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Genome rearrangement induced by Ac/Ds transposable element in plants

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Genome rearrangement induced by \textit{Ac/Ds} transposable element in plants

by

Chuanhe Yu

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Thomas Peterson, Major Professor
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Iowa State University
Ames, Iowa
2009

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ABSTRACT

Traditional \( \textit{Ac/Ds} \) transposable elements have been used in gene isolation and functional analysis in a variety of plants. Alternative transposition refers to transposition events involving the ends of different transposons; alternative transposition can induce a variety of genome rearrangement, including deletion, duplication, and inversion. The underlying aim of this thesis study is to gain a greater understanding of the alternative transposition process and the genome rearrangements thereby generated.

Composite or closely linked maize \( \textit{Ac/Ds} \) transposable elements can induce chromosome breakage, but the precise configurations of \( \textit{Ac/Ds} \) elements that can lead to chromosome breakage are not completely defined. Here, we determined the structures and chromosome breakage properties of 14 maize \( \textit{p1} \) alleles: each allele contains a fixed fractured \( \textit{Ac} \) (\( f\textit{Ac} \)) element and a closely linked full-length \( \textit{Ac} \) at various flanking sites. Our results show that pairs of directly or reversely oriented \( \textit{Ac/Ds} \) termini can induce chromosome breakage, whereas elements arranged in a macrotransposon configuration do not. Among the structures that can lead to chromosome breaks, breakage frequency is inversely correlated with the distance between the interacting \( \textit{Ac/Ds} \) 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.

The alternative transposition model predicts that a variety of chromosome rearrangements could be generated. Indeed, Barbara McClintock reported that the \( \textit{Ac/Ds} \) transposable element system can generate major chromosomal rearrangements (MCRs). However, evidence that alternative transposition can directly generate chromosome rearrangements was not previously reported. In this study, we identified a series of chromosome rearrangements derived from maize lines containing pairs of closely-linked \( \textit{Ac} \) transposable element termini. Molecular and cytogenetic analyses showed that the MCRs in these lines comprised 17 reciprocal translocations and two large inversions. The breakpoints of all 19 MCRs are delineated by \( \textit{Ac} \) termini and characteristic 8 bp target site duplications, indicating that the MCRs were generated by precise transposition reactions involving the \( \textit{Ac} \)
termini of two closely-linked elements. This alternative transposition mechanism may have contributed to chromosome evolution, and may also occur during V(D)J recombination resulting in oncogenic translocations.

To investigate the types and frequencies of the genome rearrangements caused by a pair of reversed Ac/Ds termini, we screened 100 mutant alleles induced by an intact Ac and a fractured Ac (fAc) structure at the maize p1 locus from two alleles P1-rr11 and P1-rr910. The rearrangement types were characterized by PCR pattern analysis and/or by direct sequencing. The results show that 20/100 mutant alleles display deletion patterns and 52/100 exhibit inversions, translocations or local rearrangements. Sequence analysis results show the majority of another set of 17/100 is flanking Ac or fAc small deletion. Interestingly one deletion allele, with only one nucleotide distance between Ac and fAc, is not competent for alternative transposition, evaluated by chromosome breakage frequency. 11/100 alleles are possible simple Ac excision or unknown mechanism. The reversed ends inserting to sister chromatid model was proposed to explain how the flanking Ac/fAc deletion alleles were generated from their parental lines.

Finally, to assess the extent to which alternative transposition can be applied in non-maize plants, we tested the ability of Ac/Ds alternative transposition to induce genome rearrangements in rice plant. In this study, we transformed a reverse-orientated Ac/Ds ends construct together with an Ac transposase gene in cis into rice (Oryza sativa ssp. japonica cv. Nipponbare). A green fluorescence protein (GFP) marker between the reversed Ds termini was used for efficient screening of rearrangement events. Molecular analyses indicate that a total of 25 independent rearrangements were obtained from three different chromosome loci. The rearrangements include chromosome deletions and inversions, and one translocation. Most of the deletions occurred within the T-DNA region, but two cases removed 72.5 kilobase pairs (kb) and 79 kb of rice genomic DNA flanking the transgene. The 79 kb deletion can be maintained as a heterozygote, but appears to be homozygous lethal as no homozygous deletion plants could be recovered. In addition to deletions, a variety of inversions were obtained from one transgene locus. The inversions range from very small (within the transgene DNA) to over 1 million base pairs (Mb) in size. For a subset of
inversions both breakpoints were sequenced, and all of these contained the typical 8 base pairs (bp) Ac/Ds targeted site duplication (TSD), confirming their origin as transposition products. Together, our results indicate that alternative Ac/Ds transposition can be an effective tool for functional genomics and chromosome manipulation in rice.
McClintock discovered the Ac(activator) / Ds(Dissociation) system in maize

