Ac Insertion Site Affects the Frequency of Transposon-Induced Homologous Recombination at the Maize *p1* Locus

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ABSTRACT

The maize *p1* gene regulates the production of a red pigment in the kernel pericarp, cob, and other maize floral tissues. Insertions of the transposable element *Ac* can induce recombination between two highly homologous 5.2-kb direct repeat sequences that flank the *p1* gene-coding region. Here, we tested the effects of the *Ac* insertion site and orientation on the induction of recombination at the *p1* locus. A collection of unique *p1* gene alleles was used, which carry *Ac* insertions at different sites in and near the *p1* locus, outside of the direct repeats, within the direct repeat sequences, and between the direct repeats, in both orientations. Recombination was scored by the numbers of colorless pericarp sectors (somatic frequency) and heritable mutations (germinal frequency). In both the somatic and germinal tests, the frequency of homologous recombination is significantly higher when *Ac* is inserted between the direct repeats than when *Ac* is inserted either within or outside the repeats. In contrast, *Ac* orientation had no significant effect on recombination frequency. We discuss these results in terms of the possible mechanisms of transposon-induced recombination.

IT has been widely reported that transposable elements can induce genome rearrangements, such as deletions, duplications, and inversions, in both eukaryotes and prokaryotes (reviewed in Saedler and Gierl 1996). In *Escherichia coli*, transposons Tn3 and Tn7 can increase the frequency of homologous recombination in nearby regions (Kondo *et al.* 1989; Hagemann and Craig 1993). Also, IS10 intermolecular transposition stimulates homologous recombination between the donor and acceptor molecules at the transposition site (Eichenbaum and Livneh 1995). In *Saccharomyces cerevisiae*, homologous recombination between Ty elements produces deletions, inversions, and translocations (reviewed in Boeke 1989). In Drosophila, transposition of the *P* element leads to increased recombination frequencies in both the male germline (Hiraizumi 1971; Kidwell and Kidwell 1976) and somatic cells (Sved *et al.* 1990) and to the generation of a variety of chromosome rearrangements (Svoboda *et al.* 1995; Preston *et al.* 1996). Additionally, *P*-element excision promotes efficient gene conversion and enables efficient site-specific gene replacement (Engels *et al.* 1990; Gloor *et al.* 1991; Lankenau *et al.* 1996; Preston and Engels 1996).

In plants, initial reports indicated that maize *Ac* or *Ds* transposable element insertions either decreased (McClintock 1953) or had no effect (Fradkin and Brink 1956) on meiotic recombination in the region flanking the element. At the maize *bz1* locus, the insertion of a *Ds* element reduced the intragenic recombination frequency about fourfold (Dooner and Kermicle 1986). Also, a *Mu1* insertion in *bz1* did not stimulate intragenic meiotic recombination (Dooner and Ralston 1990). In a study of meiotic recombination at the maize *a1* locus, insertion of an inactive *Mu1* element reduced recombination rates in a nearby 377-bp interval by ~50% (Xu *et al.* 1995). In this latter case, the *Mu1* insertion was inactive and, in the hemizygous condition, may have interrupted pairing or branch migration of the Holliday junction (Biswas *et al.* 1998). More recently, Dooner and Martinez-Ferez (1997) reported that an *Ac* insertion could destabilize a tandem duplication in the maize *bz* locus, but the same insertion did not stimulate meiotic recombination or homology-dependent repair.

In contrast, several reports have demonstrated a marked ability of transposons to stimulate premeiotic homologous recombination. Transposon-induced recombination has been associated with insertion of transposable elements at the maize *p1* and *knotted* loci (Athma and Peterson 1991; Lowe *et al.* 1992). The *p1* gene encodes a *Myb*-homologous transcriptional regulator of flavonoid pigmentation in kernel pericarp and cob (Styles and Ceska 1977, 1989; Grotewold *et al.* 1994). The *p1* coding region is flanked by two 5.2-kb direct repeat sequences (Lechelt *et al.* 1989), and recombination between the two repeats can be easily detected as a loss of *p1* gene function giving rise to colorless sectors on maize kernels (Athma and Peterson 1991). In a previ-

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ous study, it was shown that insertion of the maize transposable element *Ac* in the *p1* gene increases the frequency of homologous recombination ~100-fold (Athma and Peterson 1991). Similarly, the *Mu1* transposon is reported to increase recombination ~100–2000- fold at the *Kn1-O* tandem direct duplication locus in maize (Lowe *et al.* 1992). In the absence of a transposon insertion, the homologous recombination rates are very low at both the *p1* and *knotted* loci. Further studies indicated that a *Mu* element insertion in the *Kn1-O* locus stimulates meiotic recombination and gene conversion frequencies (Mathern and Hake 1997). In transgenic tobacco, the *Ac* element was reported to induce somatic recombination between homologous ectopic sequences (Shalev and Levy 1997). Moreover, in a transgenic Arabidopsis system, an active *Ds* transposon can enhance intramolecular homologous recombination >1000-fold over the spontaneous recombination frequency (Xiao and Peterson 2000). Transposon excision is thought to generate transient DNA double-strand breaks (DSBs) that may initiate recombination (Szostak *et al.* 1983). Indirect evidence in plants suggests that a DSB is most often repaired by nonhomologous end-joining (NHEJ, also termed illegitimate recombination; Coen *et al.* 1989; Morton and Hooykaas 1995; Scott *et al.* 1996; Rinehart *et al.* 1997). However, our previous results (Athma and Peterson 1991; Xiao and Peterson 2000) show that homologous recombination can occur as an alternative to the NHEJ pathway when homologous repeat sequences flank the transposon. To study the mechanism of transposon-induced recombination in plants, we tested the effects of transposon position and orientation on the frequency of recombination observed at the maize *p1* locus. The results indicate that position of the transposon within the *p1* locus, but not its orientation, strongly affects the recombination frequency.

