The Maize Transposable Element Ac Induces Recombination Between the Donor Site and an Homologous Ectopic Sequence

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ABSTRACT

The prominent repair mechanism of DNA double-strand breaks formed upon excision of the maize Ac transposable element is via nonhomologous end joining. In this work we have studied the role of homologous recombination as an additional repair pathway. To this end, we developed an assay whereby β-glucuronidase (GUS) activity is restored upon recombination between two homologous ectopic (nonallelic) sequences in transgenic tobacco plants. One of the recombination partners carried a deletion at the 5' end of GUS and an Ac or a Ds element inserted at the deletion site. The other partner carried an intact 5' end of the GUS open reading frame and had a deletion at the 3' end of the gene. Based on GUS reactivation data, we found that the excision of Ac induced recombination between ectopic sequences by at least two orders of magnitude. Recombination events, visualized by blue staining, were detected in seedlings, in pollen and in protoplasts. DNA fragments corresponding to recombination events were recovered exclusively in crosses with Ac-carrying plants, providing physical evidence for Ac-induced ectopic recombination. The occurrence of ectopic recombination following double-strand breaks is a potentially important factor in plant genome evolution.

THE transposable element Activator (Ac) of maize and the related nonautonomous Dissociation (Ds) elements belong to the same class of elements as the En/Spm of maize, P of Drosophila, Tcl of nematodes, Tn10 and Tn7 of Escherichia coli, all sharing a conservative “cut-and-paste” mechanism of transposition (see reviews in SAEKLER and GIERI, 1996). According to this model, a double-strand break (DSB) is created at the donor site upon the element excision. Healing of the broken donor can occur through a number of different pathways. In E. coli, where DSB repair via nonhomologous end-joining is not efficient, broken DNA at Tn7 or Tn10 donor sites is repaired via homologous recombination (HR) (HAGEMANN and CRAIG, 1993; EICHENBAUM and LIVNEH, 1995). Similarly, HR is involved in the donor site repair following P (ENGELS et al. 1990) and Tcl (PLASTERK, 1991) excision. Based on genetic and molecular characterization of P and Tcl donor site repair, a model was proposed that includes the following successive events: DSB formation upon element excision, gap enlargement via exonucleolytic degradation exposing 3' single-strand regions, and invasion of a sequence homologous to the donor site to be used as a template for gap repair (ENGELS et al. 1990; PLASTERK, 1991; NASSEF et al. 1994). The template for donor site repair can be supplied by either the sister chromatid, the allelic sequence on the homologue, a nonallelic (ectopic) sequence (ENGELS et al. 1990) or an oligonucleotide (BANGA and BOYD, 1992). Such a gap repair mechanism can account for the apparent lack of correlation between excision and reinsertion and for the effect of an allelic sequence on the reversion frequency at the donor site.

Although in plants HR is not efficient, there are indications that it is induced upon DSB formation: (1) Irradiation was found to enhance the rate of HR (TOVAR and LICHTENSTEIN, 1992; PUCHTA et al. 1995). (2) Genomic DSB, catalyzed by the yeast I-SceI endonuclease, induces HR between a T-DNA template and a tobacco genomic sequence (PUCHTA et al. 1996). (3) An Ac/Ds insertion was shown to increase crossover between markers flanking the insertion site (MCCLINTOCK, 1953; GREENBLATT, 1981). (4) Ac excision from the maize P locus was found to induce recombination between large direct repeats flanking Ac donor site (ATHMA and PETERSON, 1991). Similarly, the maize Mutator element was shown to increase recombination between tandem repeats at the Kn1-O locus (LOWE et al. 1992).

The data suggesting that Ac induces HR are in conflict with other works showing that an Ac or Ds insertion either reduces (MCCLINTOCK, 1953) or has no effect (FRADKIN and BRINK, 1956) on crossover in the region flanking the element. Moreover, the correlation between excision and reinsertion of Ac/Ds (GREENBLATT, 1981; CHEN et al. 1992) and the lack of apparent allelic effect on the rate of Ds excision suggest that gap repair with an homologous template plays a minor role in donor site repair following Ac excision. Finally, the molecular analysis of excision footprints has led to models where DSBs at the donor sites are repaired by end-
joining (or illegitimate recombination), following exonuclease and DNA-polymerase activities (COEN et al. 1989; SAEDLER and GERL 1996; SCOTT et al. 1996).

