Spatial Configuration of Transposable Element Ac Termini Affects Their Ability to Induce Chromosomal Breakage in Maize

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Composite or closely linked maize (Zea mays) Ac/Ds transposable elements can induce chromosome breakage, but the precise configurations of Ac/Ds elements that can lead to chromosome breakage are not completely defined. Here, we determined the structures and chromosome breakage properties of 15 maize p1 alleles: each allele contains a fixed fractured Ac (fAc) element and a closely linked full-length Ac at various flanking sites. Our results show that pairs of Ac/fAc elements in which the termini of different elements are in direct or reverse orientation can induce chromosome breakage. By contrast, no chromosome breakage is observed with alleles containing pairs of Ac/fAc elements in which the external termini of the paired elements can function as a macrotransposon. Among the structures that can lead to chromosome breaks, breakage frequency is inversely correlated with the distance between the interacting Ac/Ds termini. These results provide new insight into the mechanism of transposition-induced chromosome breakage, which is one outcome of the chromosome-restructuring ability of alternative transposition events.

INTRODUCTION

McClintock discovered the Activator/Dissociation (Ac/Ds) transposable element system during her investigation of chromosome breakage in maize (Zea mays) in the late 1940s (McClintock, 1946, 1947, 1948, 1949, 1950). She identified one stock in which chromosome breakage occurred frequently at a particular site in the short arm of maize chromosome 9; she designated this locus Dissociation (Ds) due to its ability to break chromosomes. She also found that chromosome breakage at Ds requires the presence of another factor, Activator (Ac), in the genome. In the presence of Ac, chromosome-breaking Ds elements (Ds, state I) can convert to a different state (Ds, state II) that rarely causes chromosome breakage (McClintock, 1949). State II Ds elements have been cloned from a number of loci; sequence analysis revealed that many of these were internally deleted versions of Ac (Weil et al., 1992; Yan et al., 1999; Conrad et al., 2007). A smaller number of State I Ds elements have also been cloned; their structures include doubleDs (a state II Ds inserted into another copy of the same element in the opposite orientation; Doring et al., 1984; Weck et al., 1984) and sesquiDs (an internal deletion derivative of doubleDs; Martinez-Ferez and Dooner, 1997).

The Ac element is 4565 bp in length and encodes a 3.5-kb mRNA that can be translated to an 807-amino acid transposase. The transposase uses a pair of Ac/Ds 5’ and 3’ ends as substrates; two Ac 5’ ends or two Ac 3’ ends are not capable of transposition. Functional tests showed that 238 and 209 bp from the Ac 5’ and 3’ ends, respectively, are required for efficient Ac/Ds excision (Coupland et al., 1988, 1989). These sequences contain 11-bp terminal inverted repeats and multiple copies of subterminal hexamer motifs (AAACGG or similar) to which the Ac transposase binds (Kunze and Starlinger, 1989; Bravo-Angel et al., 1995; Becker and Kunze, 1996, 1997; Kunze and Weil, 2002). Ac/Ds elements transpose via a cut and paste mechanism: the donor element is excised physically and usually reintegrates at a new location in the genome. The Ac/Ds element excision often leaves a footprint (minor sequence change) at the donor site, and reinsertions are flanked by 8-bp target site duplications at the new locus (Kunze and Weil, 2002).

By PCR and sequence analyses, English et al. (1993) and Weil and Wessler (1993) showed that doubleDs or two Ds elements in opposite orientation can cause chromosome breakage via alternative transposition reactions involving a 5’ Ds end from one chromatid and a 3’ Ds end from the sister chromatid. Excision of these Ds ends and ligation of the sequences flanking them leads to formation of a chromatid bridge, which is broken in the next mitotic division. Dissection of the doubleDs structure and functional testing in tobacco (Nicotiana tabacum) showed that a pair of 5’ and 3’ Ds termini in direct orientation are sufficient to mediate chromosome breakage (English et al., 1993, 1995); the same configuration is present in chromosome-breaking
structures, including doubleDs, sesquiDs (Martinez-Ferez and Dooner, 1997), and twoDs elements in opposite orientation in the maize wx1 locus (Weil and Wessler, 1993). In addition, some pairs of closely linked Ac/Ds or Ac/fAc (fracturedAc; a terminally deleted sequence containing only the 5′ or 3′ end of Ac) elements near the maize bz1 locus can also induce chromosome breakage, but their relative orientations were unknown (Ralston et al., 1989; Dooner and Belachew, 1991; Weil and Wessler, 1993; Martinez-Ferez and Dooner, 1997). A model for chromosome breakage based on transposition of a partially replicated macrotransposon was proposed (Ralston et al., 1989).

To determine how the orientation of Ac/Ds termini can affect chromosome breakage, we isolated and studied maize alleles that contain multiple transposition insertions in and near the maize p1 locus. The p1 gene encodes a Myb-like transcription activator that regulates the synthesis of red phlobaphene pigments in maize floral organs, including kernel pericarp and cob glumes. The patterns of expression in pericarp and cob glumes are indicated by the p1 allele suffix, for example, P1-rr specifies red kernel pericarp and red cob, P1-ww specifies white (colorless) pericarp and white cob, and P1-wr specifies white pericarp and red cob. Insertion of the Ac element into a P1-rr allele can produce P1-vv (specifies variegated pericarp and variegated cob color) or P1-ovov (specifies orange variegated pericarp and orange variegated cob) alleles (Athma et al., 1992). Aided by the tendency of Ac/Ds elements to undergo transposition to closely linked sites (Greenblatt 1984), we isolated a series of 15 maize p1 alleles that contain various configurations of Ac termini. For each allele, the frequency of chromosome breakage was estimated by loss of a distal marker gene. In addition, the presence of chromosome bridges and fragments indicative of chromosome breakage was confirmed in a subset of alleles. The results provide new insight into the mechanism of Ac/Ds-induced chromosome breakage and the configurations of Ac/Ds termini that can undergo alternative transposition reactions and thereby potentially alter genome structure.