MATERIALS AND METHODS

Terminology, maize stocks, and analysis of mutants: The maize *p1* gene encodes a transcription factor that regulates phlobaphene pigmentation of the pericarp, cob, and other floral organs (Styles and Ceska 1972, 1989; Grotewold *et al.* 1994). The pericarp is the outer layer of the mature kernel; it is derived from the ovary wall and therefore is of maternal origin. Maize *p1* alleles are conventionally identified by a suffix that indicates their expression in pericarp and cob: *P1-rr* specifies red pericarp and red cob, *P1-wr* specifies white (colorless) pericarp and red cob, *P1-ww* specifies white (colorless) pericarp and cob, *P1-vv* specifies variegated pericarp and cob, and *P1-ovov* specifies orange-variegated pericarp and cob. This study utilized six *p1* alleles, each containing *Ac* insertions at different sites in or near the *p1* locus (Figure 1); all the alleles were derived directly or indirectly from *P1-vv* (Emerson 1917) via intragenic transposition of *Ac*. The alleles *P1-ovov-Val* (F. A. Valentine, unpublished results), *P1-rr-11:666*, *P1-ovov-1114*, and *P1-ovov-12:1-1* were derived from *P1-vv*, whereas the *P1-9D36A* and *P1-9D47B* alleles were derived from *P1-ovov-1114* (Peterson 1990; Athma *et al.* 1992). Genetic crosses and mutant screens were performed as described previously (Athma and Peterson 1991).

Frequency of colorless sectors: Colorless sectors were scored on ears from plants in which the *P1::Ac* insertion allele was heterozygous with a *P1-wr* allele. The colorless sector frequency was calculated from the percentage of kernels with visible colorless sectors, regardless of the size of the sector. Sector size reflects the developmental stage at which a loss-of-function mutation occurs; the later in development a mutation occurs, the smaller the resulting sector. The average colorless sector frequency of each allele was determined by counting the number of kernels with colorless sectors divided by the estimated total number of kernels. For each allele, sectors were scored on 32 randomly picked ears, except for the *P1-ovov-12:1-1* allele for which 16 ears were used. The number of kernels per ear was estimated as the product of the number of kernel rows times the mean number of kernels per row. *P* values of the *t*-tests were generated by Microsoft Excel 97.

DNA isolation, Southern blot hybridizations, and PCR: The approximate site of *Ac* insertion in each allele was determined by genomic Southern hybridization. Genomic leaf DNA was isolated from seedling leaves of individual plants as previously described (Cocciolone and Cone 1993), digested with restriction enzymes *Sal*I or *Eco*RI, electrophoresed through 0.75% agarose gels, and transferred to nylon membranes (Micron Separations, Westborough, MA; Sambrook *et al.* 1989). Blots were hybridized with random-prime-labeled (Pharmacia, Piscataway, NJ) *p1* locus DNA fragments 15 and 8B (Figure 2). Together, these probes detect a 26-kb contiguous region encompassing the *p1* gene and its flanking sequences. The exact *Ac* insertion sites were then determined by PCR amplification, using primers in the *Ac* sequence and a second primer homologous to *p1* gene sequences. Genomic DNA templates were amplified by PCR for 35 cycles as follows: denaturation at 94° for 30 sec; annealing at 59° for 30 sec; and elongation for 1 min at 72°. PCR products were cloned into pT7Blue T-vector (Novagen) and sequenced at the Iowa State University DNA sequencing and synthesis facility.

RESULTS

***Ac* insertion sites of different *p1* alleles:** To assess the effect of the *Ac* insertion site on the frequency of homologous recombination, we used a collection of six *p1* alleles that carry *Ac* elements inserted at various sites in and near the *p1* gene. The position of *Ac* insertion in each allele was approximated by Southern blot hybridization and then precisely determined by PCR amplification and sequencing of *Ac/p1* junction fragments. *Ac* insertion site positions are depicted in Figure 2. The *P1-rr-11:666* allele contains *Ac* inserted outside the *p1* locus 5.2-kbp direct repeat, at position 6757 bp 5′ of the *P1-rr* transcription start site. The *P1-9D47B* allele has *Ac* inserted within the 5.2-kbp *P1-rr* direct repeat, at position 5024 bp 5′ of the *P1-rr* transcription start site. Four of the alleles contain *Ac* inserted at various sites between the two 5.2-kbp direct repeats: at 49 bp 5′ of the transcription start site (*P1-ovov-Val*); in intron 1 at 473 bp downstream of the transcription start site (*P1-9D36A*); and at two sites within the large second intron (*P1-ovov-1114* and *P1-ovov-12:1-1*), at positions 4338 and 4490 bp, respectively, downstream of the transcription start site (Athma *et al.* 1992). *Ac* insertions