To better assess the role of HR in Ac donor site repair, we have developed a nonselective assay in transgenic tobacco that allows us to monitor HR in the region flanking an Ac or a nonmobile Ds element through blue sectors, which reflect the repair of a defective β-glucoronidase (GUS) gene. We found that Ac excision induces HR between genomic sequences located at ectopic (nonallelic) sites by at least two orders of magnitude. In agreement with these findings, recombinant DNA molecules were detected only in Ac but not in Ds-carrying plants. These data show that in addition to the nonhomologous end-joining repair pathway, HR is used for donor site repair following Ac excision. To our knowledge, this is the first indication of ectopic HR in plants.

MATERIALS AND METHODS

Plasmid construction: Digestions with restriction enzymes, ligations and plasmid preparations were performed according to standard protocols and manufacturer’s instructions (SAM-BROOK et al. 1989). Plasmid pJD300, kindly provided by V. WALBOT, was used as a positive control for assaying GUS activity. This plasmid is a Bluescript derivative carrying the GUS reporter gene driven by the cauliflower mosaic virus 35S promoter fused to the tobacco mosaic virus translational enhancer Ω (GALLIE et al. 1987). The transcription termination fragment of pJD300 is derived from the Nopaline synthase (nos) gene of Agrobacterium tumefaciens. To test whether Ac induces HR, we built a series of constructs, derived from pJD300, schematically described in Figure 1. Recombination partners were constructed, each with a different deletion in the GUS gene. One partner (3'ΔGUS), carried a 7 kb base pair (bp) (SspI-EcoRI) deletion in the 3' region of GUS, spanning ~500 bp within the GUS gene, and the nos transcription termination fragment. The 3'ΔGUS (laboratory number pGS001) plasmid was prepared by digestion with EcoRI, blunting with Klenow, followed by partial SspI digestion and self-ligation of the 4.5-kilobase (kb) fragment. The second partner consisting of a deletion in the 5' end of GUS with either Ac or Ds inserted at the deletion site. For this purpose pJD300 was linearized at the SalI site located between 3'GUS and the gene. The deletion was created by treating with BamHI, the ends were blunted with Klenow and a 15-bp KpnI-SphI adapter was synthesized and ligated to the deleted vector fragment. This yielded the 3'ΔGUS (pGS003) plasmid. The BamHI deletion in pGS003 consisted of a 12-bp deletion, which included the ATG initiation codon of GUS. This plasmid as well as pGS001 were tested for GUS activity in transient assays and found to be GUS inactive (data not shown). The KpnI-SphI adapter was also ligated into the blunt Sali site of pJD300. The resultant plasmid pGS002 was found to be GUS active in transient assays (data not shown). Two plasmids, derivatives of pGS002, were constructed to test Ac ability to excise and to confirm Ds lack of mobility. The Ac element used in this study is derived from the wx-m7 allele (CHOMET et al. 1987). It was isolated together with some Waxy-flanking nucleotides from plasmid pJP411, generously given by H. DONNER (KEL-LER et al. 1993), and subcloned into pBluescript II KS (Bluescript) giving rise to plasmid pNA002. An Ac-containing KpnI-SphI fragment was isolated from pNA002 and subcloned into the same sites in the adaptor of pGS002 plasmid giving rise to GUS:Ac (pGS004). A Ds-containing plasmid GUS:Ds (pGS005) was derived from GUS:Ac by filling in the unique SstII site, located in the coding region of Ac. In these plasmids GUS activity can be restored upon element excision. Ac- or Ds-containing KpnI-SphI fragments were isolated from pNA002, or from pGS005, and subcloned into the same sites in the adaptor of pGS003 giving rise to the two recombination partners, 3'ΔGUS:Ac (pGS008) and 3'ΔGUS:Ds (pGS009), respectively. In these plasmids GUS activity cannot be restored upon element excision.