RESULTS

Isolation and Molecular Analysis of New Maize p1 Alleles

The maize p1-vv9D9A allele contains a full-length Ac insertion and a fractured Ac (fAc; 2039 bp of the 3′ portion of the Ac element) inserted in intron 2 of the p1 gene (Figure 1A). The Ac element is located 112 bp downstream of the fAc in p1-vv9D9A, and the 3′ end of fAc and the 5′ end of Ac are in direct orientation (Zhang and Peterson, 1999, 2005). P1-nr11 is derived from p1-vv9D9A by transposition of Ac to a site 13,175 bp upstream of fAc, and insertion such that the 5′ end of Ac and the 3′ end of fAc are in reversed orientation (Zhang and Peterson, 2004; Zhang et al., 2006; Figures 1A and 1B). To test how the spatial configuration of a pair of Ac/Ds ends affects chromosome breakage, we studied a series of new p1 alleles in which Ac had transposed to a new location in or near the p1 gene. We hypothesized that changes in position and/or orientation of Ac would affect p1 expression and thus could be detected as changes in the kernel pericarp pigmentation phenotype.

From p1-vv9D9A (specifies variegated pericarp), we selected 28 new unstable P1-rr alleles and one P1-ovov allele (with unstable red and orange variegated pericarp, respectively); from P1-nr11 (unstable red pericarp), we obtained four new p1-vv alleles (variegated pericarp) (Figure 1A). We then used a gel-based polymorphism assay termed transposon-display (Van den Broeck et al., 1998) to determine the positions of the transposed Ac elements in each new allele. Briefly, genomic DNA from plants containing each allele was endonuclease digested, ligated with adaptor oligonucleotides, and PCR amplified using oligonucleotide primers complementary to Ac/Ds subterminal and adaptor sequences (see Methods). The PCR products produced by each allele were analyzed by PAGE; polymorphic bands representing potential newly transposed Ac elements were identified by comparison with the banding patterns produced by stocks containing the progenitor alleles (p1-vv9D9A and P1-nr11) or background reference alleles (p1-ww and p1-wr). Polymorphic bands (one to four bands from each allele; average size ~300 bp) were excised from the gel and sequenced.

As expected, all of the p1-vv- and P1-ovov-type derivative alleles contain an Ac insertion within the p1 gene, whereas 7 of 27 P1-rr alleles analyzed contain an Ac insertion in the vicinity of the p1 gene (P1-nr904, P1-nr459, P1-nr910, P1-nr905, P1-nr908, P1-nr458, and P1-nr460; Figure 1B; see Supplemental Data Set 1 online). PCR analysis confirmed that all of the alleles contained the original fAc insertion in the same location as that in p1-vv9D9A. The results of transposon display were confirmed by PCR analysis using primers complementary to Ac and the Ac-flanking sequences and by genomic DNA gel blot analysis (see Supplemental Figure 1 online). Among the remaining 20 P1-rr alleles analyzed, five alleles did not exhibit polymorphic bands; 10 alleles yielded polymorphic bands, but sequencing indicated that these represented Ac insertions outside the 36 kb flanking the p1 gene, and five alleles likely represented somatic Ac insertions (see Methods).

Altogether, we determined the locations and orientations of transposed Ac elements present in 15 different p1 alleles (Figure 1B). The 3′ end of fAc and the 5′ end of Ac are in direct orientation in five alleles (P1-nr459, P1-vv577, P1-vv595, P1-vv1171, and P1-vv1172) and in reversed orientation in two alleles (P1-nr910 and P1-ovov454). Additionally, five alleles (P1-ovov455, P1-nr905, P1-nr908, P1-nr458, and P1-nr460) have the transposed Ac situated downstream of the fAc element, such that the 5′ end of Ac and the 3′ end of fAc could function together as a macrotransposon. Closely linked pairs of Ac/Ds elements in the maize bz1 locus have been shown previously to undergo macrotransposition (Dowe et al., 1990; Huang and Dooner, 2008). The five potential macrotransposons we identified at the maize p1 locus would range in size from 6.8 to 21.7 kb and would contain the 2039-bp fAc element, the 4565-bp full-length Ac, and various segments of included p1 genomic sequence. Finally, P1-nr904 has a complex structure consisting of a full-length Ac insertion upstream of the p1 gene and two fAc insertions in intron 2 of the p1 gene: the original fAc in the same position as in p1-vv9D9A and a second, new fAc containing only the 685 bp 5′ portion of Ac. The second fAc is located in the same position as that of the full-length Ac in p1-vv9D9A. Thus, the P1-nr904 allele contains both reverse- and direct-oriented Ac ends.
Acrotermini in Either Direct or Reversed Orientation Can Cause Chromosome Breakage

The maize dek1 gene is required for differentiation of the kernel aleurone, the outermost single-cell layer of the endosperm (Lid et al., 2002). Maize stocks containing functional alleles of the Dek1, C1, and R1 genes produce dark purple colored aleurone cells. The Dek1 gene is located on chromosome 1S, 0.8 Mb distal to p1; hence, a break at the p1 locus will result in loss of Dek1 and generate a colorless aleurone sector. To test whether the new p1 alleles described here can induce chromosome breakage, pollen from plants homozygous for each p1 allele was crossed onto silks of tester plants of genotype dek1/Dek1 C1 R1 and the kernels visually checked for colorless sectors (dekk1/Dek1 heterozygous plants were used because homozygous dek1 plants are severely stunted). Frequent colorless sectors characteristic of Dek1 loss were observed on the ears produced in crosses with certain p1 alleles (Figure 2). These include alleles with Ac and fAc present in either direct orientation (P1-rr459, p1-vv577, p1-vv1171, p1-vv1172, p1-vv595, and p1-vv9D9A) and in reversed orientation (P1-rr11, P1-rr910, and P1-ovov454) (Table 1). These results suggest that the presence of an Ac 5' end and Ac 3' end in either direct or reversed orientation is sufficient to induce chromosome breakage.