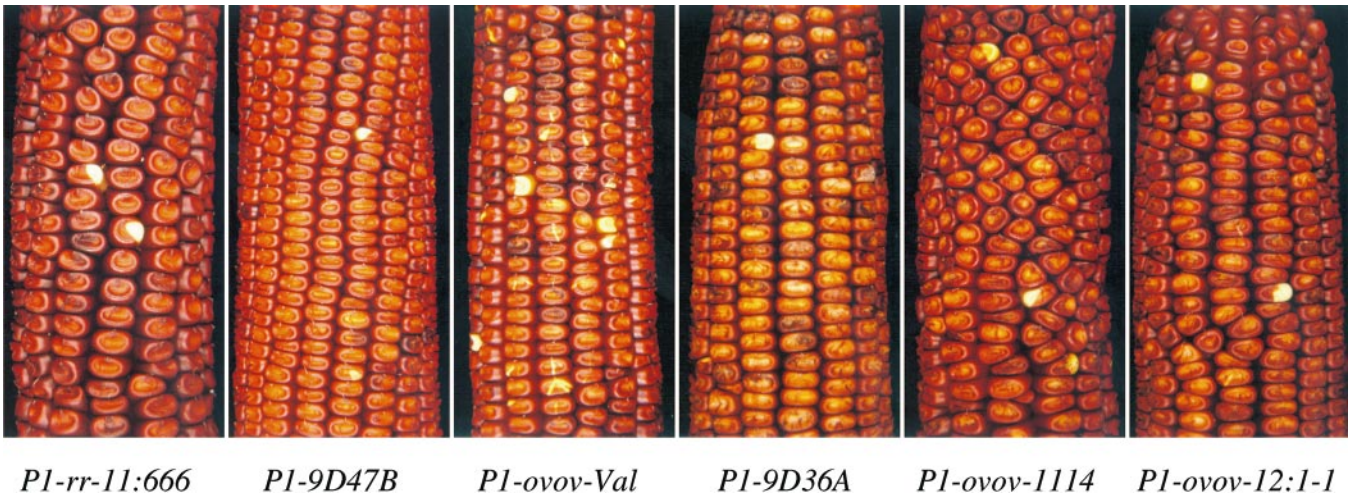

FIGURE 1.—Ear phenotypes of *p1* alleles used in this study. From left to right: *P1-rr:11:666*; *P1-9D47B*; *P1-ovov-Val*; *P1-9D36A*; *P1-ovov-1114*; *P1-ovov-12:1-1*. Each allele is in heterozygous condition with allele *P1-wr*, which specifies colorless pericarp and red cob. Cell clones with losses of *p1* function are visible as colorless pericarp sectors; numbers of these sectors are given in Table 1.

within the *p1* gene introns exhibit an orientation-dependent effect on *p1* expression; when *Ac* is inserted in the same transcriptional orientation as *p1*, transcripts that initiate from the *p1* gene promoter terminate within the *Ac* element. Hence, in the *P1-vv* allele, *p1* gene expression is blocked by *Ac* except in the cell clones in which *Ac* has excised. The resulting kernel pigmentation phenotype consists of colorless pericarp with red sectors (LECHELT *et al.* 1989). In contrast, when *Ac* is inserted in the opposite transcriptional orientation relative to the *p1* gene, *Ac* does not markedly interfere with *p1* transcription and the *Ac* insertion is apparently spliced out of the *p1* transcript (PETERSON 1990). This orientation-dependent effect has been documented in the case of *Ds* insertions in the maize *waxy* locus (WESSLER *et al.* 1987). Whereas the *P1-ovov-Val* allele gives orange-variegated pericarp, even though *Ac* is inserted in the same transcriptional orientation as the *p1* gene, in this case, *Ac* is located 49 bp upstream of the *p1* transcription start site and hence would not be expected to exhibit the orientation-dependent phenotypic effect observed with *Ac/Ds* insertions in transcribed regions.

Comparison of colorless sector frequency of different *p1* alleles: Each of the six *p1* alleles studied here specifies red or orange-variegated pericarp pigmentation (Figure 1); when these alleles are heterozygous with a colorless pericarp allele (either *P1-ww* or *P1-wr*), loss-of-function mutations produce easily visible colorless pericarp sectors (Figure 1). A colorless pericarp sector can result from any somatic mutation that eliminates *p1* gene function. Because *Ac* frequently transposes to linked sites (GREENBLATT 1984; DOWE *et al.* 1990; PETERSON 1990; GROTEWOLD *et al.* 1991; ATHMA *et al.* 1992; MORENO *et al.* 1992), some sectors may result from intragenic transpositions to other sites in the *p1* locus essential for P function. In a previous study, it was found that the great majority of colorless sectors (49 out of 52 analyzed) arising from the *P1-ovov-1114* allele were *p1* deletions caused by recombination between the long 5.2-kb direct repeats flanking the *P1-rr* gene

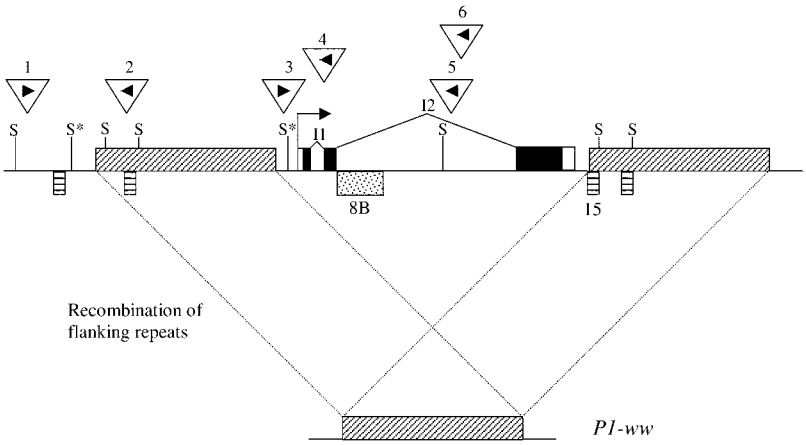

FIGURE 2.—(Top) *Ac* insertion sites in different maize *p1* gene alleles. (Bottom) Schematic diagram showing *Ac*-induced homologous recombination. (▨) 5.2-kb direct repeats; (→) *p1* gene transcription start site; (▽) *Ac* insertion; (▶) *Ac* transcription direction; (▤) fragment 15; (▩) fragment 8B; (■) exons; (□) 5′ leader sequence and 3′ untranslated region; I1 and I2, intron 1 and intron 2; S, *Sal*I site; S*, methylated *Sal*I site; 1, *P1-rr:11:666*; 2, *P1-9D47B*; 3, *P1-ovov-Val*; 4, *P1-9D36A*; 5, *P1-ovov-1114*; 6, *P1-ovov-12:1-1*.

