DOI: 10.1534/genetics.106.065185

Two Unlinked Double-Strand Breaks Can Induce Reciprocal Exchanges in Plant Genomes via Homologous Recombination and Nonhomologous End Joining

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Manuscript received August 31, 2006
Accepted for publication October 6, 2006

ABSTRACT

Using the rare-cutting endonuclease I-*Sce*I we were able to demonstrate before that the repair of a single double-strand break (DSB) in a plant genome can be mutagenic due to insertions and deletions. However, during replication or due to irradiation several breaks might be induced simultaneously. To analyze the mutagenic potential of such a situation we established an experimental system in tobacco harboring two unlinked transgenes, each carrying an I-*Sce*I site. After transient expression of I-*Sce*I a kanamycin-resistance marker could be restored by joining two previously unlinked broken ends, either by homologous recombination (HR) or by nonhomologous end joining (NHEJ). Indeed, we were able to recover HR and NHEJ events with similar frequencies. Despite the fact that no selection was applied for joining the two other ends, the respective linkage could be detected in most cases tested, demonstrating that the respective exchanges were reciprocal. The frequencies obtained indicate that DSB-induced translocation is up to two orders of magnitude more frequent in somatic cells than ectopic gene conversion. Thus, DSB-induced reciprocal exchanges might play a significant role in plant genome evolution. The technique applied in this study may also be useful for the controlled exchange of unlinked sequences in plant genomes.

EFFICIENT repair of double-strand breaks (DSBs) in genomic DNA is important for the survival of all organisms. Basic mechanisms of DSB repair in somatic plant cells have been elucidated in recent years (for reviews see Gorbunova and Levy 1999; Reiss 2003; Puchta 2005; Schuermann *et al.* 2005). DSBs are mainly repaired by nonhomologous end joining (NHEJ). The repair can be associated with deletions but also insertions due to copying genomic sequences from elsewhere into the break (Gorbunova and Levy 1997; Salomon and Puchta 1998). Species-specific differences of NHEJ have been reported and an inverse correlation of deletion size to genome size has been postulated, indicating that NHEJ might contribute significantly to the evolution of genome size (Kirik *et al.* 2000). DSB repair by homologous recombination (HR) might influence genome organization as well. Whereas homology present in allelic (Gisler *et al.* 2002) or ectopic positions (Shalev and Levy 1997; Puchta 1999a) is hardly used for repair, the use of homologous sequences in close proximity to the break is frequent (Orel *et al.* 2003). Especially efficient is a "single-strand annealing" mechanism that leads to sequence deletions between direct repeats (Xiao and Peterson 2000; Siebert and Puchta 2002).

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For the study of the DSB repair consequences in recent years mainly experimental systems were used, which are based on the induction of a unique break at a specific genomic site. However, if cells are exposed to radiation more than one DSB might occur in the genome at a given time. Moreover, it is generally assumed that during a replication cycle of a eukaryotic genome a number of DSBs occur that have to be repaired. In such a situation several DNA ends are present in the nucleus, the repair of which might interfere with the others. An interesting question is whether the ends of the breaks could be joined vice versa, resulting in a reciprocal exchange of sequences. Alternatively further rearrangements of the genome might occur. Under certain circumstances reciprocal exchanges might be viable and transferred to the germ line. Indeed, recent genome analysis indicates that translocations occur regularly during the evolution of plant genomes (*e.g.*, for Arabidopsis, Arabidopsis Genome Initiative 2000; Blanc *et al.* 2000; for Brassica, Udall *et al.* 2005). In this report we describe the setup of an experimental system that allowed us to directly demonstrate that reciprocal exchanges can be induced by unlinked DSBs in plant genomes.

MATERIALS AND METHODS

DNA constructs: The construction of plasmid pTS was described before (Puchta *et al.* 1996). For the construction

of pTL the plasmid pUCPLBR+Z was used (Puchta *et al.* 1996). This plasmid contains, besides a pUC backbone within a polylinker, a *bar* gene next to the right border and the "overdrive" sequences of Agrobacterium strain C58. Using the oligonucleotides 5′-GCCGCCCGGGTATTACCCTGTTATCCCT AGTGCTGAAGTGCTT ATTATCTAAG-3′ and 5′-GCCGCTCG AGTGGCAGGATATATTGTGGTGTAAACAATTCCCCATGGA GTCA AAGATTC-3′ we amplified from the plasmid pTZAH271 (Hrouda and Paszkowski 1994) a fragment containing an I-*Sce*I recognition sequence next to the intron, the 5′ exon, and the 35S promoter of the kanamycin-resistance gene, as well as a left border sequence, and we cloned the fragment into the *Sma*I and *Xho*I sites of pUCPLBR+Z. The resulting plasmid was cut by *Hpa*I and the fragment carrying the marker genes was cloned into the *Pvu*II site of a borderless derivative of pBin19 (Bevan 1984). The resulting binary pTL was then electroporated into Agrobacterium for plant transformation.

The I-*Sce*I expression vector pCISceI (Puchta *et al.* 1996) contains a synthetic I-*Sce*I ORF under the control of the cauliflower 35S promoter (Puchta *et al.* 1993) between T-DNA borders.

Plant transformation: Molecular characterization of the transgenic line 1-12 was described before (Puchta 1999a). The line was produced from *Nicotiana tabacum* L. cv. Petite Havana line SR1 via Agrobacterium-mediated transformation with the plasmid pTS. Line 1-12 contains a single functional copy of the transgene in its genome. 1-12 seedlings homozygous for the transgene were retransformed with an Agrobacterium strain containing the vector pTL. Vacuum infiltration of tobacco seedlings and plant regeneration were done as described (Salomon and Puchta 1998). Segregation of the selfed transformants was tested by germinating the seeds on MS medium supplemented with 20 μg phosphinotricin per milliliter.