Additional crosses with dekk1/Dek1 testers were done using plants of three other genotypes: (1) p1-vv, (2) P1-rr::fAc, and (3) P1-rr::fAc, r1-navajo::Ac. The p1-vv allele contains a single Ac insertion in p1 (Emerson, 1917; Xiao and Peterson, 2002), the P1-rr::fAc allele contains a single fAc insertion in P1 (derived from p1-vv9D9A by excision of Ac), and the P1-rr::fAc, r1-navajo::Ac stock contains the preceding fAc insertion plus a second one in r1 (Dellaporta et al., 1988). The cross with p1-vv tests whether a single Ac insertion in the p1 gene can induce chromosome breakage, while the cross with P1-rr::fAc and P1-rr::fAc, r1-navajo::Ac indicates whether fAc can induce breaks, either alone or in the presence of an unlinked Ac element. Previous studies have shown that a single Ac element does not induce chromosome breakage at a significant frequency (Dooner and Belachew, 1991). Our results further confirm this conclusion, as no colorless sectors were observed on the dek1/Dek1 ears crossed with p1-vv. In addition, no evidence of chromosome breaks was observed with fAc alone or fAc in the presence of an Ac element in trans.

Ac Macrotransposition Does Not Induce Chromosome Breakage

Interestingly, five p1 alleles (P1-ovov455, P1-rr905, P1-rr908, P1-rr458, and P1-rr460) that contain both a fAc and a nearby

Figure 1. Ac Transposition Generates Multiple p1 Alleles.

(A) Kernel phenotypes of representative p1 alleles and their molecular structures. The solid black boxes indicate p1 gene exons 1, 2, and 3 (left to right). The thick red lines represent Ac (two arrowheads) or fAc (one arrowhead). The closed and open red arrowheads represent the 5' and 3' termini of Ac/fAc, respectively.

(B) Locations of Ac and fAc insertions in p1 alleles used in this study. The solid black boxes indicate p1 gene exons 1, 2, and 3 (left to right). Triangles with allele numbers represent Ac/fAc insertion sites in each allele. Open triangles indicate Ac elements in the same transcriptional orientation as the p1 gene (5' to 3', left to right); closed triangles indicate Ac insertions in the opposite orientation. The P1-rr904 allele is marked with an asterisk because it contains an intact Ac insertion and an additional 685 bp fAc insertion in the same position as that of the Ac element in p1-vv9D9A.
transposed Ac did not elicit chromosome breakage. These alleles differ from the previous cases in that the transposed Ac is inserted downstream of the fAc element, with the 3' ends of both Ac and fAc in the same orientation. In this configuration, the Ac 5' end could potentially interact with the fAc 3' end to form a macrotransposon. To test whether macrotransposition could be detected in somatic cells, we performed PCR analysis using oligonucleotide primers flanking each potential macrotransposon. Primers PA-A13 and PP1' flank the putative 6.8-kb macrotransposon in P1-ovov455 (Figure 3A). As shown in Figure 3B, PCR of P1-ovov455 genomic DNA (lane 2) produced a 0.74-kb band, which matches the size expected following macrotransposon excision. Similarly, we detected bands of 0.36, 1.15, and 1.44 kb representing the predicted products of excision of the 9.7-, 16.5-, and 21.7-kb macrotransposons in P1-rr905, P1-rr458, and P1-rr460 alleles, respectively (Figure 3, lanes 5, 7, and 9). A 1.57-kb band expected from macrotransposon excision in the P1-rr908 allele (lane 4) was not detected, possibly due to inefficient PCR amplification. In addition to detecting macrotransposon excision in somatic DNA, we also isolated one allele containing a germinal excision of the 9.8-kb macrotransposon in the P1-rr905 allele. This derivative allele was of P1-ww phenotype, as would be expected because macrotransposon excision would result in deletion of the p1 gene exon 3. Together, these results are consistent with previous reports of excision of Ac/Ds macrotransposons of various sizes (Dowe et al., 1990; Huang and Dooner, 2008). Importantly, our results show that macrotransposition per se is not sufficient to induce chromosome breakage.

The Influence of Distance on Chromosome Breakage

In previous analyses of chromosome breakage in maize, researchers have counted the numbers of individual marker-loss sectors appearing on a particular tissue, such as kernel aleurone (Dooner and Belachew, 1991; Weil and Wessler, 1993). However, this method was unsuitable for our materials because some alleles exhibit extremely high levels of sectors that preclude accurate quantitation (Figure 2, Grades 3 and 4). Precise quantitation is also confounded by the fact that breakage events that occur early in development produce large Dek1-loss sectors within which no additional events can be scored. A similar problem was addressed by Emerson (1929) in analyzing the frequency of somatic mutation in variegated pericarp maize. Emerson proposed that variegation grades be established by comparison of experimental ears with a set of standard ears.