TABLE 1

***Ac* insertion position, orientation, and the frequencies of colorless sectors in different alleles**

Allele	*Ac* position	*Ac* orientation	Total no. of kernels screened	No. of colorless sectors	Average colorless sector (%)
P1-rr-11:666	Outside repeats	*vv*[a]	21,858	529	2.42 ± 0.21[c]
P1-9D47B	Within one repeat	*ovov*[b]	21,635	357	1.65 ± 0.11
P1-ovov-Val	Between repeats	*vv*	22,462	813	3.62 ± 0.87
P1-9D36A	Between repeats	*ovov*	20,572	364	1.77 ± 0.15
P1-ovov-1114	Between repeats	*ovov*	19,837	990	4.99 ± 0.50
P1-ovov-12:1-1	Between repeats	*ovov*	10,010	377	3.77 ± 0.32

[a] *Ac* in *vv* orientation, transcription direction of the *Ac* element is same as *p1* gene.
[b] *Ac* in *ovov* orientation, transcription direction of the *Ac* element is opposite to *p1* gene.
[c] Standard error.

(Athma and Peterson 1991). Thus, the number of colorless pericarp sectors provides an indication of the frequency of homologous recombination between the *p1* locus flanking repeats. In the absence of *Ac* at the *p1* locus (*P1-rr-4026*) the colorless sector frequency is 0.031% (Athma and Peterson 1991). This frequency is increased from 50- to 150-fold in the *Ac* insertion alleles characterized here (Table 1). Additionally, we characterized an allele (*P1-9D27B*) containing a *Ds* insertion generated by an internal deletion in the *Ac* element in the *P1-ovov-1114* allele; hence, this *Ds* element is at the same site as the *Ac* element in the *P1-ovov-1114* allele. In the absence of *Ac*, this allele exhibited two colorless sectors in 2008 kernels (0.1%); in the presence of an *Ac in trans*, this allele produced 65 colorless sectors among 2025 kernels (3.2%). These results confirm the finding of Xiao and Peterson (2000) that *Ac* transposase is essential for induction of recombination by a *Ds* element.

To determine the possible effects of *Ac* position and orientation on recombination frequency, we used a *t*-test for significant differences in the colorless sector frequencies between alleles. The results (Table 2) indicate that the six alleles fall into three statistical groups: Group 1 comprises the alleles *P1-ovov-Val*, *P1-ovov-1114*, and *P1-ovov-12:1-1*, with colorless sector frequencies ranging from 3.6 to 5.0%. Group 2 comprises the *P1-rr-11:666* allele (colorless sector frequency 2.4%). Finally, Group 3 comprises alleles *P1-9D47B* and *P1-9D36A*, with 1.6 and 1.8% colorless sector frequency, respectively. However, if the alleles are grouped on the basis of their insertion location, the average colorless sector frequency is higher for *Ac* elements inserted between the two direct repeats (group A, ~3.5%) than for *Ac* elements inserted outside and within the repeats (group B, ~2.0%). A *t*-test analysis shows that the frequencies of colorless sectors of these two groups are significantly different (P value = 2.48×10^{-5}). Overall, these results show that *Ac* insertions between the two direct repeats induce a higher frequency of recombination than *Ac* insertions outside or within the repeats.

Developmental timing of recombination: While the numbers of sectors indicate frequency of recombination, sector size indicates the developmental time at which mutation occurs. As shown in Table 3, colorless sectors ranged in size from <¼ kernel to sectors covering more than six kernels. In all six alleles, smaller sectors were much more numerous than larger sectors; this most likely reflects the fact that more cells are available for mutation at later stages of development. We also considered the possibility that sector size could affect the frequency measurements of different alleles, because the area available for mutation is slightly altered by the fact that once an area has mutated it is no longer available for mutation (Anderson and Eyster 1928). We found that larger sectors (>1 kernel) were observed only in the *P1-9D36A*, *P1-ovov-Val*, *P1-ovov-1114*, and *P1-ovov-12:1-1* alleles. Thus, the potential complication of

TABLE 2

***P* values of pairwise *t*-test comparisons of colorless sector frequencies of different alleles**

P value	*P*–9D47B*	*P1-ovov-Val*	*P*–9D36A*	*P1-ovov-1114*	*P1-ovov-12:1-1*
P1-rr-11:666	0.002	0.188	0.016	<0.001	0.002
P1-9D47B		<0.001	0.535	<0.001	<0.001
P1-ovov-Val			0.043	0.176	0.867
P1-9D36A				<0.001	<0.001
P1-ovov-1114					0.046

TABLE 3

Sizes of colorless sectors associated with different alleles

Allele	No. of sectors of indicated size								
	<¼ k	¼–½ k	½–1 k	1 k	2 k	3 k	4 k	5 k	>6 k
P1-rr-11:666	444	25	43	0	0	0	0	0	0
P1-9D47B	313	9	33	1	0	0	0	0	0
P1-ovov-Val	642	39	49	1	0	0	1	0	1
P1-9D36A	298	27	59	2	1	0	0	2	0
P1-ovov-1114	880	28	35	4	3	0	1	1	4
P1-ovov-12:1-1	332	9	31	0	0	0	1	0	1

k, kernel.

sector size actually did not affect the conclusion that alleles with *Ac* inserted between the two direct repeats have more colorless sectors than alleles with *Ac* inserted outside or within the direct repeat.