In 1944, McClintock did an experiment to investigate the behavior of a ruptured chromosome end. She utilized a large terminal inverted duplication on maize chromosome 9 short arm, on which a few well known visible markers (Bz, Sh, Wx) are located. A crossover, between the terminal fragment of the inverted duplication and a normal maize chromosome 9, leads to formation of a dicentric chromosome 9 during meiosis. The dicentric chromosome will break and form a broken chromosome with a sticky end. Two sticky ends, one from the male parent and another from the female parent, were included in a fertilized egg cell (Figure 1). After replication, each broken chromosome will always join with its sister chromatids by sticky ends and form a dicentric chromosome. The dicentric chromosome will form a bridge in the next anaphase. The bridge is broken at a position between the two centromeres. In the telophase, two broken chromosomes will join together and form a dicentric chromosome again. Because each newly-formed dicentric chromosome will physically break during cell division process, the sticky ends will form again. McClintock called this process chromosome breakage-fusion-bridge (BFB) cycle (McClintock 1956).
Figure 1 The chromosome breakage-fusion-bridge cycle. 1. The fertilized zygote nucleus contains two broken-end chromatids, one from pollen and one from egg cell. The crosses mark the broken ends of each chromosome. 2. After chromosome replication each pair of sister chromatids fuses at the position of the sticky end. 3. In the first anaphase of the zygotic division, these two chromosomes give rise to bridge configurations as the centromeres of the sister chromatids pass to opposite poles. Breaks occur in each bridge at some position between the centromeres. 4. In the telophase nuclei, two chromosomes, each with a newly broken end, are present. The crosses mark the broken ends of each chromosome. 5. Fusion of the two broken-end chromosomes occurs at each telophase nucleus, establishing a dicentric chromosome. 6. In the next prophase, each sister chromatid is dicentric. 7 At the subsequent anaphase, several types of configurations may result from separation of the sister centromeres. Two types are shown. In the right side of 7, breaks occur in each bridge at some position between the centromeres. The subsequent behavior of the broken ends, from telophase to telophase, is the same as that given in 4 to 7. (Modified from (Jones 2005; McClintock 1956) )

McClintock analyzed several hundred progeny plants derived from the chromosome BFB lines. Some plants did not germinate; some progeny exhibited visible mutant phenotypes which McClintock attributed to the BFB-induced chromosome fragment deficiency. Because the chromosome breakage phenomenon is a stress condition, some transposable elements may be stimulated from a silenced to an active state. McClintock paid much attention to one line which showed unstable phenotype. Because the unstable phenotype is associated with loss of multiple loci on maize chromosome 9, she checked the chromosome structure using cytogenetic techniques. She found frequent chromosome breaks at a Dissociation (Ds) locus, which lead to loss of markers distal to the locus on 9S. She termed this locus state I Ds, which is characterized by frequent dicentric sister chromatids and chromosome breakage. In addition to chromosome breakage, she also found some chromosome rearrangements including deletion, duplication, inversion and ring chromosome associated with Ds. The state I Ds could also transpose to other sites, could be lost, or sometimes could be changed to a non-chromosome breakage state (State II Ds). All of these changes occurred only in presence of another factor (Ac) (McCLINTOCK 1950). McClintock’s pioneering work on the Ac/Ds system changed people’s view from that of a static genome to a dynamic genome.
Molecular structure of Ac/Ds transposon and chromosome breakage structure

In the 1980s, several examples of McClintock’s Ac/Ds elements were cloned and sequenced. The Ac element is 4565 bp long and contains imperfect 11-bp terminal inverted repeat sequences (TIRs). Transcription of the Ac element produces a 3.5 kb mRNA, and this mRNA can be translated into an 807-amino acid transposase, the key enzyme for Ac/Ds transposition. Many state II Ds elements are derived from Ac by internal deletion. In addition to the TIRs, Ac and Ds also share about 250-bp subterminal sequences, which include the transposase binding motif (AAACGG hexamer) (Sutton et al. 1984; Yan et al. 1999). 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 leaves a footprint (minor sequence change) at the donor site, and reintegration produces 8-bp target site duplication (TSD) at the new locus (Kunze and Weil 2002). The Ac/Ds transposon was classified as a member of the hAT transposon superfamily based on similarities of the encoded transposases.