### Table 1. Chromosome Breakage Frequency Exhibited by Different Alleles

<table>
<thead>
<tr>
<th>Allele Name</th>
<th>Distance between the 3' End and the 5' End of Ac/fAc (nt)</th>
<th>Individual Ear Breakage (Grade Total Ear Number)</th>
<th>Average Breakage Grade (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Directly oriented</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac/fAc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1-r904</td>
<td>6,809</td>
<td>0,0,0,0,0,0,0,0 (8)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>P1-rr455</td>
<td>17,840</td>
<td>2,1,1,1,1 (4)</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td>p1-w577</td>
<td>13,175</td>
<td>1,1,1,1,1,1 (6)</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>p1-w1171</td>
<td>16,538</td>
<td>1,1,1,1,1 (4)</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>p1-w1172</td>
<td>21,741</td>
<td>1,1,1,1,1 (4)</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>p1-w959</td>
<td>823</td>
<td>3,3,3,3,3,3,3 (7)</td>
<td>3.0 (0.0)</td>
</tr>
<tr>
<td>p1-w9D9A</td>
<td>8919</td>
<td>2,2,2,2,2,2,2,2,2 (8)</td>
<td>2.0 (0.0)</td>
</tr>
</tbody>
</table>

| Reversely oriented | | | |
| Ac/fAc | | | |
| P1-rr11 | 8919 | 2,2,2,2,2,2,2,2,2 (8) | 2.0 (0.0) |
| P1-rr910 | 13,175 | 1,1,1,1,1,1 (6) | 1.0 (0.0) |
| P1-ovov454 | 16,538 | 1,1,1,1,1 (4) | 1.0 (0.0) |

| Macrotransposon | | | |
| P1-ovov455 | 9,774 | 0,0,0,0,0,0,0,0,0 (8) | 0.0 (0.0) |
| P1-rr905 | 16,060 | 0,0,0,0,0,0,0,0,0 (8) | 0.0 (0.0) |
| P1-rr908 | 16,538 | 0,0,0,0,0,0,0,0,0 (8) | 0.0 (0.0) |
| P1-rr458 | 21,741 | 0,0,0,0,0,0,0,0,0 (8) | 0.0 (0.0) |
| Control | | | |
| p1-wv | N/A | 0,0,0,0,0,0,0 (6) | 0.0 (0.0) |
| P1-r9459Ac | N/A | 0,0,0,0,0,0,0 (6) | 0.0 (0.0) |
| P1-r9459Ac,r1-nt::Ac | N/A | 0,0,0,0,0,0,0 (6) | 0.0 (0.0) |

| Others | | | |
| | | | |

*nt, nucleotides.*

**P1-rr904** contains an intact Ac insertion and an additional 685-bp fAc insertion in the same position as that of the Ac element in p1-wv9D9A. It has three possible pairs of Ac/fAc ends.
selected for relative variegation frequency. We employed a similar approach to estimate chromosome breakage frequency among the \textit{p1} alleles tested here. Ears produced by crossing each \textit{p1} allele with the \textit{dek1/Dek1} tester line were compared with a set of five standard ears that represented various grades of \textit{Dek1}-loss sector frequency. Multiple test cross ears from each allele were produced and scored on a graduated scale of 0 (no sectors) to 4 (most frequent sectors) (Figure 2). Sector frequencies were then related to configurations of element ends (direct or reversed) and distances between interacting ends (Table 1). Among the alleles with a pair of \textit{Ac/fAc} ends in direct orientation, \textit{p1-vv9D9A} exhibits a high relative frequency of colorless sectors (Grade 3.0); it also has the shortest distance between the \textit{Ac/fAc} termini (2151 bp). In comparison, allele \textit{P1-rr459} exhibits a low relative frequency of colorless sectors (Grade 1.3), and it has the greatest distance (17,840 bp) between the \textit{Ac/fAc} ends. The

\textbf{Figure 3.} Detection of Somatic Macrotransposon Excision by PCR. 

(A) Molecular structures of \textit{p1} alleles containing possible macrotransposons. Five alleles (\textit{P1-ovov455}, \textit{P1-rr905}, \textit{P1-rr908}, \textit{P1-rr458}, and \textit{P1-rr460}) have the \textit{Ac} situated downstream of the \textit{fAc} element, such that the 5′ end of \textit{Ac} and the 3′ end of \textit{fAc} could function together as a macrotransposon. The large closed and open arrowheads represent the 5′ and 3′ termini of \textit{Ac/fAc}. The positions of oligonucleotide primers used for PCR are indicated by the small arrows. The figure is drawn to scale, except that an 8.5-kb segment (indicated by / /), including the \textit{p1} 5′ region, exon 1, intron 1, exon 2, and part of intron 2, is not shown. 

(B) Results of PCR analysis of somatic macrotransposon excision. Primers used are shown at top in the two rows above the brackets. Alleles tested are shown above each lane: Pvv, \textit{p1-vv}; 455, \textit{P1-ovov455}; 11, \textit{P1-rr11}; 908, \textit{P1-rr908}; 458, \textit{P1-rr458}; 905, \textit{P1-rr905}; and 460, \textit{P1-rr460}. Total genomic DNA from young leaves of plants homozygous for the indicated alleles was used as template. Bands corresponding to expected macrotransposition excision products are indicated by arrows and apparent sizes. For each pair of primers, \textit{P1-rr11} is used as a negative control and to identify probable nonspecific or background amplification products, for example, the ~0.33-kb band observed in lanes 1, 2, and 3; the ~0.3-kb band in lane 3; the ~0.65-kb band in lane 8; and the 0.70-kb band in lanes 9 and 10. In addition, the standard \textit{p1-vv} allele, which contains a single \textit{Ac} element between the binding sites of primers PA-A13 and PP1′ (lanes 1 to 3), was used as a positive control. PCR of this allele produced a 0.80-kb band (lane 1), which is the size expected from \textit{Ac} excision.
same relationship is observed for alleles with Ac/fAc ends in reversed orientation (Table 1).