All plasmids described above were subcloned into binary vectors to produce transgenic plants. The HindIII-BgII fragment of pJD300 was subcloned into the same sites of the pGA492 binary vector (AN 1987) giving rise to pGS006. The HindIII-PstI fragment containing the 3'GUS of pGS001 was subcloned into the HindIII-filled-in ClaI sites of the same binary vector giving rise to pGS007. The 7-kb PstI-BgII GUS:Ac containing fragment of pGS004 was subcloned into the HindIII-BamHI sites of the same pGA492 binary vector giving rise to pGS010. The 7-kb PstI-BgII fragment of 3'ΔGUS:Ac (pGS008) was subcloned into the PstI-BamHI sites of the binary vector pCGN1548 (MCDARAD and SUMMERFELT 1990) giving rise to pGS012. Similarly, the blunted 7-kb EcoRI-XbaI fragment of 3'ΔGUS:Ac (pGS008) was subcloned into the blunted PstI-BgII sites of the pGPTV-HPT binary vector (BECKER et al. 1992) giving rise to pGS015. The 7-kb PstI-BgII fragments of GUS:Ds (pGS005) and 3'ΔGUS:Ds (pGS009) were ligated with the 14.3 kb PstI-BgII vector-containing fragment of pGS012 giving rise to pGS011 and pGS013, respectively.

Tobacco transformation: The binary vectors mentioned above were introduced by electroporation into the Agrobacterium tumefaciens LB4404 strain (HOEKEMA et al. 1983). The transformed Agrobacterium strains were used to infect leaf discs of Nicotiana tabacum and regenerate transgenic plants as described by Horsch et al. (1985). All transgenic plants carried transformation markers that confer resistance to kanamycin (100 mg/liter) or hygromycin (18 units/ml). Analyses were carried out in progenies of crosses whose parents were hemizygous for one or the other T-DNA. The number of T-DNA copies was determined on the basis of kanamycin or hygromycin resistance frequency in T2-selfed seedlings and by Southern blot analysis (data not shown).

GUS assays: GUS activity in transgenic plants was determined according to the fluorimetric or histochemical procedures (JEFFERSON et al. 1987). Tissues of transgenic plants were stained with the X-Gluc (5-bromo-chloro-3-indoly-β-D-glucoronide from Molecular Probes) substrate and subsequently cleared as described (BEECKMAN and ENGGER 1994). Pollen staining was performed with a solution consisting of 250 mM Mannitol, 100 mM (pH 7) phosphate buffer and 1% DMSO. Pollen cells were stained at the day of anthesis for 1 hr at 37°C under sterile conditions.

Microcalli were done using mesophyll protoplasts isolated from leaves of double hemizygous F1 plants of the 3'GUS × 5'GUS:Ac and 3'GUS × 5'GUS:Ds combinations as described (FRIDLENDER et al. 1996). The protoplasts were placed in tissue-culture plates containing 10 ml of the VKM X 2 proliferating medium for 6 days in a tissue-culture chamber, under low light conditions (AVT and GALUN 1985). On the sixth day, when microcalli consisted of four to 16 cells, GUS activity was assayed by X-Gluc staining and clearing was done as described for seedlings. A total of 500,000 microcalli were screened, and recombinant blue cells were counted under an inverted Axiovert 35 Zeiss microscope.

PCR and Southern blotting: Genomic DNA samples were isolated as described (DELLAPORTA et al. 1983). The following conditions were used in all PCR reactions: denaturation at
92° for 1 min, annealing at 58° for 55 sec and elongation for 50 sec at 72° for 30 cycles. To isolate recombinant and excision products, two rounds of PCR amplification with nested primer pairs were done using primers a, b, c and d shown in Figure 1. In the first round, primers a (5'-ATGAGCGGAGAATTAAGGGAG3') and b (5'-GTCGGGTCGAGTTTACGCG3') were used. As illustrated in Figure 1, this step could not amplify a product from pGS007, as primer b is in the deleted region of GUS in pGS007 and primer a is in the right border region unique to the pGPTV binary vector. This step could not amplify a product from pGS013 or pGS015 if excision had not occurred. A 5-µl sample of the first round was used as a template in the second round of amplification carried out with primers c (5'-GCCGTTTTACGTTTGGAACTG3') and d (5'-GCGGATCCAGACTGAATG3') in pGEM-T vector system (Promega) and sequenced using the T7 or SP6 primers.