In a second series of experiments, F_2 seedlings of the transgenic lines IRC1, -7, and -10 were inoculated with an Agrobacterium strain harboring the binary vector pCISceI as described (Puchta 1999b). A selection using 50 μg kanamycin per milliliter of medium was applied. The surviving calli were put on selective medium without hormones to induce shoot regeneration and the plants were regenerated.

Plant DNA extraction and Southern analysis: DNA extraction from leaf tissues and calli was done as described (Salomon and Puchta 1998). Southern blotting of *Hin*dIII- or *Eco*RV-digested DNA using the hybridization membrane "Hybond N+" (GE Healthcare Europe, Freiburg, Germany) was performed as described (Salomon and Puchta 1998). The DNA probes were labeled using a random-priming labeling kit (Megaprime DNA labeling system RPN1607; GE Healthcare Europe) and [α-^{32}P]dCTP (GE Healthcare Europe GmbH). As DNA template a *kanamycin*-specific probe was isolated as a 1.9-kb *Hin*dIII fragment from pCAH5 (Hrouda and Paszkowski 1994), and a *bar*-specific probe was purified as a *Bgl*II fragment from p(UC)PCBR (Puchta *et al.* 1996).

PCR and sequence analysis: Genomic DNA was analyzed via PCR using the primers KIH2 (5′-CGTGGCTGGCCTCCTTCA ATGTAA-3′) and KIR1 (5′-GTGACAACGTCGAGCACA GCTG CG-3′) for detection of the junction A–D (Figure 1) and subsequent sequencing. Primers Hyg1 (5′-ATGTCCTGCGGGTA AATAGC-3′) and BarS (5′-TACATCGAGACAA GCACGGT-3′) were used to provide evidence for the junction C–B and sequencing reactions. Primers I-SceI-FW3 (5′-GATGCTTACAT CCGTTCTC-3′) and I-SceI-RV2 (5′-CAGGAAAGTTTCGGAG GAG-3′) were used to analyze the recombinant lines regarding the presence of a functional I-*Sce*I cassette. The PCR reactions and the direct sequencing of the amplification products were carried out as described (Hartung and Puchta 2000).

RESULTS

Experimental setup: To characterize DSB-induced rearrangements in the genome we developed an experimental system that enabled us to address a number of questions with a single setup. The system is based on two independent T-DNAs, each carrying an I-*Sce*I site (Figure 1). When both sites are present in the same genome and I-*Sce*I is expressed, two breaks should be induced simultaneously. To detect the joining of unlinked DSB ends a selection marker was used. Close to the I-*Sce*I sites of both constructs nonfunctional parts of a kanamycin resistance gene are cloned. This gene is split by an artificial intron into two exons (Hrouda and Paszkowski 1994; Puchta *et al.* 1996). One T-DNA (pTL) contains the promoter with the 5′ part of the gene and the intron whereas the other T-DNA (pTS) contains the same intron and the 3′ end of the gene. After I-*Sce*I expression the gene function can be restored (joining ends A and D in Figure 1) either by homologous recombination between the two intron sequences or by nonhomologous end joining in such a way that the two intron sequences are joined in tandem. Thus, a kanamycin-resistance gene with a bigger intron arises. Since this larger intron contains both functional donor and acceptor splicing sites—as in the case of the "original" intron—after splicing a functional neomycin phosphotransferase protein is produced. Thus, restoration of the kanamycin resistance can occur via HR or NHEJ, and due to the setup a direct comparison of the frequencies of both pathways is possible.

Kanamycin resistance arises if the two unlinked ends A and D (Figure 1) are rejoined. It is, of course, important to know what happens to the two other ends (C and B in Figure 1). There are two possibilities: either the other ends are simultaneously joined in a reciprocal reaction (as shown in Figure 1) or further rearrangements occur at the broken ends. As there are no homologies between these ends, a direct linking can be achieved only via NHEJ. The newly produced junction C–B can easily be detected by PCR using primer-binding sites on the respective T-DNAs. In our previous studies on DSB repair by homologous recombination we used the transgenic tobacco line 1-12 that carries a single copy of the transgene pTS. In these studies homologous DNA repair was achieved using either the homology from an incoming T-DNA (Puchta *et al.* 1996; Puchta 1998; Reiss *et al.* 2000) or that from an ectopic transgene (Puchta 1999). As 1-12 was well characterized and DSB induction could be performed reproducibly, we decided to use this line for the current study, too. Moreover, we could directly estimate the frequency of translocations in relation to ectopic gene conversion as determined in an earlier study using the same line (Puchta 1999a). Transgenic seedlings of the line 1-12, homozygous for a single-copy transgene of pTS, were transformed via Agrobacterium with the binary vector