Overall, the chromosome breakage frequency is inversely related with the distance between the pair of Ac/fAc ends for both reverse- and direct-oriented Ac termini. This result is consistent with previous studies of chromosome breakage induced by Ac/Ds insertions in the vicinity of the maize bz1 locus (Dooner and Belachew, 1991). Among the p1 alleles tested here, the highest relative frequency of chromosome breakage is induced by P1-rr904, which contains, in addition to the original fAc and a transposed Ac, a second fAc insertion consisting of the 5' Ac end in direct orientation with the 3' fAc (Figure 1). Thus, P1-rr904 contains both direct and reversed Ac/fAc termini; possibly, the presence of both reversed and direct Ac/fAc ends, and/or of multiple ends in close proximity, may produce an additive effect on the chromosome breakage frequency (Table 1).

To determine whether alternative transposition between elements located on different chromosomes could be detected, we tested genotypes that combined a 3' fAc at the p1 locus on chromosome 1 with Ac elements at two different loci. The first was the r-najo-m1::Ac allele, which contains a full-length Ac element inserted in the r1 gene on chromosome 10 (Dellaporta et al., 1988). The second Ac source was the p1-vv5145 allele, which contains a trans-active, transposition-defective Ac element inserted in the p1 locus on the homolog. The p1-vv5145::Ac element has a 1-bp deletion in the Ac 3' terminal inverted repeat, but the 5' end is normal and so should be competent to participate in alternative transposition reactions (Xiao and Peterson, 2002). Because the P1-rr::fAc allele conditions red kernel pericarp, any alternative transposition events involving the 3' fAc in the p1 gene and either r-najo-m1::Ac or p1-vv5145::Ac should generate a deletion of P1-rr resulting in a colorless pericarp sector. Screens of ~100 ears of both genotypes yielded very few colorless sectors. One whole-kernel colorless sector was obtained from the P1-rr::fAc/- r-nj-m1/- screen, but molecular analysis showed no evidence that it originated by alternative transposition. We conclude that alternative transposition between pairs of unlinked elements is very rare, at least for the loci tested here.

### Cytogenetic Detection of Chromosome Breakage

Previous research has shown that Ac transposase can recognize directly oriented Ac 5' and 3' ends from different sister}

### Table 2. Frequencies of Telophase I Cells with a Bridge (B) and a Fragment (F)

<table>
<thead>
<tr>
<th>fAc Allele</th>
<th>Distance between Ac and fAc (kb)</th>
<th>No. of Cells with a B and F/Total No. of Cells</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-rr11 (two plants)</td>
<td>13.1</td>
<td>4/118</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3/114</td>
<td>2.7</td>
</tr>
<tr>
<td>P1-rr910 (two plants)</td>
<td>8.9</td>
<td>3/103</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6/110</td>
<td>5.5</td>
</tr>
<tr>
<td>P1-rr904 (three plants)</td>
<td>9.1</td>
<td>0/102</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/102</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/105</td>
<td>0.0</td>
</tr>
<tr>
<td>P1-rr459 (three plants)</td>
<td>17.8</td>
<td>3/120</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/108</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3/100</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The frequencies indicate the percentage of cells that have a bridge (B) and a fragment (F). Plants in which no cells with bridges and fragments were found may have been homozygous for normal chromosomes due to meiotic segregation in the previous generation. Alternatively, plants in which no cells with bridges and fragments were found may result from random variation due to the small sample size. The results of χ² statistical tests indicate that there is no significant difference in the frequency of chromosome breakage between any two alleles (P > 0.05) (see Supplemental Table 1 online).
chromatids as substrates for transposition; excision of these Ac ends is followed by ligation of the sister chromatids (English et al., 1993, 1995; Weil and Wessler, 1993; Martinez-Ferez and Dooner, 1997; Zhang and Peterson, 1999). The predicted junctions of sister chromatid sequences were detected in DNA of somatic tissues by PCR (Weil and Wessler, 1993) and in germinal derivatives containing inverted duplications (Zhang and Peterson, 1999); however, cytogenetic evidence for chromatid fusions has not been reported. Therefore, we examined chromosomes of pollen mother cells in plants containing five different alleles (p1-vv9D9A, P1-rr11, P1-rr910, P1-rr904, and P1-rr459) that showed Dek1-loss events. A significant proportion of the telophase I cells were found to contain chromatid bridges and acentric fragments (Figure 4; see Supplemental Figure 2 online). The proportion of cells containing bridges and fragments ranged from 2.5 to 5.5% (Table 2); in general, the bridge/fragment frequency appeared to be inversely proportional to the distance between the Ac and fAc elements. However, because the sample size was small, it cannot be concluded that this difference is actually correlated to the distance between the Ac and fAc elements. In some plants, no bridges and fragments were detected. This is most likely due to meiotic segregation: one parent of the plants analyzed was heterozygous for the chromosome-breaking allele and the other parent carried normal chromosomes. The plants in which no bridges or fragments were found may have been homozygous for normal chromosomes. In summary, the expectation that alternative transposition generates chromatid fusions that result in chromosome bridges and fragments is confirmed.