**RESULTS**

**An assay for ectopic recombination:** To assay Ac-induced HR, transgenic plants were made with the constructs described in MATERIALS and METHODS. A recombination assay was developed, based on reactivation of the GUS gene using constructs described in Figure 1. Crosses were made between T1 plants transformed with the 3'ΔGUS and T1 plants transformed with 5'ΔGUS:Ac or 5'ΔGUS:Ds. Ac is expected to generate a DSB near the 5' deletion site upon excision, while Ds is not expected to excise. This Ac-induction of DSB is expected to trigger HR. GUS reactivation could occur if the 12-bp deletion in the 5'ΔGUS is repaired by a gene conversion-like mechanism using the intact 5' region of the 3'ΔGUS partner as a template. Alternatively, one crossover product containing the NPTII region, the 35S promoter, the intact ATG of the 3'ΔGUS construct and the intact 3' region of GUS from the 5'ΔGUS could also produce an active GUS enzyme. The reciprocal crossover product containing the right border from the T-DNA in 5'ΔGUS, the 35S promoter and the CAT gene in 3'ΔGUS would not produce an active enzyme. Eight different genetic combinations, involving parents derived from independent transformation events, were analyzed for the 3'ΔGUS × 5'ΔGUS:Ac cross (pGS007 × pGS012 or pGS007 × pGS015), and nine combinations were analyzed for the 3'ΔGUS × 5'ΔGUS:Ds cross (pGS007 × pGS013). The position of the 3'ΔGUS, 5'ΔGUS:Ac or 5'ΔGUS:Ds constructs in the various transgenes has not been mapped. However, it is reasonable to assume they are located on different chromosomes in the various transgenic plants. In case they are located on the same chromosome they cannot be at allelic positions or on the same homolog in F1 plants. This is evident from the fact that F1 plants from the various crosses contain two nonallelic T-DNAs (the 3'Δ or the 5'Δ), which, being contributed by a different

![Diagram](image-url)
ectopic HR by at least two orders of magnitude. Fluorometric analysis of GUS activity in seedlings extracts confirmed the results from X-Gluc staining, i.e., background GUS levels in parents or $3\Delta GUS \times 5\Delta GUS:Ac$ crosses and increased activity in $3\Delta GUS \times 5\Delta GUS:Ac$ crosses (data not shown). The physical characterization of HR events is described below.

**Physical evidence for ectopic recombination:** To physically characterize the observed ectopic HR events, we searched for germlinal recombination in F$_1$ seedlings derived from the $3\Delta GUS \times 5\Delta GUS:Ac$ crosses. Of 73,000 double hemizygote F$_2$ seedlings, we failed to find germlinal recombinant plants using a viable staining screen for GUS (MARTIN et al. 1992). Therefore, we used PCR to amplify recombinant molecules in somatic tissues. Two primer pairs were used for nested PCR amplification: primer pair a-b (see Figure 1) was used in the first round of amplification, and primer pair c-d was used in the second one. Ac excision and subsequent repair by end joining of the donor is expected to give rise to the amplification of a fragment of $\sim$680 bp (Figure 1). In this end-joining fragment, the Waxy sequence flanking Ac is expected to be a mixture of fragments carrying excision footprints (SAEDLER and NEVERS 1985; SCOTT et al. 1996). On the other hand, if HR repair of Ac-induced DSB is done using the $3\Delta GUS$ as a template, a recombinant 606-bp fragment, corresponding to a conversion-like event should be amplified (Figure 1). To determine the identity of the amplified fragments, the EtBr-stained gel containing PCR products from the second round of amplification was blotted and hybridized with two different probes (Figure 3). A Waxy oligoprobe (pr2 in Figure 1) hybridized to the 680-bp end-joining fragment only in lanes where genomic DNA from $3\Delta GUS:Ac$ or $3\Delta GUS \times 5\Delta GUS:Ac$ F$_1$ plants was used as template. No visible bands were observed in NN plants, $3\Delta GUS$, $5\Delta GUS:Ds$ or $3\Delta GUS \times 5\Delta GUS:Ds$ in EtBr-stained gels (data not shown) and in Southern blot (Figure 3), either because of lack of the target sequence or because of the incapacity of Ds to excise. A faint band of $\sim$580 bp was observed with $3\Delta GUS:Ac$ in addition to the major end-joining 680-bp band (Figure 3A, lane 7). The origin of this fragment, which did not hybridize to the 35S probe, is not known. Both the 680-bp end-joining and the 606-bp recombination fragments were observed with the 35S probe (pr1 in Figure 1) in the $3\Delta GUS \times 5\Delta GUS:Ac$ crosses (Figure 3B, lanes 8 and 9). However, only the 680-bp end-joining fragment was observed for the $3\Delta GUS:Ac$ template (Figure 3B, lane 7). This is expected because, with the $5\Delta GUS:Ac$ line, recombination cannot occur since no recombination partner is available. The 606-bp band was observed only with the 35S probe (pr1). It was amplified only with $3\Delta GUS \times 5\Delta GUS:Ac$ and was cleavable with Sall (data not shown), suggesting that it is a recombination product. This molecule was not observed with the $3\Delta GUS \times 5\Delta GUS:Ds$ crosses despite