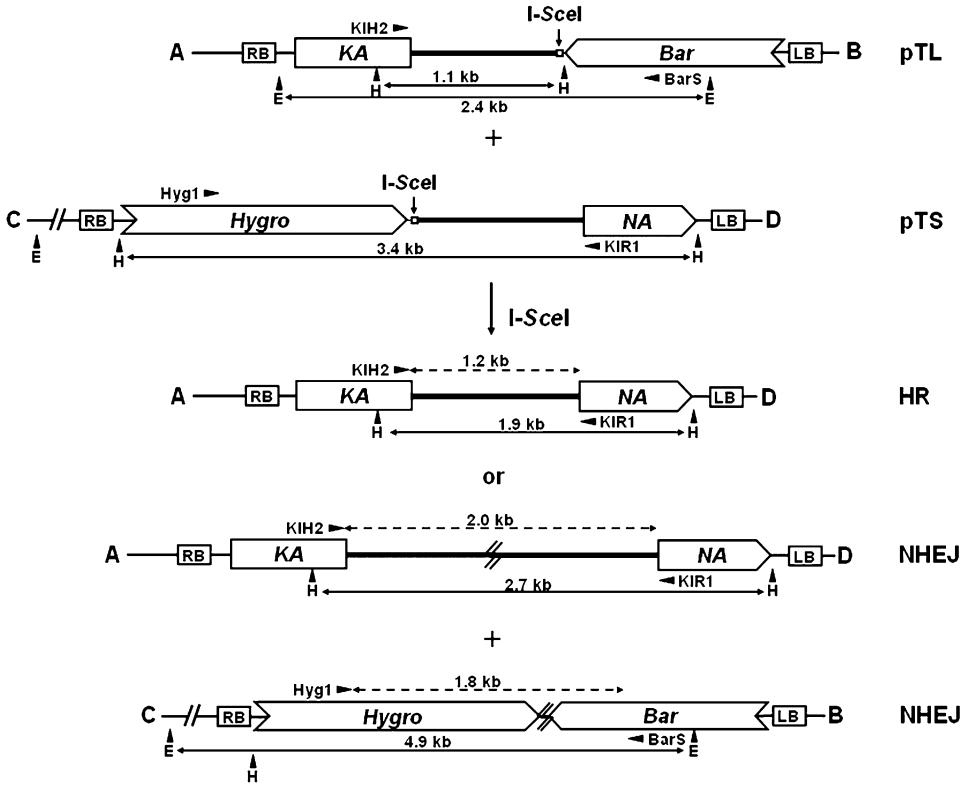

FIGURE 1.—Schematic map of the T-DNAs pTS and pTL. Possible outcomes of the recombination reaction are depicted (for reasons of clarity neither promoter nor terminator sequences are shown). HR, homologous recombination; NHEJ, nonhomologous end joining. The triangles represent the primers used for the PCR amplification of the recombined junctions. A 1.2-kb fragment will be detected if the transgene halves A and D are joined by HR. In the case that NHEJ took place, a 2.0-kb fragment is amplified (dashed arrows). The new junction between halves C and B can be detected as a 1.8-kb PCR fragment. E (*Eco*RV) and H (*Hin*dIII) are restriction sites used for Southern blotting (see Figure 3). A 1.9-kb *Hin*dIII restricted specific fragment is indicative of HR between transgenes A and D, whereas a 2.7-kb fragment is indicative of NHEJ. The newly joined transgenes C and B are detected as a 4.9-kb fragment in *Eco*RV-digested genomic DNA with a *bar*-specific probe. The position and length of the probes used for membrane hybridization are depicted as dashed arrows. RB, right border; LB, left border.

pTL. Plants were regenerated, and lines carrying one copy of the transgenic sequences at a single locus were identified by means of segregation analysis and Southern blotting. Three lines were chosen for further analysis: IRC1, IRC7, and IRC10.

Induction of recombination: F_2 seedlings of all three lines that were homozygous for pTS and hemizygous for pTL were inoculated with an Agrobacterium strain that harbored on its T-DNA an I-*Sce*I-ORF under the control of a CaMV 35S promoter to achieve transient expression of the enzyme (PUCHTA 1999b). After 3 days, the seedlings were put on callus-inducing medium that contained kanamycin for the selection of cells with DSB-induced translocations. As a control, seedlings were inoculated with an Agrobacterium strain that did not contain the I-*Sce*I ORF. Altogether 1000–2000 seedlings were inoculated with the I-*Sce*I ORF per line, and 400–500 seedlings were used as controls. The results of the inoculation are depicted in Table 1.

We did not expect to obtain resistant calli for all lines. Depending on the orientation of the transgenes in the chromosomes to the centromeres, translocations between chromosome arms could lead to di- or acentric chromosomes. Such rearrangements might not be passed through cell division and thus would not result in viable calli. On the other hand, if the exchange is limited to chromosome ends or results in a sequence inversion within a chromosome, the recombined progeny might be viable. Indeed, calli arose from only one of the three lines (IRC1) after inoculation with the I-*Sce*I ORF. No kanamycin-resistant calli arose in the controls. In total, 84 calli could be isolated from which DNA for PCR analysis was obtained.

PCR analysis of recombinant junctions: In a first set of experiments we determined whether the restoration of the kanamycin-resistance gene was due to HR or due to NHEJ. Using the primers KIH2 and KIR1 (see Figure 1 and MATERIALS AND METHODS), amplification of a 1.2-kb band was indicative of HR, and a 2.0-kb band was

TABLE 1

Recombination events leading to the restoration of the kanamycin-resistance gene with and without DSB induction by I-*Sce*I expression

Line	I-*Sce*I	No. of seedlings (10^2)	PCR positive: kanamycin-resistant calli	1.2 kb (HR)	2.0 kb (NHEJ)
IRC1	+	11.4	84	33	51
	−	4.7	0		
IRC7	+	19.8	0		
	−	5.5	0		
IRC10	+	19.8	0		
	−	4.3	0		

indicative of NHEJ. In 33 cases the short and in 51 cases the longer band could be detected. Thus, in ~40% of the cases the joining was mediated by HR. To further sustain the interpretation that the smaller PCR fragment arose indeed due to HR, six of the smaller PCR fragments were sequenced. All of them carried the same intron sequence without any mutations as expected for HR. We also sequenced seven longer PCR fragments. Here, in three cases the I-*Sce*I site was conserved due to a simple ligation of the ends, and in the four other cases deletions of 2, 8, 12, and 28 nucleotides occurred during the joining process. In all cases the deletions were within or included the 18-mer I-*Sce*I restriction site.

