez-Ferez 1997). Thus the effect might be specific for somatic tissue.

It is becoming more and more apparent, especially for plants, that there are major differences between homologous recombination in somatic and meiotic cells. Our previous studies on the repair of DSBs in somatic plant cells by means of homologous sequences supplied by T-DNA molecules led us to the conclusion that the classical DSB repair model suggested for meiotic recombination (Szostak *et al.* 1983) does not seem to be appropriate for the description of homologous recombination in somatic cells. In our first study we

were able to demonstrate that in one third of the cases the DSB was not repaired by the use of homologous sequences at both ends, despite their presence (Puchta *et al.* 1996), a fact that cannot easily be explained by the DSBR model. This finding was further confirmed by comparing the recombination frequency of repair constructs that harbor homologies to one or both ends of the break (Puchta 1998). The repair construct with homology to only one end of the break was more than half as efficient as the construct with the two ends. This clearly demonstrates that the two ends of the DSB can be repaired independently of each other. One-ended homologous recombination events have been detected before in bacteria (*e.g.*, Takahashi *et al.* 1992; Yamamoto *et al.* 1992) and eukaryotes. For eukaryotes, the synthesis-dependent strand annealing (SDSA; Nassif *et al.* 1994) and the one-sided invasion (OSI; Villemure *et al.* 1997) models of recombination were suggested to describe independent interactions of both ends of the break. A combination of the two latter models was also proposed for the description of DSB repair in plant cells (Puchta 1998).

The results presented in this article demonstrate that DSB-induced ectopic recombination is much less efficient than DSB-induced homologous T-DNA integration with the same donor sequences. This could be due to different factors, like the number of available copies, the topology of the DNA (the T-DNA is transferred into the nucleus as single-stranded linear molecule), and its putative chromatin structure. Nevertheless, the repair models suggested for homologous T-DNA integration can also be applied to the recombination reaction between ectopic sequences in the genome (see Figure 7). Three (1-12 A 1, 1-12 B 1, 1-12 B 2) of the four characterized events are in accordance with such a model, whereas the classical DSBR model can be used to explain only one (1-12 A 1) of the four events. In the case of 1-12 A 1, a gene conversion without crossover was detected. A direct indication for the existence of a mechanism as described by the DSBR model would have been supplied, should the gene conversion have been accompanied by a reciprocal crossover. Such a product would not have been in accordance with the SDSA model. However, one cannot exclude the possibility that, due to the (unknown) orientation of homologies to the centromeres, a putative crossover product might have been unstable, resulting either in cell death or further genomic rearrangements. Also in support of the SDSA model being the major pathway of homologous DSB repair in plants is the fact that gene conversion within an inverted repeat integrated into the tobacco genome was not found to be associated with crossover (Tovar and Lichtenstein 1992).

Due to its complicated rearranged structure, it is difficult to determine precisely the recombination reactions that took place in the recombinant line 1-12 A 2. The occurrence of major chromosomal rearrangements has recently been reported for one case of T-DNA integration in *Arabidopsis thaliana* (Nacry *et al.* 1998). The authors postulated the involvement of a DSB in the reaction. In fact, recently we were able to demonstrate a direct link between DSB repair and T-DNA integration (Salomon and Puchta 1998). Thus, both cases might represent a further class of recombination reaction in which a homologous interaction might be accompanied by major genomic rearrangements. The restoration of the kanamycin gene in line 1-12 A 2 indicates that a one-sided invasion step by homologous sequences took place during the recombination reaction. This interaction is at least in accordance with the proposed model for DSB repair in plants (Puchta 1998). However, it is formally also possible that the rearrangement found in line 1-12 A 2 is due to a putative crossover intermediate that could not be resolved properly.

The results of this study clearly indicate that spontaneous homologous recombination between ectopic sequences seems to be extremely rare. Taking into account the total number of cells of a seedling, in at least 10^9 cells no noninduced ectopic recombination event could be detected in the experiments described (note that in Table 1 only the number of transformed cells and not the total number of cells are taken into account). In mammalian cells the frequency of spontaneous interchromosomal recombination was estimated to be $<3.6 \times 10^{-9}$ (Godwin *et al.* 1994). The spontaneous frequency of intrachromosomal recombination in mammalian cells was reported to be up to 4 orders of magnitude higher than that of interchromosomal recombination (Shulman *et al.* 1995; Baker *et al.* 1996). Thus, in contrast to spontaneous recombination in yeast (Lichten and Haber 1989), spontaneous recombination between ectopic sites is rare in animals and plants. The presence of a DSB-induced homologous recombination between ectopic sequences by several orders of magnitude, but even these events seem to be extremely seldom (see Table 2). If we assume that in about one-tenth of the cells a DSB is induced after transient expression of I-*Sce*I, the frequency of recombination would be 0.5 to 1×10^{-4}. This assumption is based on our previous result that a 10-fold surplus of I-*Sce*I ORF to the repair construct is needed for optimal induction of recombination (Puchta *et al.* 1996). Shalev and Levy (1997) found a frequency of 2.4×10^{-4} for ectopic recombination per cell in microcalli, comparable to our result. However, one has to postulate that *Ac* excision, inducing the reaction, would have happened in almost all of the cells of the particular transgene lines used in their study. In any case, the number of events detected in this and the former study indicate that ectopic recombination is not a major pathway in DSB repair in plants. It might, *per se*, already help that repetitive sequences occurring in ectopic positions do not destabilize plant genome integrity. Furthermore if ectopic recombination occurs, the mechanism might exclude crossovers that might