**Genetic evidence for Ac-induced ectopic recombination:** F$_1$ seedlings were stained histochemically with X-Gluc. Blue sectors were detected in the cotyledons (A), true leaves (B), and in roots (C) of 15-day-old seedlings. Similar staining was detected in the pollen (D) and in microcalli derived from protoplasts isolated from F$_1$ plants (E and F).

**Figure 2.—** In situ staining for GUS activity in double hemizygous F$_1$ plants for $3\Delta GUS:Ac$ and $3\Delta GUS$. Tissues were stained at various developmental stages according to the histological assay described by JEFFERSON (JEFFERSON et al. 1987). GUS$^+$ sectors were detected in the cotyledons (A), true leaves (B), and in roots (C) of 15-day-old seedlings. Similar staining was detected in the pollen (D) and in microcalli derived from protoplasts isolated from F$_1$ plants (E and F).
the fact that the $5'\Delta GUS:Ac$ and $5'\Delta GUS:Ds$ constructs are almost identical in sequence. This supports the notion that the formation of the 606 bp is excision dependent rather than sequence dependent.

To further confirm the nature of the 680-bp religation and 606-bp recombination bands, the PCR products corresponding to these bands were cloned and sequenced. The sequences of three independent clones of the 606-bp fragment, obtained from two different F1 combinations, were found identical, corresponding to a recombinant molecule between the T-DNA right border of $5'\Delta GUS:Ac$ (pGS015) and the region around SalI in $3'\Delta GUS$, which served as a template for gap repair (see Figure 4A). Note that the T-DNA right border of $5'\Delta GUS:Ac$ (pGS015) is different in sequence compared to the right border of $3'\Delta GUS$ (Figure 1) and that no such chimeric molecule existed previously in our laboratory. Sequencing of three clones of the ~680-bp fragment revealed typical Ac excision footprints (SCOTT et al. 1996) as shown in Figure 4B. This confirms that the 680-bp band, which hybridizes to the Waxy oligoprobe, is truly an end-joining product. The physical evidence shown here, for Ac-induced formation of recombinant molecules—conversion-like products—is in agreement with the genetic data obtained by GUS staining (Table 1), suggesting that ectopic HR does not occur, or is very rare, in the absence of Ac-induced DSB. Although additional recombination products (via crossing over) might also be Ac induced, our preliminary attempts at long range PCR to amplify such products failed (data not shown).

**The frequency of ectopic recombination** The frequency of ectopic recombination was estimated by various means and in different tissues. Pollen grains from double hemizygoate plants from the $3'\Delta GUS \times 5'\Delta GUS:Ac$ or the $3'\Delta GUS \times 5'\Delta GUS:Ds$ crosses were stained with X-Gluc for GUS activity. Twelve pollen grains with strong blue color (Figure 2D) were found out of a population of $5 \times 10^5$ pollen grains in the $3'\Delta GUS \times 5'\Delta GUS:Ac$ cross while no blue pollen grains were found in a population of $3 \times 10^6$ grains in the $3'\Delta GUS \times 5'\Delta GUS:Ds$ cross. This represents a recombination frequency of $2.4 \times 10^{-3}$ in pollen cells.

Microcalli derived from mesophyll protoplasts were used to estimate the frequency of somatic ectopic recombination. GUS activity was assayed by X-Gluc staining in four to 16 cells microcalli of F1 $3'\Delta GUS \times 5'\Delta GUS:Ac$ plants. A total of ~500,000 microcalli were stained and screened for the presence of blue cells as shown in Figure 2E and F. The frequency of blue cells was $2.4 \times 10^{-4}$.