To further analyze the recombination events at the molecular level we regenerated fertile plants from a row of calli. To determine if, in addition to the kanamycin-resistance gene (A and D in Figure 1), the other ends of the induced DSBs were also joined we tried to amplify putative junctions between C and B via PCR using the primers Hyg1 and BarS. Altogether 19 different recombinants were tested and we were able to obtain amplification products in 16 cases, which were all sequenced. Analysis demonstrated that in three lines (IRC1 7, IRC1 9, and IRC1 12) a functional I-*Sce*I site was present at the new junction. Interestingly, in two of these cases the A–D junction also contained a functional I-*Sce*I site (lines IRC1 9 and IRC1 12), demonstrating that in these cases both DSBs were rejoined by simple ligation with the respective other unlinked I-*Sce*I half sites. In all other cases the new junction was formed by NHEJ. As the I-*Sce*I expression cassette used for transient expression might have stably integrated in some lines we tested by PCR whether an I-*Sce*I ORF was present in the respective recombinants. Indeed, in 11 of 16 lines tested this was the case. The continuous production of the restriction enzyme might have excluded the isolation of simple ligation events in these lines. Interestingly, both IRC1 9 and IRC1 12 indeed did not contain an I-*Sce*I gene in their genome.

In 10 cases short deletions at the I-*Sce*I site occurred from 2 to 38 bp. Figure 2 depicts several of the junctions. Microhomologies of one to five nucleotides could be identified at several of the junctions. In two cases insertions of 28 or 61 bp were found, accompanied by deletions of four and eight nucleotides within the I-*Sce*I site, respectively. The 61-bp insert was copied into the break, using parts of the CaMV 35S terminator located 42 bp downstream of the DSB as a matrix (see Figure 2). The origin of the smaller insertion could not be elucidated as no clear homology to known sequences could be found in GenBank searches. For lines IRC1 14, 52, and 65 we were not able to detect a C–B-specific band.

Germinal transmission of the translocations: To test the germinal transmissibility of the obtained genome rearrangements the seeds obtained from the regenerated recombinant plants were sown on MS medium containing kanamycin and checked for segregation. In 13 of 19 cases a Mendelian segregation of the kanamycin resistance was found (Table 2), indicating that the progeny inherited a single copy of the rejoined transgene. In two cases all seedlings were kanamycin resistant, indicating that apparently two independent translocations occurred within the respective genomes (IRC1 7 and 14). Such events were possible as besides the homozygous transgene C–D in a quarter of the inoculated seedlings the transgene A–B was also in a homozygous state. In four remaining cases half or less of the seedlings were kanamycin resistant (lines IRC1 18, 27, 47, and 65). We germinated individual seedlings of the respective lines without antibiotic selection and checked via PCR with the primers KIH2 and KIR1 if the lack of resistance was due to the absence of the kanamycin resistance gene or due to a silencing phenomenon. Indeed we obtained the kanamycin-resistance gene-specific fragment in three-quarters of the seedlings (χ^2-test, $P = 0.05$; data not shown), which demonstrates that the lack of resistance was not due to an aberrant segregation of the kanamycin-resistance gene but due to silencing phenomena.

To test if the newly formed junction C–B was also inherited in a Mendelian fashion we performed PCR with the primers Hyg1 and BarS on kanamycin-resistant seedlings of the lines IRC1 2, 4, 16, 21, 43, 47, and 73. In the case that both junctions were not linked genetically, we expected that three-quarters of the seedlings containing the A–D junction should also contain the C–B junction. For each recombinant line 20 seedlings were tested. Indeed, in all cases tested we detected the fragment as predicted for independent Mendelian segregation (χ^2-test). If we take into account that in all cases tested the reciprocal exchange of the broken ends led to the same kind of genome rearrangement, we will be able to pool the data (in total we obtained a PCR signal from 107 of 140 seedlings) and conclude that the two newly formed junctions are not linked and segregate independently with a 3:1 relation with very high probability ($\chi^2 = 0.152381$).

Southern blot analysis: For a more detailed analysis we performed Southern blots for a row of recombinants. For this purpose we used single plants originating from the selfed progeny of the recombinant lines. The analyzed plants contained the recombined fragments partly in the absence of the parental bands. In a first set of experiments we sustained our PCR data on the restoration of the kanamycin-resistance gene. After extraction DNA was digested by *Hin*dIII to easily discriminate between HR and NHEJ events. In the original line 1-12 (Figure 3A, lane 3) and parental line IRC1 (lane 4) a 3.4-kb *kanamycin*-intron-specific fragment can be cut out of the transgene of pTS. The parental line IRC1 contains also a 1.1-kb *kanamycin*-intron-specific fragment resulting from the transgene pTL (Figure 1; Figure 3A, lane 4). In the case that the broken ends A and D are joined by HR a new 1.9-kb fragment should arise,