Sequence analysis of state I Ds indicates that it contains a “double Ds” structure, with one inserted in the reverse direction into the other (Doring et al. 1984; Martinez-Ferez and Dooner 1997). The double Ds elements are not the only chromosome-breaking structures. Two Ds elements in opposite orientation in Wx1 locus and two closely linked Ac/Ds or Ac/fAc elements can also mediate chromosome breakage (Dooner and Belachew 1991; Martinez-Ferez and Dooner 1997; Ralston et al. 1989). By PCR and sequencing analysis, English et al (English et al. 1993; English et al. 1995) and Weil and Wessler (Weil and Wessler 1993) determined that double Ds or two Ds in opposite orientation causes chromosome breakage via an alternative transposition mechanism: Ac transposase binds to a 5’ Ds end from one sister chromatid, and a 3’ Ds end from the other sister chromatid. Excision of these Ds ends and joining of the sequences flanking them leads to formation of a chromatid bridge, which would be broken in the upcoming anaphase. By dissecting the double Ds structure in tobacco, English et al showed that a pair of 5’ Ds and 3’ Ds ends in a direct orientation is sufficient to mediate chromosome breakage (English et al. 1993); both the double Ds and two Ds in opposite orientation in the Wx1 locus contain this
configuration of $Ds$ ends. The two closely-linked $Ac/Ds$ or $Ac/fAc$, either in direct or reverse orientation, can lead to chromosome breakage (DOONER and BELACHEW 1991; HUANG and DOONER 2008).

**Maize $p1$ gene structure and $p1$ alleles’ terminology**

The maize $p1$ gene has been used for a number of years as a model to study gene regulation and transposon biology. The $p1$ gene encodes a R2R3 Myb-homologous transcriptional activator that regulates the synthesis of a red phlobaphene pigment in maize floral organs (GROTEWOLD et al. 1994). The two-letter suffix of $p1$ alleles indicate their expression in pericarp and cob, i.e., $P1$-$rr$ specifies red pericarp and red cob; $P1$-$wr$ specifies white pericarp and red cob; and $P1$-$ww$ specifies white pericarp and white cob (CHOPRA et al. 1996). Characters placed after the two-letter suffix indicate the culture number of origin of each allele; alleles with the same phenotype but different culture numbers may have different gene structures. The standard $p1$-$vv$ (variegated pericarp and variegated cob) allele described by Emerson (Emerson 1917) contains an $Ac$ insertion in the second intron of a $p1$ gene. From $p1$-$vv$, we obtained a spontaneous derivative termed $P1$-$ovov1114$ (orange-variegated pericarp and orange-variegated cob), in which the $Ac$ element had undergone an intragenic transposition to a nearby site in the $p1$ gene and inserted in the opposite orientation (Peterson 1990). From $P1$-$ovov1114$, we obtained a subsequent derivative termed $p1$-$vv9D9A$, which contains a closely-linked full-length $Ac$ and a fracture $Ac$ ($fAc$) in a direct orientation.

**Alternative transposition and genome rearrangement**

Chromosome breakage is not the only outcome of the alternative transposition process; if the excised transposon ends in direct orientation reinsert into the chromatid bridge, a deletion and a reciprocal inverted duplication would be generated. Such deletions and inverted duplication have been isolated in transgenic tobacco carrying a pair of $Ds$ ends in direction orientation (ENGLISH et al. 1995). Presumably, they were generated via the alternative transposition mechanism.
Maize \( p1 \) alleles with a fractured \( Ac \) (\( fAc \), 2039 bp of the \( Ac \) 3’ portion) inserted in intron 2 and a whole \( Ac \) inserted nearby have been used to study alternative transposition. One such allele is \( p1-vv9D9A \); in \( p1-vv9D9A \), both \( Ac \) and \( fAc \) inserted in intron 2 of the \( p1 \) gene, and the 3’ end of \( fAc \) and the 5’ end of \( Ac \) are in direct orientation. A twin sector was isolated from a \( p1-vv9D9A \) ear; molecular analysis showed that one of the alleles from the twinned sector carries a deletion, while the other carries a reciprocal inverted duplication. The structures of the twinned alleles suggest that they were generated by an alternative transposition reaction, and the characteristic footprint and target site duplication were identified in the twinned alleles (Zhang and Peterson 1999). Such a transposition reaction, in which a pair of \( Ac \) ends from different sister chromatids are involved, was designated sister chromatid transposition. In independent sister chromosome transposition reactions, the excised \( Ac/fAc \) ends could reinsert into different sites in the chromatid bridge to generate deletions with different sizes. We indeed isolated a set of nested deletions anchored at the \( p1 \) locus. The deletions range in size from 12.5 kb to >4.6 cm. All the deletion alleles begin exactly at the insertion site of the \( fAc \) or \( Ac \) in the \( p1 \) gene and extend to sites proximal to \( p1 \) (Zhang and Peterson 2005).