PCR experiments also provide rough estimates on the frequency of Ac-induced ectopic recombination (Figure 3). These experiments were carried out using 500 ng genomic template in the first round, corresponding to ~33,000 tobacco genomes (C value = 15.5 pg) (BENNETT and SMITH 1976). All the PCR reactions, where genomic DNA prepared from the $3'\Delta GUS \times 5'\Delta GUS:Ac$ combinations was used as template, gave rise to the 606-bp recombination band; hence at least one recombinant molecule was present in the template. This further supports the estimates that Ac-induced ectopic recombination occurred in $10^{-3}$–$10^{-4}$ of the cells.

**DISCUSSION**

**Evidence for Ac-induced ectopic recombination** We have developed a direct assay for Ac-induced HR be-
between two nonallelic recombination partners that shared 2 kb of homology (0.5 kb upstream of Ac excision site and 1.5 kb downstream of it, as shown in Figure 1). This assay has provided both genetic (GUS reactivation) and physical evidence that Ac induces recombination between homologous sequences located at ectopic sites. The findings that GUS reactivation occurred only with Ac in \( \Delta GUS \times \Delta GUS:Ac \) crosses but not with an almost identical \( Ds \) element provided the genetic evidence for recombination. The physical evidence is that a 606-bp PCR fragment was obtained exclusively in all the \( \Delta GUS \times \Delta GUS:Ac \) combinations tested, and its sequence confirmed that it is a recombinant molecule. The 606-bp fragment has the size and sequence expected for such a recombination product and was not obtained in any of the crosses carrying \( Ds \) (\( \Delta GUS \times \Delta GUS:Ds \)), whose sequence is almost identical to Ac. This fragment was not amplified either in \( \Delta GUS:Ac \), i.e., in the absence of the \( \Delta GUS \) template, or in the other controls (Figure 3B). Therefore, its formation is both excision (DSB)- and template-depends, as expected for a recombination product. This argues against the possibility that the 606-bp recombinant fragment was formed by a PCR artifact. Moreover, neither this fragment nor a similar molecule had existed in our laboratory, hence excluding a PCR contamination.

**DSBs induce homologous recombination in plants:** DNA DSBs have been shown previously to induce recombination in plants using assays based on different types of homologous partners: (1) Somatic crossover between homologous chromosomes was induced by DNA-damaging agents such as \( \gamma \)-irradiation (Carlson 1974). (2) Extrachromosomal HR assays were performed between plasmids introduced into plant cells. These assays showed that HR is induced by DSBs intro-
duced in vitro with restriction enzymes (Engels and Meyer 1992) or in vivo with site-specific recombinases (Puchta et al. 1993). Single-strand annealing was proposed as the major pathway for extrachromosomal recombination in plants (Puchta et al. 1994). (3) Homologous integration of a T-DNA sequence into a chromosomal site was shown to be increased by the formation of a DSB in the chromosomal target (Puchta et al. 1996). (4) Intrachromosomal recombination between large direct repeats was induced upon Ac excision at the Plocus in maize (Atha and Peterson 1991). Similarly, Mutator was shown to increase recombination between tandem repeats at the Kn1-O locus (Lowe et al. 1992). Intrachromosomal recombination was also found to be induced by DNA-damaging agents (Tovar and Lichtenstein 1992; Puchta et al. 1995). Recently, the HO endonuclease was shown to increase intrachromosomal recombination by 10-fold in Arabidopsis (Chiarazzi et al. 1996). (5) Sister-chromatid exchange was shown to be induced by DNA-damaging agents (Schartzman 1987), suggesting that recombination between sister chromatids can play a role in DNA repair. It was also shown that Ac excision can induce gap repair using a sister chromatid as a template (E. Rubin and A. A. Levy, unpublished data).