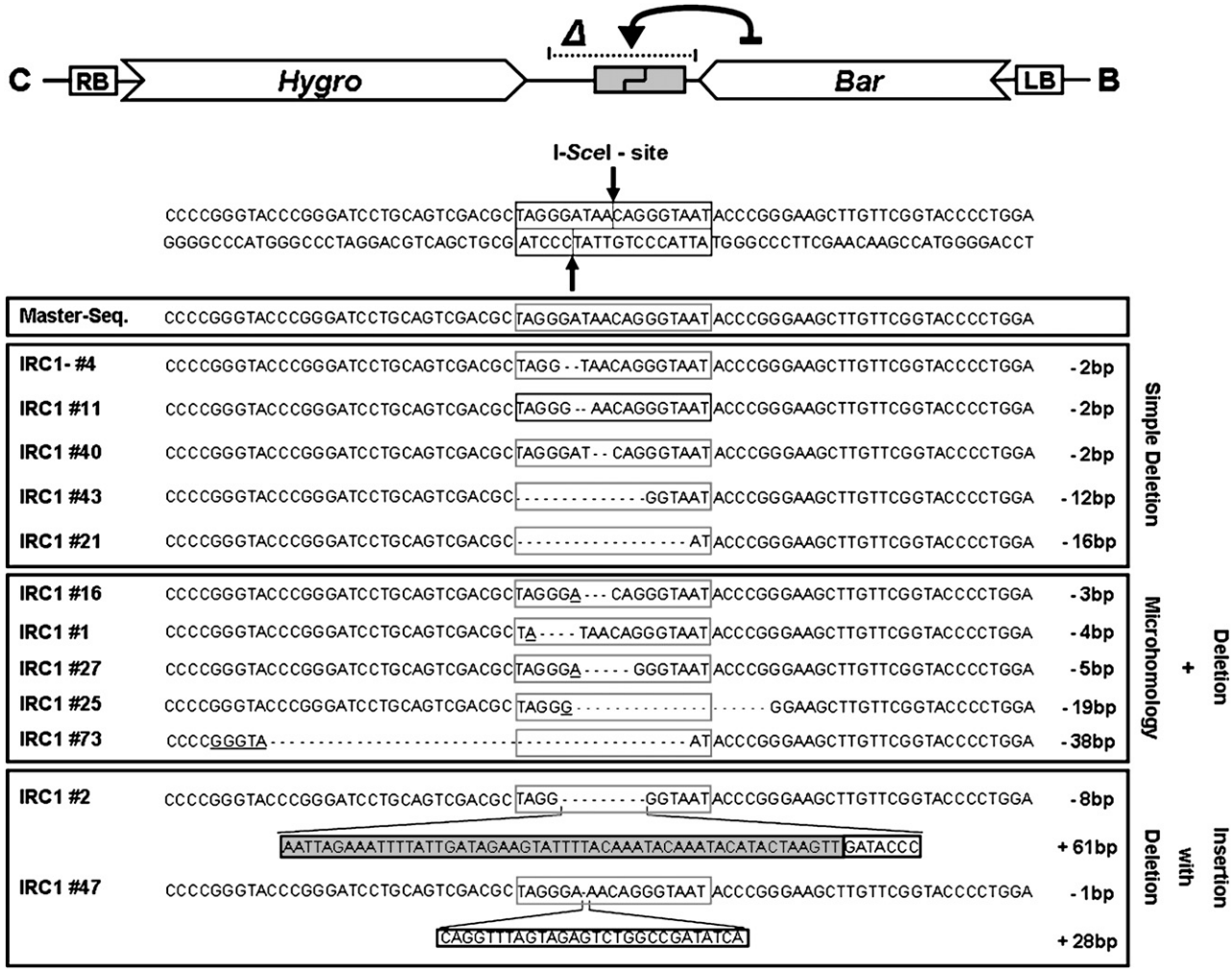

FIGURE 2.—Compilation of junctions originating from transgenes B and C in which end joining between the two I-*Sce*I sites had occurred. Assuming that a simple ligation occurred leading to the repair of the two free ends B and C a master sequence was designed that is shown at the top wherein the functional I-*Sce*I site is marked by a shaded frame. Three different classes of junctions are found: in five cases, the I-*Sce*I site was partly destroyed by simple deletions of 2–16 bp. In four recombinant IRC1 lines, deletions of 3–38 bp accompanied the use of microhomologies (underlined letters) at the newly formed junctions of 1–5 bp, a hallmark of DSB repair via NHEJ. A third class of repair events is represented by lines IRC1 2 and IRC1 47 in which insertions (in solid frames) of 61 and 28 bp were detected. These insertions accompanied small deletions of 8 and 1 bp, respectively. Fifty-four nucleotides of the 61-bp insertion of line IRC1 2 (shaded boxes) originate from a part of the transgenic sequence 38 bp downstream of the I-*Sce*I site as depicted above. The sequence is in the same orientation as its original template, which is part of the 3′ end of the 35S terminator. The origins of the last 7 of 61 bp inserted in line IRC1 2 and the 28-bp insertion in line IRC1 47 are unknown.

whereas in the case of NHEJ due to the presence of two intron sequences in tandem, a 2.7-kb fragment should be visible (Figure 1). In the case of lines ICR1 7-1 and 12-2 the digest of a progeny plant containing only the recombined junction results indeed in the expected 2.7-kb band indicative of NHEJ (Figure 3, lanes 5 and 7). As expected, in another plant of the progeny of the same line (ICR1 12-1), in which parental and recombined transgenes did not segregate (Figure 3, lane 6), in addition to the 2.7 kb, the 3.4- and the 1.1-kb band are visible as in the original line IRC1 (lane 4). In the case of the plants IRC1 25-1 and IRC1 52-1 (Figure 3, lanes 8 and 9, respectively) a 1.9-kb band was indicative of homologous recombination. The size was identical to a fragment of the line 1-12c (Figure 3, lane 2). Here the fragment arose due to restoration of a kanamycin-resistance gene by gene targeting after I-*Sce*I expression in line1-12 (Figure 3, lane 3) (described in PUCHTA *et al.* 1996). In total, we tested 11 lines for restoration of the kanamycin-resistance gene via Southern blotting (data not shown) and in all cases the results (five HR and six NHEJ events) were in accordance with our previous PCR data.