Comparison of germinal mutations of different alleles: As noted above, the colorless pericarp sectors scored in the somatic assay can result from mutations other than recombination of the flanking repeats. To assess the types of mutations generated, we isolated a number of heritable mutants derived from each insertion allele and analyzed these mutants by Southern blot hybridization (Figure 3). The results allowed us to compare the frequencies of germinally transmitted recombination events produced by different alleles (Table 4). From the *P1-rr-11:666* allele, 5 *P1-ww* mutants were recovered, but none were derived from homologous recombination. From the *P1-9D47B* allele, 7 *P1-ww* mutants were obtained, and only 1 resulted from recombination. In contrast, from the four alleles (*P1-ovov-Val*, *P1-9D36A*, *P1-ovov-1114*, and *P1-ovov-12:1-1*) with *Ac* insertions between the two 5.2-kb direct repeats, 85 heritable *P1-ww* mutants were obtained, and 61 of these were deletions generated by recombination of the flanking repeats. The other 24 *P1-ww* mutants were generated by intragenic *Ac* transposition or other transposition-related rearrangements (data not shown). These results suggest that the somatic assay is a reasonable indicator of recombination frequency for the alleles that carry *Ac* insertions between the repeats, but that it may overestimate the true recombination frequency for alleles that carry *Ac* inserted within or outside the direct repeats.

Effect of *Ac* orientation: The six alleles characterized here contain *Ac* insertions in one of two orientations with respect to the *p1* gene: the VV group (*P1-rr-11:666* and *P1-ovov-Val*) in which *Ac* is inserted in the same transcriptional orientation as the *p1* gene and the OVOV group (*P1-9D47B*, *P1-9D36A*, *P1-ovov-1114*, and *P1-ovov-12:1-1*) with *Ac* inserted in the opposite orientation. The average colorless sector frequency among both the VV and OVOV group alleles is ~3.0%. More importantly, comparisons among the alleles with *Ac* insertions between the direct repeat sequences show no significant differences in somatic recombination frequency between *P1-ovov-Val* and *P1-ovov-1114*, nor between *P1-ovov-Val* and *P1-ovov-12:1-1* (Table 2). There is a significant difference in somatic recombination frequency between *P1-ovov-Val* and *P1-9D36A*, but it is unclear whether this is related to the orientation of the *Ac* insertion in *P1-9D36A* (see DISCUSSION). Additionally, comparison of germinal mutation frequencies also

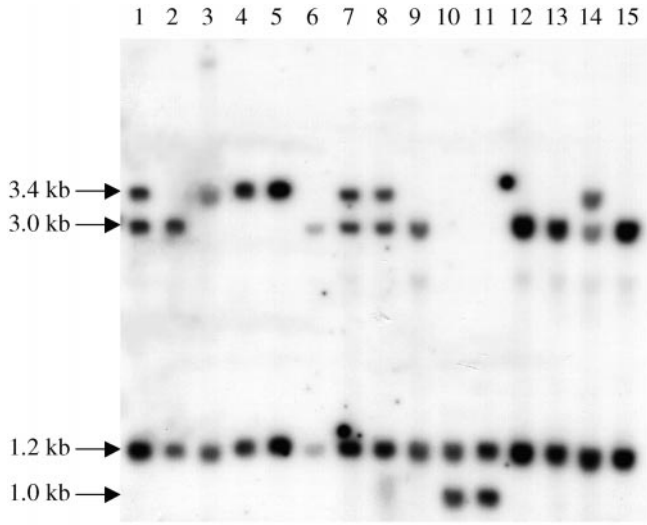

FIGURE 3.—Representative Southern blot analysis of new *p1* mutations. Genomic DNA samples from individual plants were digested with *Sal*I and hybridized with *p1*-specific probe P15, a genomic fragment from the *P1-rr* allele (Figure 2; LECHELT *et al.* 1989). Lane 1, *P1-rr* gives bands of 3.4, 3.0, and 1.2 kb; the 1.2-kb band is a doublet arising from the duplicated sequences flanking the *P1-rr* gene. Lane 2, *P1-ww-1112* is deleted for the 3.4-kb band and one copy of the 1.2-kb fragment; this mutation is the archetype of the class arising via recombination of the *p1*-flanking duplications (ATHMA and PETERSON 1991). Lanes 3–5, *p1* mutations derived from the *P1-rr-11:666* allele; lanes 6–15, *p1* mutations derived from the *P1-9D36A* allele. The banding patterns in lanes 6, 13, and 15 identify these as mutations arising by recombination. The banding patterns in the other lanes do not match those of *P1-ww-1112* (lane 2), and thus these mutations have other types of lesions in the *p1* locus.

TABLE 4

Frequencies of germinally transmitted recombination events produced by different alleles

Allele	Total no. of independent mutant sectors[a]	Total no. of *P1-ww* mutants	No. of *P1-ww* mutants from recombination	Ratio of *P1-ww* from recombination[b]
P1-rr-11:666	60	5[c]	0	0
P1-9D47B	54	7[c]	1	0.14
P1-ovov-Val	73	29	24	0.83
P1-9D36A	71	23	15	0.65
P1-ovov-1114	83	24	16	0.67
P1-ovov-12:1-1	33	9	6	0.67

[a] For each allele, ~100–300 ears were screened for large sectors of kernels with colorless pericarp phenotype. Values given indicate the number of sectors identified for each allele. Most sectors were from different ears and hence were derived from independent mutations. In a few cases, well-separated sectors from the same ear were selected and were considered as most likely arising from independent mutations. Kernels from within the colorless pericarp sectors were planted, and heritable *P1-ww* mutant alleles were obtained following the genetic screen described by Athma and Peterson (1991).

[b] Ratio of *P1-ww* from recombination = (number of *P1-ww* mutants derived by homologous recombination)/(the total number of *P1-ww* mutants).

[c] The low numbers of *P1-ww* mutants recovered from *P1-rr-11:666* and *P1-9D47B* alleles are due to two effects: First, the efficiency with which mutant alleles can be recovered from a mutant sector is proportional to the size of the sector (Anderson and Eyster 1928); the mutant sectors from the *P1-rr-11:666* and *P1-9D47B* alleles were generally smaller than those produced by the other alleles, and hence fewer heritable mutations were obtained. Second, among the mutations that were obtained from these alleles, most produced a variegated phenotype associated with transposition of *Ac* to another site in the *p1* gene (Athma *et al.* 1992).

shows no significant effect of *Ac* orientation on the frequency of homologous recombination (Table 4).