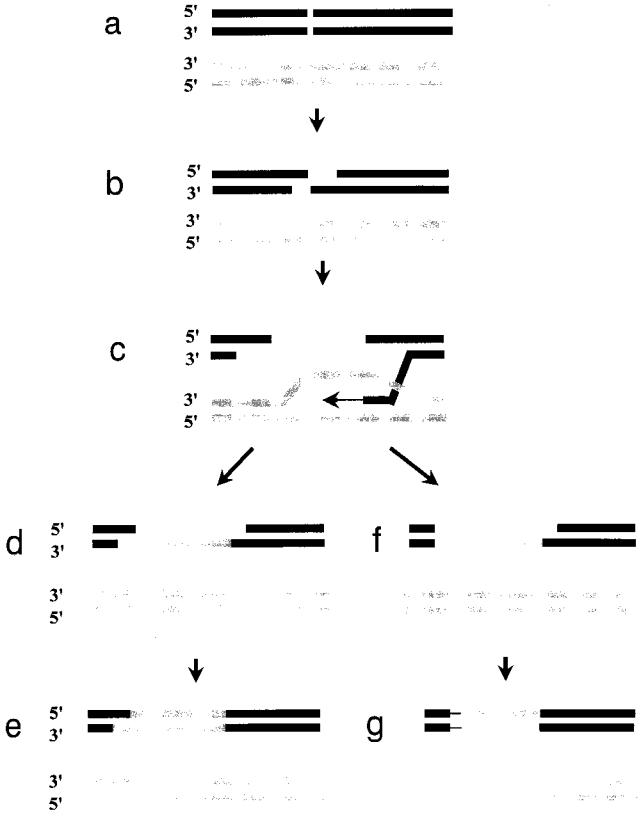

Figure 7.—Model for the repair of genomic DSBs by ectopic homologous sequences in somatic plant cells (modified from Puchta 1998). After induction of a double-strand break in the recipient molecule (a), 3′-single-strand overhangs are produced via exonuclease-catalyzed digestion (b). By forming a D-loop, a free 3′ end invades the homologous double-stranded donor and repair synthesis occurs (c). For the further processing of the intermediate, two possible outcomes can be envisaged: if the 3′ of the recipient molecule is elongated up to the homology of the second 3′ end of the DSB (c, left) and the single strands anneal (d), the molecule can be repaired, resulting in a gene conversion without loss of information (e; as in 1-12 A 1). If, on the other hand, the elongated 3′-end of the recipient molecules does not find complementarity at the 3′ end of the DSB (f, right), the break is closed via illegitimate recombination (g, dark line), a process frequently accompanied by insertions or deletions (as in 1-12 B 1).

result in major rearrangements between different plant chromosomes.

Interestingly, there seems to be no strict separation between homologous and illegitimate recombination mechanisms in somatic plant cells. We were able to demonstrate previously that DSBs can be repaired by a combination of illegitimate and homologous recombination (Puchta *et al.* 1996; Puchta 1998). Recently the SDSA model was applied to explain transposon-induced deletions (Rubin and Levy 1997) and the repair of DSBs by illegitimate recombination in plant cells (Gorbunova and Levy 1997). We were able to show that different kinds of genomic sequences can be copied into a DSB in the tobacco genome. Apparently only a few base pairs of homology between the donor locus and the invading 3′-end of the DSB are required to start a copying reaction (Salomon and Puchta 1998). Thus, a similar combination of SDSA and OSI models of recombination (Figure 7) can be applied for the capture of nonhomologous as well as of homologous ectopic sequences. Comparing the frequencies of such recombination events in our two-assay systems indicates—also in accordance with its relaxed requirement for homologous base pairs—that the capture of ectopic sequences occurs much more often via illegitimate recombination than via homologous recombination.

It is interesting to speculate about the main pathway in DSB repair in somatic plant cells. Recently we were able to demonstrate that genomic DSBs can be efficiently repaired by illegitimate recombination in somatic plant cells (Salomon and Puchta 1998). However, as an alternative to illegitimate recombination, ectopic homologous recombination (this article), intrachromosomal homologous recombination (Chiurazzi *et al.* 1996), and sister chromatid exchange (for mammals see Natarajan *et al.* 1985), it is possible to repair DSBs by the use of allelic sequences of the homologue (see also Moynahan and Jasin 1997 for mammalian cells and Hu *et al.* 1998 for recent indications in plants). We are currently setting up a system to elucidate the role of this pathway in DSB repair in plants.

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