With a second set of blots we sustained our previous PCR findings that a new C–B junction was produced during the recombination reaction containing both bar and hygromycin-resistance genes. There are two *Eco*RV sites in pTL, one next to the right border (transgene half A) and one in the bar-resistance gene (Figure 1). In the case of an *Eco*RV digest probed with a *bar*-specific fragment, a 2.4-kb specific band can be seen on the blot

TABLE 2

Segregation of the kanamycin-resistant seedlings of the recombined transgene lines

Line	Resistant	Sensitive	χ^2-test
IRC1 1	32	11	+
IRC1 2	55	18	+
IRC1 4	58	24	+
IRC1 7	40	0	–
IRC1 11	57	23	+
IRC1 12	41	15	+
IRC1 14	75	0	–
IRC1 16	64	25	+
IRC1 18[a]	10	41	–
IRC1 21	59	19	+
IRC1 25	31	15	+
IRC1 27[a]	7	79	–
IRC1 35	61	17	+
IRC1 40	33	15	+
IRC1 43	74	21	+
IRC1 47[a]	46	45	–
IRC1 52	66	29	+
IRC1 65[a]	52	54	–
IRC1 73	70	22	+

A χ^2-test was used to test Mendelian segregation ($P < 005$).
[a] PCR analysis revealed that the kanamycin-resistance gene was silenced in part of the progeny of the respective lines and that segregation of the restored resistance gene occurred indeed in a Mendelian fashion (see RESULTS).

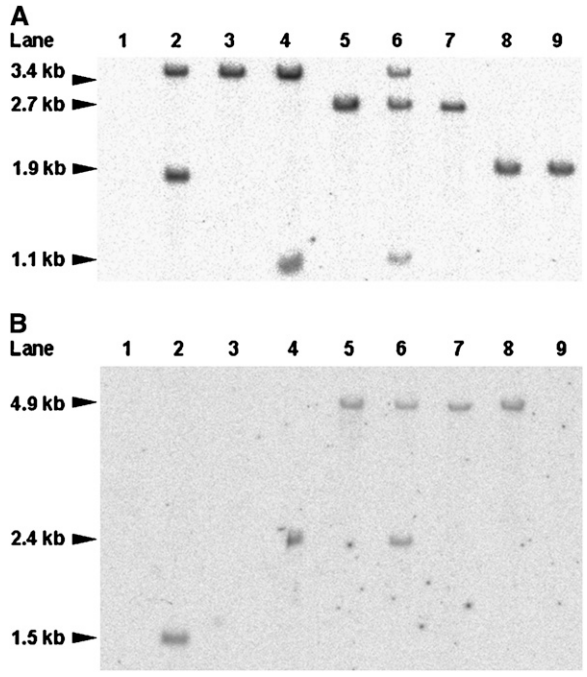

FIGURE 3.—Southern blot analysis with restriction-digested DNA of the plant lines. Lane 1, wild type; lane 2, line 1.12c; lane 3, line 1_12 homo; lane 4, line IRC1; lane 5, line IRC1 7-1; lane 6, line IRC1 12-1; lane 7, line IRC1 12-2; lane 8, line IRC1 25-1; lane 9, line IRC1 52-1. (A) *Hin*dIII-restricted DNA hybridized with a *kanamycin*-intron-specific probe. Wild-type tobacco was used as a negative control (lane 1), whereas lanes 2 and 3 represent positive controls that have been described earlier (see PUCHTA *et al.* 1996). Both in the original line 1-12 (which is homozygous for pTS) and in the parental line IRC1 (lane 4) a 3.4-kb *kanamycin*-intron-specific fragment can be detected. In line IRC1 also a 1.1-kb band can be visualized that corresponds to an *Hin*dIII fragment of pTL (see Figure 1). The fragments of the parental line IRC1 are still detectable in the recombinant line IRC1 12-1 (lane 6), and the additional 2.7-kb fragment represents the new joint between transgene halves A and D via NHEJ (see Figure 1). The other lines shown here contain only the recombinant junctions A–D: whereas both lines IRC1 7-1 and 12-2 (lanes 5 and 7, respectively) contain a 2.7-kb fragment, which is representative of NHEJ between transgene halves A and D, for both lines IRC1 25-1 and 52-1 only a 1.9-kb fragment is visible that represents HR-mediated joints between A and D (lanes 8 and 9, respectively). (B) *Eco*RV-digested DNA hybridized with a *bar*-specific probe. The controls (lanes 1–3) are the same as those described in A. For the parental line IRC1 (lane 4) a *bar*-specific 2.4-kb band is detectable, representing an *Eco*RV fragment of pTL (see Figure 1). This parental band can also be seen in line IRC1 12-1 (lane 6), which also harbors a completely new 4.9-kb band representative of the recombinant junction between transgene halves C and B (see Figure 1). All other recombinant lines shown here (lanes 5 and 7–9) possess only the new 4.9-kb fragment, which is unique for the NHEJ-mediated joint between transgenes C and B.