DISCUSSION

The aim of this study is to test the influence of the position and orientation of *Ac* elements inserted at the maize *p1* locus on the induction of homologous recombination in nearby sequences. The results indicate that *Ac* insertions in the 8.2-kbp interval between the 5.2-kbp direct repeats can significantly increase recombination frequencies when compared to insertion within or outside of the direct repeats. These results were initially observed in a somatic assay and were even more pronounced when germinal mutations were examined. Here, we discuss the implications of these results for proposed mechanisms of DSB repair and transposon-induced recombination in plants.

Homologous recombination initiated by a double-strand break: In yeast, it is well documented that DNA DSBs promote mitotic recombination (Orr-Weaver *et al.* 1981; Osman *et al.* 1996). In plants, the frequency of intrachromosomal homologous recombination can be enhanced by X-ray, gamma-ray, and UV irradiation (Tovar and Lichtenstein 1992; Lebel *et al.* 1993; Puchta *et al.* 1995) and by chemical agents that induce DSBs, such as methylmethanesulfonate and mitomycin C (Puchta *et al.* 1995). Moreover, homologous recombination in plants is enhanced by *in vivo* induction of DNA double-strand breaks by a site-specific endonuclease (Puchta *et al.* 1993; Chiurazzi *et al.* 1996). In Drosophila, the *P* element can undergo precise excision in a homology-dependent process requiring P transposase (Engels *et al.* 1990). In *Caenorhabditis elegans*, the frequency of precise reversion following excision of the *Tc1* element is much higher when heterozygous with a wild-type homologous chromosome (Plasterk 1991). Both of these examples indicate that DSBs generated by transposon excision can be repaired using the homologous locus as a template. The precise mechanism of *Ac/Ds* transposition is unknown, but genetic evidence indicates that it occurs via a "cut and paste" mechanism (Greenblatt and Brink 1962; Chen *et al.* 1992). Together, these studies are consistent with the hypothesis that transposon-induced homologous recombination is initiated by a DNA double-strand break generated by transposon excision.

Mechanism of transposon-induced recombination in plants: *Ac* insertion site and recombination frequency: A substrate with two direct repeats separated by an internal sequence was used to study DSB-induced intrachromosomal recombination in *S. cerevisiae*. The recombination frequency was >10 times higher when the DSB was induced in the internal sequence than at a site outside the repeats. In both cases, >90% of the recombinants were of the deletion type, in which the internal sequence was deleted but one direct repeat was retained (Prado and Aguilera 1995). This recombination product is similar to what we observed at the maize *p1* locus. Similar results were obtained in the yeast *Schizosaccharomyces pombe*, where 99.8% recombinants were of the deletion type when the DSB was generated between the direct