DISCUSSION

Researchers have previously identified and characterized chromosome-breaking (State I) Ds elements and a number of chromosome-breaking configurations of multiple linked Ac/Ds insertions. However, questions have remained regarding

![Figure 5. Chromosome Breakage Models.](image-url)

**A** Model for chromosome breakage by directly oriented Ac/Ds termini (sister chromatid transposition). The two lines indicate sister chromatids joined at the centromere (oval). The closed and open triangles represent the 5’ and 3’ termini of Ac/fAc. The black X indicates the footprint generated by transposition. (1) Ac transposase (open circles) recognizes the 3’ and 5’ termini of Ac/fAc on different sister chromatids. (2) Cleavage by transposase occurs at Ac or fAc termini. (3) The cleavage results in excision of the entire chromosome arm distal to the p1 gene. (4) A chromatid bridge is formed. The bridge will break in the subsequent anaphase. An acentric chromosome fragment is also produced.

**B** Model for chromosome breakage by reverse oriented Ac/Ds structure. (1) Ac transposase recognizes the reverse oriented 3’ and 5’ termini of Ac/fAc on the same chromatid. (2) Cleavage by transposase occurs at the Ac or fAc terminus. (3) Excised 5’ and 3’ termini of Ac/fAc insert into a target site in the sister chromatid (black arrow). (4) A chromatid bridge is formed. The bridge will break in the subsequent anaphase. An acentric chromosome fragment is also formed.

[See online article for color version of this figure.]
Chromosome Breakage by Ac/Ds Transposition

precisely which configurations of Ac/Ds termini are capable of inducing chromosome breakage. In particular, the potential role of macrotransposition in chromosome breakage has not been clear. Here, we used the unique advantages of the maize p1 locus to investigate the structural requirements for Ac/Ds-induced chromosome breakage. In previous work, we isolated an allele (p1-vv9D9A) that contains a full-length Ac element and an fAc element (2039 bp from the 3′ end of Ac) inserted in intron 2 of the p1 gene. Using a simple visual screen for changes in kernel pericarp pigmentation, we isolated an allelic series in which the Ac element in p1-vv9D9A had transposed to a nearby site, while the fAc remained fixed in position. In each allele, the 5′ end of Ac and the 3′ end of fAc are present in either direct or reversed orientation; in addition, some alleles contain segments of genomic DNA flanked by Ac/fAc elements that could potentially behave as macrotransposons. We estimated the frequency of chromosome breakage induced by each allele using a visual assessment of losses of the linked marker gene Dek1. Our results show that a pair of Ac ends in either tandem or reversed orientation can cause chromosome breakage and that the chromosome breakage frequency is inversely proportional to the distance separating the Ac termini. Interestingly, significant chromosome breakage was not detected when the Ac/fAc termini are present in macrotransposition configurations. In addition, we confirmed the presence of chromosome bridges and fragments in a subset of alleles using cytogenetic methods. These results help to define models for chromosome breakage based on alternative transposition reactions.

Chromosome Breakage Occurs Following Transposition-Induced Fusion of Sister Chromatids

Conventional transposition reactions involve the 5′ and 3′ termini of a single transposon. Macrotransposition reactions involve the external termini of a composite element (macrotransposon). The net result of conventional transposition or macrotransposition is a change in position of the element in the genome.

By contrast, alternative transposition reactions involve the 5′ and 3′ termini of different elements in either direct or reversed orientation. Alternative transposition can lead to major changes in genome structure (Huang and Dooner, 2008; Zhang et al., 2009). Our results show that alleles capable of undergoing alternative transposition reactions induce significant chromosome breakage as a direct consequence of fusion of sister chromatids. In the case of directly oriented Ac/fAc termini (sister chromatid transposition), excision of the element termini followed by ligation of the flanking sequences produces a covalent linkage between the two sister chromatids (Figure 5A). If the excised Ac ends fail to reinsert into the genome or reinsert at a site other than the chromatid bridge, the two chromomeres in the sister chromatids will move to opposite poles in the succeeding anaphase resulting in breakage of the chromatid bridge (Figure 5A).

In the case of reverse-oriented Ac/fAc termini, Ac transposase can recognize the 5′ and 3′ ends on the same chromatid as substrates (Zhang and Peterson, 2004; Zhang et al., 2006). In this scenario, insertion of the excised Ac ends into a site in the sister chromatid would form a chromatid bridge that would be broken in the subsequent anaphase (Figure 5B). In addition, insertions into other chromosomes can generate reciprocal translocations (Zhang et al., 2009), half of which would be expected to be dicentric. These latter cases would also be scored as chromosome breakage events, although the frequency of interchromosomal transposition is low (Huang and Dooner, 2008; Zhang et al., 2009).

The alternative transposition model proposes that chromatid fusion occurs as a direct and immediate consequence of sister chromatid transposition (Figure 5A); hence, all sister chromatid transpositions should generate chromatid bridges. The only exceptions would be those cases in which the excised transposon ends reinsert back into a proximal site in the chromatid bridge, in which case reciprocal deletion/duplication chromatids are generated (Zhang and Peterson, 1999). By contrast, only a subset of reversed Ac ends transposition events (i.e., those that reinsert into the sister chromatid) would produce chromatid or chromosome bridges (Figure 5B); hence, sister chromatid transposition should yield a higher frequency of chromosome breakage than reversed Ac ends transposition. However, we did not observe a significant difference in chromosome breakage frequencies from directly oriented Ac termini compared with reverse-oriented termini (Table 1). Possibly, this may be due in part to a tendency for the Dek1-loss assay to overestimate the frequency of chromosome breakage by reversed-ends transposition. Following reversed-ends transposition, the excised Ac ends could reinsert into sites distal to the dek1 locus. Depending on the orientation with which the transposon ends reinsert, either a deletion or inversion is generated (Zhang et al., 2006, 2009). Deletions would produce a Dek1-loss sector that would resemble a chromosome breakage event. In this way, loss of dek1 is not accompanied by chromosome breakage. In general, assays based on loss of a nearby marker gene would tend to overestimate the frequency of chromosome breakage induced by reversed-ends Ac transposition.