(for IRC1, lane 4, Figure 3B). On the other hand, an *Eco*RV site is present in the genomic plant DNA in IRC1 close to the right border (RB) of pTS (transgene half C; Figure 1). If the two broken ends C and B are joined, a new 4.9-kb *Eco*RV-specific fragment should arise combined with the loss of the 2.4-kb fragment. Indeed, as shown in the blot for lines IRC1 7-1, IRC1 12-2, and IRC1 25-1 (Figure 3B, lanes 5, 6, and 8, respectively), this new 4.9-kb band can be detected in the recombinants. In the case of line IRC1 12-1 (lane 6) both the parental (2.4 kb) and the newly recombined (4.9 kb) bands are present. Interestingly we could not detect any *bar*-specific fragment in the case of line IRC1 52-1 (lane 9). This line is one of the three recombinants from which we were not able to obtain a PCR signal for the C–B junction. The absence of *bar*-specific sequences hints at the occurrence of a larger deletion event at the broken end B during the recombination reaction. In total, nine lines were tested that, according to our PCR analysis, carried a newly formed C–B junction. In all of them the 4.9-kb fragment could be detected.

DISCUSSION

Using an experimental system that was based on two unlinked transgenes we were able to demonstrate that after DSB induction reciprocal recombination can occur within the plant genome. This study was purposely performed in tobacco, as we assumed that due to its amphidiploid nature the organism might tolerate more genomic variations than a classical diploid plant species. It has been shown before for mammals, Drosophila, and yeast that DSB repair can result in reciprocal exchanges (RICHARDSON and JASIN 2000; EGLI *et al.* 2004; YU and

Gabriel 2004; Maggert and Golic 2005). Via FISH analysis the reciprocal translocation of chromosome arms by HR and NHEJ was sustained for embryonic stem cells (Richardson and Jasin 2000; Elliott *et al.* 2005). Reciprocal exchange mediated in yeast by NHEJ (Yu and Gabriel 2004) and in Drosophila by HR (Yu and Gabriel 2004; Maggert and Golic 2005) was documented. Here, we not only did demonstrate that a similar phenomenon occurs in plants, but also were able to compare within the same setup the frequencies of NHEJ and HR in mediating such translocations. The high rate of HR (~40% of the events) found is reminiscent of a recent study we performed on the repair of a single DSB in tobacco (Siebert and Puchta 2002). In the latter study in almost half of the cases the breaks would be repaired by HR, if sufficient homology was available near the break site—an experimental situation similar to the one created in this study. In a recent study in mammalian cells the group of Maria Jasin was able to demonstrate for mouse cells that dependent on the number of mismatches within the homologous sequence the mode of linkage can be influenced. Whereas in the majority of cases in the presence of perfect homology HR was used, 20% of the divergence in sequences resulted almost exclusively in NHEJ (Elliott *et al.* 2005).

The fact that HR can be so efficient in our experimental set is most probably due the break being rejoined via a single-strand annealing (SSA) mechanism of recombination. The SSA model was first suggested for extrachromosomal recombination between plasmids in mammalian cells (Lin *et al.* 1984, 1990). After induction of a DSB, the free double-stranded ends are resected by a single-strand-specific exonuclease, leaving behind 3′-single-stranded overhangs. These single strands can anneal with each other at regions of complementarity, overhanging nonhomologous sequences are digested or alternatively single-stranded gaps could be refilled by means of repair synthesis, and in a last step the double strand is restored by religation of the remaining nicks. This model has been applied for describing extrachromosomal recombination in Xenopus oocytes (Maryon and Carroll 1991a,b) and yeast (Fishman-Lobell *et al.* 1992). In plants three different approaches unambiguously demonstrated that extrachromosomal recombination proceeds efficiently via single-strand annealing (Puchta and Hohn 1991; Bilang *et al.* 1992; De Groot *et al.* 1992; for review see Puchta and Meier 1994). Our recent study demonstrated that SSA is also a major pathway for the repair of genomic DSBs (Siebert and Puchta 2002). Interestingly, a similar mechanism is used for NHEJ: the majority of junctions contain small patches of homologous nucleotides between reaction partners, which is best explained via the operation of a "SSA-like" mechanism (Lehman *et al.* 1994; Nicolas *et al.* 1995; Mason *et al.* 1996; Gorbunova and Levy 1997; Salomon and Puchta 1998; for review see Gorbunova and Levy 1999; see also Figure 2). Thus SSA and SSA-like mechanisms might be the most prominent mode for the rejoining of broken DNA molecules in higher eukaryotes irrespective of whether two closely linked or two unlinked break ends are joined.

Our results also demonstrate that two simultaneously induced DSBs can be repaired in the same genome by HR and NHEJ. This indicates that the same cell at a given time point is competent to repair DSBs by different pathways. Thus, different proteins required for HR and NHEJ might be recruited to break sites simultaneously.

Reciprocal exchanges: Due to our experimental setup the rejoining of the two unlinked broken ends A and D resulting in kanamycin resistance was selected for (Figure 1). However, for most recombinants analyzed we could demonstrate that the two other ends C and B were also joined—independent of whether A and D were joined by NHEJ or by HR. In 16 of 19 recombinants tested a C–B fragment could be detected via PCR. We further analyzed a total of 9 of these lines via Southern blot and confirmed the reciprocal recombination reaction with the appearance of a new indicative 4.9-kb *bar*-specific band (Figure 3B). Thus, at least most events analyzed are reciprocal recombinants. Indeed, in the case of the recombinant line IRC1 52-1 we failed not only to detect a C–B junction via PCR, but also to demonstrate via Southern blot the presence of a *bar*-specific transgene sequence. As we obtained a *hygromycin*-specific signal (data not shown) it might well be that the joining of the transgene halves C and B resulted in a reciprocal recombination in which the *bar*-specific transgene sequences were lost before joining due to exonucleolytic degradation of the broken end B.