In this article we show for the first time the induction of ectopic HR in plants by DSB formation. We did not find evidence for ectopic recombination in $3'\Delta GUS \times 3'\Delta GUS:Ds$ crosses using the GUS reactivation assay or by PCR. Moreover, ectopic recombination in the absence of DSBs has not been reported previously in plants, suggesting that ectopic recombination in the plant genome does not occur or occurs very rarely. This is in contrast to yeast, in which HR, including ectopic recombination, is efficient (Ernst et al. 1981; Mirus and Petes 1982) and to Drosophila in which ectopic HR was reported even in the absence of transposon excision (Lim and Simmons 1994).

Despite the extensive above-quoted literature on DSBs-induced HR in plants, the question remains how efficient is HR as a DSB repair mechanism in plants, compared to the alternative nonhomologous end-joining or illegitimate-recombination pathways. In the case of Ac the frequent occurrence of revertant sectors and the extensive analysis of excision footprints (Saedler and Nevers 1985; Scott et al. 1996) suggest that nonhomologous end joining is the major DSB repair pathway. If this is the case, it is surprising that the recombinant 606-bp and the relocation ~680-bp fragments showed equal stoichiometry in Southern blots of the PCR reactions (Figure 3B). This discrepancy might be caused by the fact that PCR is not quantitative, particularly after two rounds of amplification, or that there is PCR template preference.

As discussed above, DSBs are repaired by both illegitimate and homologous recombination in plants. From the data presented here, one may extrapolate that any DSB occurring in a repetitive DNA region can induce ectopic recombination in plants. The implications of such a possibility are described below.

**Genetic and evolutionary implications of ectopic HR:** Ectopic recombination could lead to chromosomal translocations in cases of crossover or to gene conversion. In organisms such as plants or mammals, in which a high percent of the genome is repetitive, crossover between ectopic sequence would create a genomic chaos. Therefore, ectopic recombination should occur at a very low rate, or a mechanism favoring conversion over crossover should be used. In mice, ectopic HR was found at a frequency of 0.1–0.7% (Murti et al. 1994). These ectopic recombination events corresponded to gene conversion, thus providing a mechanism to maintain genome stability. Nevertheless, evidence was found for ectopic crossover, leading to chromosomal translocations (Kovaly et al. 1994; Wang et al. 1995) in humans. Such events are probably rare or are efficiently selected against. In plants, translocation lines have been isolated in several species, though, no sequence analysis of translocation breakpoints is available. Therefore, it is not known whether translocation occurred as a result of crossover between homologous ectopic sequences or as a result of nonhomologous end joining of broken chromosomes. The ectopic recombination events characterized in this work occurred at a rate of $10^{-2} - 10^{-3}$. As Ac excision is usually in the 10$^{-2}$ range (Jones et al. 1989; Levy and Walbot 1990); this suggests that, once a DSB is formed, it can be repaired using an ectopic homologous template for 0.1–10% of the events. Such frequencies make ectopic recombination in plants a potentially important factor in genome evolution. Ac/Ds insertion, or any other type of DSB within repeated DNA, could lead to shuffling of the genome if recombination is followed by crossover. This work could not determine whether conversion or crossover occurred since recovery of germinal recombination events and rescue of the GUS$^+$ calli were not successful. A system similar to the one described here, but with a selectable marker, e.g., antibiotic resistance, could allow regeneration of somatic events or screening of large plant populations for germinal events.

Given the dangers of ectopic recombination, one may speculate that the underlying mechanisms of ectopic recombination favors conversion over crossover. One such mechanism, the synthesis-dependent strand-annealing (SDSA) pathway (Formosa and Alberts 1986), has been shown to occur for the repair of P-element excision in Drosophila (Nassif et al. 1994). It has the advantage of explaining both deletions and insertions often observed at recombination sites (Puchta et al. 1996), and it can prevent reshuffling of the genome via crossover. Evidence that SDSA is active for DSB repair in plants have been recently obtained (E. Rubin and A. A. Levy, unpublished data; V. Gorbunova and A. A. Levy, unpublished data).
For practical applications, the DSB-induced HR reported here can facilitate targeted integration at any locus where an active transposable element insertion is present. Targeting could be done via T-DNA integration as described by Puchta et al. (1996). Alternatively, a new strategy of gene targeting, based on ectopic recombination, can be conceived. Under this scheme, one line where the DSB is induced at a specific locus serves as the targeted site. This line is then crossed to another line carrying an engineered construct (the template), which can recombine with the targeted loci. Resolution of such recombination via gene conversion will result in gene targeting.

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