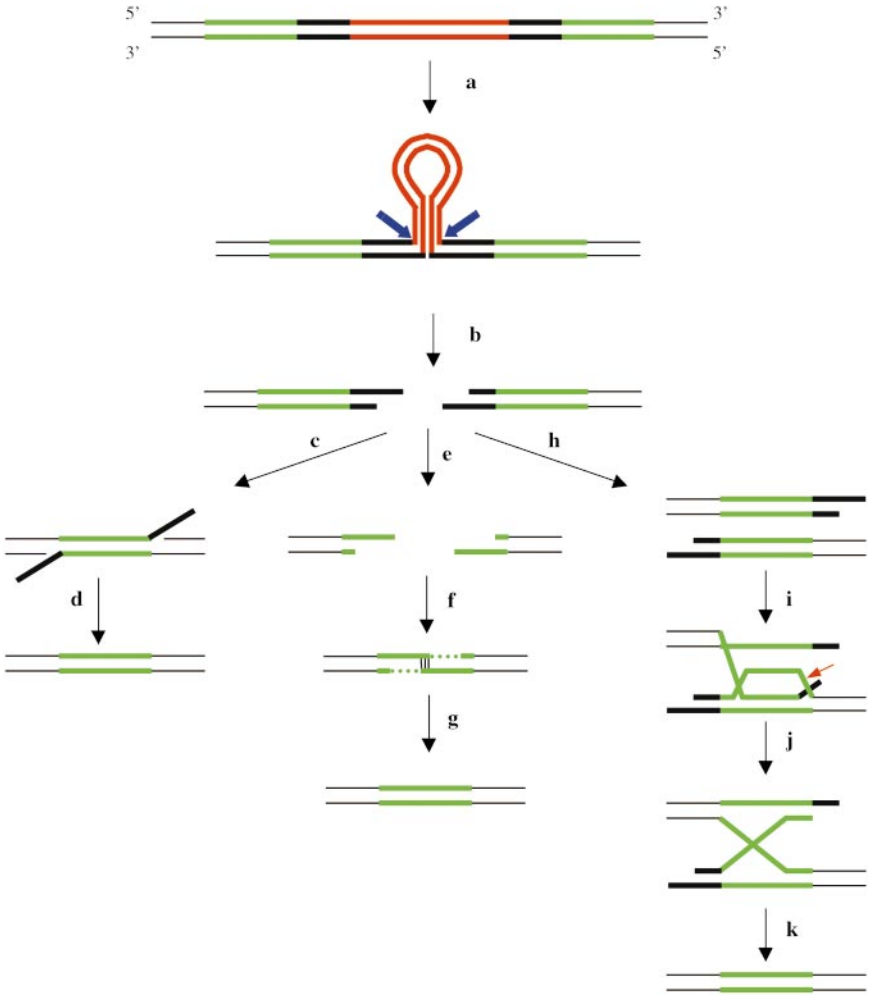

Figure 4.—Models for transposon-induced homologous recombination. Red lines represent the inserted transposon; green lines, homologous direct repeats; thick black lines, sequences internal to the direct repeats; thin black lines, other genomic sequences. (a) Transposase binds to the terminal and subterminal regions of transposon causing the association of the transposon ends and generating a DNA double-strand break. (b) Exonuclease degrades from 5′ to 3′ at the DSB site and generates 3′ single-strand DNA. The transient DSB is repaired by one of three pathways: single-strand annealing (SSA; c and d); annealing-dependent synthesis (ADS; e–g); one-sided invasion (OSI; h–k). (c) The 5′ to 3′ degradation reaches the homologous region and two 3′ single-strand homologous sequences pair with each other. (d) Heterologous DNA is digested by nuclease and gaps are ligated. (e) Both strands of DSB are degraded by exonucleases that process the 5′ end faster than the 3′ end to form a 3′ overhang. (f and g) Once 3′ end degradation reaches homologous regions, complementary strands can anneal with each other to prime DNA synthesis and fill the remaining gaps. Green dotted lines indicate newly synthesized strands. (i–k) The 3′ end invades the homologous repeat copy. The heterologous DNA at the 3′ end is removed by nuclease. (j) D-loop is nicked at the red arrow, and DNA synthesis follows to generate a Holliday structure. (k) Resolution of the Holliday junction results in deletion of the *p1* gene and retention of one copy of the direct repeat sequence.

repeats, whereas if the DSBs were generated within one repeat, the deletions were reduced to 77% (Osman *et al.* 1996). These deletions were proposed to occur via the single-strand annealing (SSA) pathway (Lin *et al.* 1984). The SSA model proposes that the free 5′ ends produced by a DSB are degraded by a 5′ to 3′ exonuclease, exposing regions of homology on both sides of the break. The 3′ single strands anneal at complementary regions, and the protruding nonhomologous ends are removed by a flap endonuclease (Figure 4, steps c and d). According to this model, the reduction in deletion frequency observed when a DSB is induced within the repeat sequences reflects the reduced chance that a free 3′ single-stranded DNA end generated by 5′ to 3′ exonucleolytic digestion will find its complementary counterpart, due to the nonsymmetrical position of the DSB.

In the case of the maize *p1* locus, the interval between the two flanking repeats is ~8.2 kbp. According to the SSA model, induction of a DSB at a site that is more centrally located within this interval would be expected to produce a higher frequency of recombination of the homologous flanking repeats compared to sites that are closer to one side of the interval, assuming that exonucleolytic degradation proceeds in both directions from the DSB at approximately equal rates. However, the recombination frequency of *P1-ovov-Val* is not significantly different from that of *P1-ovov-12:1-1* and *P1-ovov-1114* (Table 2), even though the locations of the *Ac* insertion alleles are very different: the *Ac* insertion site of allele *P1-ovov-Val* is 979 bp downstream of the end of the 5′ direct repeat of the *p1* gene, while the *Ac* insertion sites of alleles *P1-ovov-1114* and *P1-ovov-12:1-1* are 5.4 kb downstream of the end of the 5′ direct repeat of the *p1* gene. Recombination in allele *P1-ovov-Val* via the SSA model would necessitate degradation of >12 kbp on the downstream side of the DSB to expose a complementary sequence for pairing with the upstream repeat.

A variation of the SSA model is termed synthesis-dependent strand annealing (SDSA) (Formosa and Alberts 1986). In this model, the free 3′ ends resulting from a DSB invade a homologous duplex and act as primers for DNA synthesis. The newly synthesized DNA strands anneal with each other, followed by further DNA synthesis to fill remaining gaps. On the basis of the SDSA

model, we propose a model of annealing-dependent synthesis (ADS). Following formation of a DSB, both broken ends are degraded by exonucleases that process the 5′ end more rapidly than the 3′ end, resulting in 3′ overhanging strands on both sides of the break (Figure 4e). Once the 3′ end degradation reaches a region of homology (in the *p1* locus within the flanking 5.2-kbp direct repeats), the complementary strands can anneal with each other, and the 3′ ends prime DNA synthesis to fill remaining gaps (Figure 4, f and g). This ADS model can account for the observed generation of the *p1* mutants containing a single copy of the 5.2-kbp direct repeat sequence.

An alternative pathway for the repair of DSBs has been termed one-sided invasion (OSI; Belmaaza and Chartrand 1994); the OSI mechanism appears to be a major pathway in plant somatic recombination (Puchta *et al.* 1996; Puchta 1998). To explain the formation of *p1* gene deletions, the OSI model proposes that a free 3′ end generated by DSB would invade the opposite duplex. Complementary regions within the homologous repeat sequences would pair, and the nonhomologous 3′ end sequences would be removed by a flap endonuclease. Resolution of the resulting Holliday junction would result in deletion of the interval region and one repeat (Figure 4, h–k). A potential complication in the case of the *p1* locus is that the invading 3′ ends may have several kilobase pairs of heterologous sequence arising from the interval between the direct repeats, and these heterologous sequences could affect the homology search and pairing with the downstream repeat sequences.

The behavior of an allele that contains *Ac* inserted within the direct repeat sequences provides a test of the predictions of the ADS and OSI models. Formation of a DSB by excision of *Ac* from the upstream direct repeat sequence would generate a free end homologous to the downstream direct repeat, and this should be the most efficient substrate for the OSI model, whereas the same free end would be a less efficient substrate for the ADS model: Exonucleolytic degradation in both directions from the DSB would likely result in deletion of the remaining ~1 kbp portion of the upstream 5′ flanking repeat before the degradation of the downstream strand had proceeded the 12 kbp required to expose the complementary region in the downstream repeat sequence. The observed low frequency of deletions generated by the *P1-9D47B* allele is consistent with the expectations of the ADS model, but not the OSI model.