However, it cannot be concluded from our study that the joining of two unlinked ends is always correlated with the rejoining of the two other ends. Although we were able to demonstrate this for at least most of the events analyzed, our study was based on the selection of events resulting in genomes that could proceed through mitosis. It might well be that other nonreciprocal events occurred; however, the resulting products could not be passed to the progeny. This might be true for lines IRC7 and IRC10, where transient expression of I-*Sce*I did not result in kanamycin-resistant calli. Alternatively and more likely, reciprocal recombination occurred in these lines, too. However, di- and acentric chromosomes might be produced, which were not able to pass mitosis.

Whatever kind of repair occurs, one has to keep in mind that only events that can be transferred via meiosis to the next generation will be of importance for genome evolution. Under this premise one has to state that our study clearly indicates that reciprocal recombination occurs in plants and that at least in tobacco it can also be transferred to the next generation. However, due to experimental limitations we are not able to further characterize the nature of the induced translocations. Our segregation analysis clearly demonstrates that the rejoined transgene loci of pTS and pTL are not linked.

This can be explained by a transgene integration on different chromosomes or on the same chromosome yet far apart. In the latter case a reciprocal translocation would result in an inversion within the same chromosome. As the tobacco genome consists of 48 chromosomes, it is, however, more probable that both transgenes were integrated in different chromosomes. Thus, an exchange of chromosome arms is the most likely outcome of the reaction induced in this study. This has been demonstrated for the mammalian and the Drosophila genome before (Richardson and Jasin 2000; Elliott *et al.* 2005; Maggert and Golic 2005), although an inversion was described for Drosophila, too (Egli *et al.* 2004). Unfortunately, the tobacco genome has not been sequenced yet, so we are not able to determine the location of the integration sites for pTS and pTL in the transgenic lines. Another drawback preventing a more detailed analysis is the lack of chromosome-specific probes and the similar size of most chromosomes in tobacco. Therefore, no cytological analysis of the recombinants could be performed similar to the one applied in mouse (Richardson and Jasin 2000).

Translocations in plant genome evolution: Our analysis also hints at how often reciprocal translocations can occur in plants. Before, using the same transgene line 1-12 we were able to demonstrate that in ~1 of 1000 cases a DSB could be repaired by the use of a homologous sequence in an ectopic position (Puchta 1999a). About 6000 seedlings were inoculated and four ectopic recombination events could be isolated in that study. Of these four events only one was a classical gene conversion. For line IRC1 <1200 seedlings were inoculated in this study, resulting in 84 recombination events—at least with this transgene combination. Thus, for the recombination substrate pTL used in this study, translocation occurs two orders of magnitude more often than ectopic gene conversions. As it was estimated that a DSB is repaired in 1 of 10,000 cases by ectopic homologies (Shalev and Levy 1997; Puchta 1999a), our study indicates that indeed in 1 of 100 cases two DSBs that occur simultaneously in a genome might be rejoined in the "wrong" way. In many cases the respective translocation might lead to dicentric or acentric chromosomes and thus will not be passed through cell division. However, in the case that an inversion within the same chromosome occurs or chromosome arms are exchanged, the new genotype could be transferred to the next generation. Thus, DSB-induced reciprocal exchanges might play a significant role in plant genome evolution. Indeed, translocation is a general mechanism in plant genome evolution and reciprocal exchanges have been found in the Arabidopsis genome (Blanc *et al.* 2000; Arabidopsis Genome Initiative 2000).

Possible biotechnological applications: Our results clearly demonstrate that reciprocal translocations can be induced in the plant genome after induction of two unlinked DSBs by means of a highly specific restriction endonuclease. We are convinced that the described technique has a high potential for biotechnological applications. Site-specific recombinases can be used to exchange DNA segments (for review see Ow 2002). It has been demonstrated before that site-specific recombination between tobacco chromosomes could be achieved using the *Cre* recombinase, and the translocation could be transmitted through meiosis (Qin *et al.* 1994). However, chromosomal translocations between Arabidopsis and tobacco chromosomes could not be passed through the germ line (Koshinsky *et al.* 2000). Using site-specific recombination, recognition sites of the recombinases are left behind in the genome. Thus, for the performance of multiple genomic changes a combination of different site-specific recombination systems has to be applied. The development of alternative approaches for site-specific alterations of genomes is of great interest for biotechnology. A very promising approach would be the elimination of the respective recognition sequence during the genomic manipulation. Therefore, the use of highly specific restriction endonucleases for genome manipulations might be a useful, irreversible alternative to the established site-specific techniques. Endonucleases have already been applied for other kinds of plant genome manipulations: it has been demonstrated before that rare cutting restriction endonucleases can be used for the elimination of marker genes after transformation (Siebert and Puchta 2002; Puchta 2003) and for the induction of site-specific integration (Puchta *et al.* 1996). This might become even more feasible as new methods are developed for directed evolution of homing endonucleases (Chen and Zhao 2005). Recently, the introduction of mutations in genes of Arabidopsis has been demonstrated via the application of a sequence-specific zinc-finger nuclease (Lloyd *et al.* 2005). As zinc-finger nucleases can be developed for any recognition sequence required (Carroll 2004; Durai *et al.* 2005) and were shown to drastically increase the frequency of homologous recombination in plants (Wright *et al.* 2005), one can expect that in the long run it will be possible to induce DSBs at any given site in the plant genome for various purposes, such as mutation, integration, excision, or translocation. Thus, endonucleases might become important tools for future plant gene technology.

We thank Sabrina Hettinger, Maren Nitze, Karl-Heinz Knoch, Waldtraut Wehrle, Andrea Kunze, Beate Kamm, and Christa Fricke for technical assistance and the Deutsche Forschungsgemeinschaft (Pu 137/8-1) for funding this work.

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Communicating editor: J. A. Birchler