Cytogenetic Confirmation of Chromosome Bridges and Fragments

The marker-loss assays employed by us and others provide a facile method to estimate chromosome breakage frequencies.

### Table 3. Oligonucleotide Primers and Their Sequences

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Primer Sequence (5′–3′)</th>
</tr>
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<tbody>
<tr>
<td>cd-a1</td>
<td>GTATCCACCAGGAGGCAAGCGAGATTCAC AGAATTCAAGGAGAATGT</td>
</tr>
<tr>
<td>cd-a2</td>
<td>CGACTCTACTCTC</td>
</tr>
<tr>
<td>cd-p1</td>
<td>CCCAGAGGAGGAGAAGGCA</td>
</tr>
<tr>
<td>Ac264r</td>
<td>AATACGTTAACGAACACGGAAATCATC</td>
</tr>
<tr>
<td>cd-p2</td>
<td>CCAAGGAGGAGGAGGAGGTTCAC</td>
</tr>
<tr>
<td>Ac27r</td>
<td>GGATTTCCCATCTCTTTTTCATCCCTG</td>
</tr>
<tr>
<td>PA-A13</td>
<td>ATTTGGGATTCGGCCCTG</td>
</tr>
<tr>
<td>PP1′</td>
<td>GACAGCGATTCGGCCTC</td>
</tr>
<tr>
<td>P1-15588f</td>
<td>GCTATCAACAGGACAGGGAGAGAAT</td>
</tr>
<tr>
<td>462end5</td>
<td>GCTGAAATGTGGTTCGCGCCTT</td>
</tr>
<tr>
<td>EP3-7</td>
<td>CAGCGACTTAACAGGAGACGCGAAC</td>
</tr>
<tr>
<td>605P35986</td>
<td>GCAGCATCCCCGTTACGC</td>
</tr>
</tbody>
</table>
However, we felt that it was important to confirm, using cytogenetic methods, the presence of chromosome bridges and fragments predicted by the alternative transposition models. Initially, we examined mitotic cells in seedling root tips and did observe several examples of chromosome bridges in plants containing the p1-vv9D9A and P1-rn11 alleles. However, the highly condensed mitotic maize chromosomes were somewhat difficult to visualize. By contrast, maize microsporocytes (male meiotic cells) are much larger than mitotic cells, the chromosomes can be visualized in much greater detail, and homologous chromosomes are synapsed during the first meiotic division, thus greatly facilitating cytogenetic analyses. In the case of p1-vv9D9A, sister chromatid transposition of the directly oriented Ac termini would not by itself generate a chromatid bridge in anaphase I of meiosis because this division segregates homologous chromosomes, not sister chromatids. However, sister chromatid transposition followed by a crossover between homologous chromosomes anywhere in the region between the centromere and the p1 locus (a distance of ~50 centimorgans) would generate a bridge that would be apparent at the anaphase I stage. Similarly, reversed-end transposition followed by a proximal crossover between homologs could also generate a bridge at anaphase I. Interchromosomal transposition could also generate dicentric chromosomes, but this is expected to occur at a much lower frequency (Zhang et al., 2009). In summary, this study provides cytological evidence that alternative transposition events can produce bridges and fragments at anaphase I and telophase I of meiosis.

The Potential Use of Alternative Transposition in Maize

According to our results and those reported by Huang and Dooner (2008), Ac/Ds-induced chromosome breakage can be fully explained as a consequence of alternative transposition. Although alternative transposition reactions occur somewhat less often than standard transposition (Huang and Dooner, 2008; Zhang et al. 2009), alleles containing certain Ac/Ds configurations can induce high-frequency chromosome breakage (Figure 3, Grades 3 and 4). These alleles have also been shown to induce heritable deletions, duplications, inversions and translocations (rearrangements that may be useful for functional genomic analysis or chromosome engineering) (Zhang and Peterson, 1999, 2004, 2005; Huang and Dooner, 2008). Being 2500 Mb in size and rich in transposable elements, the maize genome is significantly larger and more complex than that of Arabidopsis thaliana or rice (Oryza sativa; Messing and Dooner, 2006; Rabinowicz and Bennetzen, 2006; Schnable et al., 2009). Collections of lines carrying sequence-tagged Ds insertions are currently being developed for functional genomics applications (Ahern et al., 2009).

Based on our results, we suggest that alternative transposition reactions may offer additional potential uses for these Ds insertion lines. Early genetic experiments in maize showed that Ac/Ds elements often transpose from a replicated donor site to a nearby unreplicated target site (Greenblatt and Brink, 1962; Greenblatt, 1984). This can generate pairs of closely linked elements that we predict should be competent to undergo alternative transposition events and induce chromosome breakage; such paired elements could be identified by screening for stocks showing loss of distal chromosome markers. A similar strategy was used by Neuffer (1995) to identify stocks containing chromosome-breaking double Ds elements inserted on 10 maize chromosome arms. In this way, pairs of closely linked Ac/Ds elements could be identified and used to generate region-specific chromosome rearrangements, including deletions, duplications, inversions, and translocations (Huang and Dooner, 2008; Zhang and Peterson, 1999, 2005; Zhang et al., 2009). The ability to generate these types of major chromosomal rearrangements would complement single-gene tagging approaches and thereby expand the utility of Ac/Ds insertion lines for functional genomics research.