It should be noted that our ability to detect recombination events is based on a screen for loss of *p1* expression; hence, we would not have detected gene conversion events that restore *p1* function, if they did occur. Molecular analysis indicates that *Ds* elements may arise via double-strand gap repair following *Ac* transposition (Rubin and Levy 1997; Yan *et al.* 1999); however, such repair synthesis appears to be rare compared to the frequency of simple end-joining following *Ac* excision, regardless of whether homologous sequences are present *in cis* or *in trans* (Dooner and Martinez-Ferez 1997). Relevant to this study, Dooner and Martinez-Ferez (1997) observed no cases of conversion of the *Ac* insertion site by the adjacent duplication in the *bz(Dp26)-m1* allele, which is structurally similar to *P1-9D47B* (*i.e.*, a *cis* duplication with *Ac* inserted in one repeat copy). This suggests that gene conversion events that restore *p1* function, if they occur, are probably too infrequent to account for the low frequency of recombination observed in the *P1-9D47B* allele.

How do transposons stimulate recombination? In addition to the importance of a DSB, transposon-induced recombination may involve the recruitment of host factors by transposase. Our data from transgenic Arabidopsis show that active transposons can greatly increase intrachromosomal homologous recombination (>1000-fold higher than control; Xiao and Peterson 2000). This frequency is 10–100 times higher than that observed for direct induction of a DSB (Chiurazzi *et al.* 1996). One possible explanation for this high level of enhancement is that the efficiency of generating DSBs is different in these two experiments. Another explanation is that *Ac* transposase might recruit host factors that promote homologous recombination. There is some evidence that host plant factors are involved in transposition: First, host proteins that bind subterminal regions of *Ac* elements were found in both tobacco and maize (Becker and Kunze 1996; Levy *et al.* 1996). Second, a recessive mutation found in Arabidopsis can increase the frequency of *Ac* transposition (Jarvis *et al.* 1997). Possibly, the *Ac* transposition process may recruit host factors required for a nonhomologous end-joining and/or homologous recombination. Following transposition, a DSB generated by transposon excision may be repaired by alternative pathways depending on the sequences or chromatin structure near the break (Scott *et al.* 1996). Further experiments will be required to elucidate the relative roles of DSBs, host factors, and chromatin structure in transposon-induced recombination.

The observation that *Ac* induces recombination between directly duplicated sequences contrasts with a recent report that *Ac* does not stimulate homologous meiotic recombination in the maize *bz1* gene (Dooner and Martinez-Ferez 1997). To reconcile these apparently conflicting results, it is important to note that the transposon-induced recombination events we observed in maize (this report) and Arabidopsis (Xiao and Peterson 2000) are premeiotic. That is, these events occurred during sporophytic development to generate clonal sectors of genetically distinct cells. If these somatic sectors of mutant cells are included in the lineage that gives rise to the gametophytes, the premeiotic mutations can be transmitted to the next ge-

neration. In contrast, the events studied by Dooner and Martinez-Ferez (1997) appeared to occur at or near meiosis. The lack of effect of *Ac* excision on meiotic recombination may be due to temporal or spatial differences in the occurrence of *Ac* transposition relative to meiotic recombination. For example, *Ac* transposition is known to occur predominantly during or shortly after DNA replication (Greenblatt and Brink 1962; Chen *et al.* 1992), whereas meiotic recombination occurs after pairing of homologous chromosomes. Alternatively, *Ac* transposase may be spatially excluded from chromosomal regions that are undergoing meiotic recombination.

Anomalous mutation frequency of the *P1-9D36A* allele: Although the *Ac* insertion position in the *P1-9D36A* allele is between the two direct repeats, its colorless sector frequency is second lowest among all alleles. Nevertheless, the germinal recombination frequency of this allele is similar to that of other alleles with *Ac* inserted between the repeats. Thus, the *P1-9D36A* allele is suppressed in frequency of somatic mutations, yet it has a normal germinal mutation frequency. This mutation bias is opposite to that effected by a dominant mutation that suppresses germinal reversion of an allele of the maize *waxy* gene with a *Ds* insertion (Eisses *et al.* 1997). The observed low somatic mutation rate of the *P1-9D36A* allele could result from suppression of the activity of the *Ac* transposon. It has been demonstrated that the *Ac* element activity can vary depending on the *Ac* copy number and location (McClintock 1964; Heinlein 1995; for review see Fedoroff and Chandler 1994). Each of the alleles studied here was crossed to a *r-sc:m-3 Ds* tester stock; in this test, multiple copies of *Ac* elements delay the occurrence of *Ds* excision, resulting in small colored revertant sectors in the kernel aleurone (Kermicle 1980; Schwartz 1984). *Ac* activity of the *P1-9D36A* allele in the aleurone appeared normal; thus, suppression of *Ac* activity in pericarp cells, if it occurred, would have had to occur without affecting *Ac* activity in aleurone cells. *Ac* activity has also been shown to be inversely correlated with *Ac* methylation (Schwartz and Dennis 1986; Chomet *et al.* 1987; Schwartz 1989; Brutnell and Dellaporta 1994). However, Southern hybridizations show that the *Bam*HI site at the *Ac* 5′ end is unmethylated in the *P1-9D36A* allele (not shown); moreover, the *Ac* insertion in *P1-9D36A* is located in a region of the *p1* gene that is relatively free of DNA methylation (Das and Messing 1994; Chopra *et al.* 1998). Therefore, the low frequency of colorless sectors in the *P1-9D36A* allele cannot be attributed to increased methylation of the *Ac* element, nor to the proximity of a hypermethylated region in the *p1* locus. The basis for the anomalous somatic sector frequency of *P1-9D36A* remains to be determined.

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