METHODS

Maize Stocks

The maize (Zea mays) p1-vv9D9A allele, which is highly unstable, contains a complete Ac element and a terminally deleted Ac (Ac) in the second intron of the p1 gene (Zhang and Peterson, 1999). Excision of the intact Ac in p1-vv9D9A and reinsertion into sites nearby or within the p1 gene leads to the formation of other alleles, including P1-rn11, P1-rn910, and P1-ovov454. The r-m3::Ds allele contains a Ds element inserted in the r1 gene that is required for kernel aleurone pigmentation; Ac-induced excision of Ds from r-m3::Ds results in purple aleurone sectors (Kermicle, 1980).

Genomic DNA Extractions and DNA Gel Blot Hybridization

Young leaves of individual plants were ground in liquid nitrogen, and genomic DNA was extracted with CTAB (cetyltrimethylammonium bromide) reagent (Saghai-Maroof et al., 1984). Agarose gel electrophoresis and DNA gel blot hybridizations were performed according to Sambrook et al. (1989), except that hybridization buffers contained 250 mM NaHPO4, pH 7.2, and 7% SDS, and wash buffers contained 20 mM NaHPO4, pH 7.2, and 1% SDS. The p1 gene-specific probes 15, 6, and 8B have been described (Lechelt et al., 1989; Zhang and Peterson, 2004).

Transposon Display and PCR

Ac elements were mapped by a transposon display approach (Van den Broeck et al., 1998). Genomic DNA (5 μg) was digested with 10 units HinP1I or HpyCH4IV (Neb) at 37°C for 3 h. Oligonucleotide adaptors and primers and their sequences are shown in Table 3. Preammeadlated adaptors (cd-a1 and cd-a2; final concentration 5 mM), ATP (final concentration 1 mM), and 1 Weiss Unit ligase (NEB) were added to the reaction tubes and incubated for 8 h at room temperature. The ligation product was used directly for two rounds of PCR amplification (Eppendorf HotMaster Taq). Primers used in the first round of PCR are cd-p1 and Ac264r; primers used in the second round of PCR are cd-p2 and Ac27r. The PCR mix was heated at 94°C for 3 min to denature the DNA template, followed by 35 cycles of 20 s at 94°C, 30 s at 60°C, and 3 min at 65°C, and one cycle of 8 min at 65°C. PCR products (1 mL) were resolved on a denaturing 6% polyacrylamide-7 M urea gel at 1000 V for 4 h and then visualized by a silver stain detection system (Promega). Polymorphic bands were excised from the gel, recovered, and reamplified with cd-p2 and Ac27r. The PCR products were purified with the Pureflex gel cleanup system (Eppendorf HotMaster Taq) and sequenced by the DNA Synthesis and Sequencing Facility, Iowa State University. Among 27 new unstable red alleles analyzed in this way, seven alleles contained new heritable insertions within the p1 gene and are characterized in this article. Among the remaining 10 alleles, 10 alleles exhibited one to four polymorphic
bands, but sequencing of these bands indicated that the Ac insertions were outside the 36-kb region flanking the p1 gene and hence were not useful for this study because their exact distance and orientation with respect to fAc could not be determined. Five alleles showed no clear polymorphic band, and five alleles showed one or more polymorphic bands, indicating Ac insertions nearby the p1 gene. However, these latter five cases could not be confirmed by subsequent PCR or genomic DNA gel blot and probably represent somatic insertion events.

Cytogenetical Analysis

Immature tassels were collected from field-grown plants, fixed in a 3:1 mixture of 95% ethanol:propionic acid (v:v) at room temperature for 1 d, and then maintained at –20°C. The carmine smear technique was applied to developing sporocytes according to (Sharma and Sharma, 1965) to visualize the chromosomes in these cells.

Evaluation of Chromosome Breakage Frequency

The Dek1 gene is required for differentiation of maize kernel aleurone cells; endosperm cells lacking Dek1 function fail to differentiate into aleurone cells and thus cannot synthesize anthocyanin pigments (Lid et al., 2002). Wild-type Dek1 C1 R1 were crossed as ear parents by pollen from plants homozygous for the weak dek1-Dooner allele (Becraft et al., 2002) to generate a stock of heterozygous dek1-Dooner/Dek1 tester plants. Heterozygous dek1/Dek1 plants were used in test crosses because homozygous dek1-Dooner plants are very weak and produce few if any seed. The dek1/Dek1 plants were crossed as ear parents by pollen from plants homozygous for the set of standard ears and classified into one of five aleurone variegation grades ranging from 0 (no sectors) to 4 (most frequent sectors) as shown in Figure 2. This method is based on a description by Emerson (1929) for classification of maize kernel pericarp variegation.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: NM_001111873 (p1), NM_001158182 (c1), NM_001112058 (dek1), NM_0011112603 (r1), GU595147 (p1-n904), and GU595146 (all other p1 alleles listed in Table 1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. DNA Gel Blot Analysis of Selected p1 Alleles.

Supplemental Figure 2. Cytogenetic Detection of Chromosome Bridges and Fragments in Maize p1-vv9D9A Allele Meiotic Cells.

Supplemental Table 1. Statistical Test for Frequencies of Telophase I Cells with a Bridge and a Fragment among P1-nr11, P1-nr910, P1-nr904, and P1-nr459 Alleles.

Supplemental Data Set 1. Sequences Flanking Ac/fAc in the p1 Alleles Described Here.

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