![Figure 1](image-url)

Figure 1 Reversed ends transposition model Solid boxes indicate \( p1 \) gene exons 1, 2, and 3 (left to right). Red arrows indicate \( Ac \) or \( fAc \); the open and solid arrowheads indicate the 3’ and 5’ ends, respectively, of \( Ac/fAc \). Ovals indicate \( Ac \) transposase. Short vertical lines indicate transposon
reinsertion sites, and solid triangles indicate TSD. (A) Ac transposase cleaves at the 5' end of Ac and the 3' end of fAc. (B) Following transposase cleavage at the junctions of Ac/p1 and fAc/p1, the internal p1 genomic sequences are joined to form a 13 kb circle. The “x” on the circle indicates the site where the joining occurred, marked by a transposon footprint. The Ac 5' and fAc 3' ends are competent for insertion anywhere in the genome. (C–E) The structures expected from insertion into two possible target sites. (C) The transposon ends insert into the 13 kb circle. The Ac 5' end joins to the end adjacent to exon 1 and fAc 3' end joins to the other end. The 13 kb sequence is rearranged (segment b–c and segment d exchanged positions). (D) The transposon ends insert distal to sequence e: the Ac 5' end joins to the end adjacent to e, and the fAc 3' end joins to the other end. Segment e is inverted, and the 13 kb circle is lost. The resulting chromosome contains an inversion of sequences from the fAc 3' end to the insertion site distal to e. (E) The transposon ends insert distal to sequence e: the fAc 3' end joins to the end adjacent to e, and the Ac 5' end joins to the other end. Segment e is circularized and presumed lost; the 13 kb circle is also lost. The resulting chromosome contains an interstitial deletion from the Ac 5' end to the insertion site distal to e (adapted from ZHANG and PETERSON 2004) (also see animation at http://www.public.iastate.edu/~jzhang/Transposition.html)

The P1-rr11 allele, derived from p1-vv9D9A allele, contains a full-length Ac and the same fAc as that in p1-vv9D9A; the Ac is about ~13-kb upstream the fAc, and the 5’ end of the Ac and the 3’ end of the fAc are in reversed orientation. The pair of Ac ends in reversed orientation is competent for transposition. Transposition reactions involving a pair of Ac/Ds ends in reversed orientation is designated as reversed Ac/Ds ends transposition (Figure 1). Local rearrangements, small inversions, and small deletions have been identified as products of reversed Ac/Ds ends transposition in P1-rr11 (ZHANG and PETERSON 2004). The p2 gene, a p1 paralog, is ~60 kb upstream p1, in tandem direct orientation. Four functional chimeric alleles carrying promoter, exon 1 and exon 2 from p2 and exon 3 from p1 were isolated; molecular analysis indicated they were produced via reversed Ac ends transposition mechanism (ZHANG et al. 2006).

**Alternative transposition in other organisms**

Alternative transposition is not limited to the Ac/Ds transposable element system. The bacterial transposons Tn10/IS10 have been observed to induce frequent deletions and inversions (ROSS et al. 1979). Tn10 is a composite bacterial transposon. It is 9300-bp in
length, with 1400 bp inverted repeats at its ends which are termed IS10-right and IS10-left elements and internal sequences (Foster et al. 1981). Each IS10 element has the inverted repeat and is capable of transposition. If the transposition involved the outside termini of the two IS10 elements, the net result will be simple excision and reinsertion. If the transposition involved the inside ends of the IS10 elements, the products will be deletion or inversion. The formation of deletions and inversions occurs by a mechanism which is analogous to that of reversed Ac/Ds ends transposition (Shen et al. 1987). Recently, other bacterial transposons such as Tn5 and ISSha1 also were shown to produce inversion/deletion rearrangements in bacterial genomes via a similar mechanism (Goryshin et al. 2003; Watanabe et al. 2007).

In Drosophila, two P elements from homologous chromosomes can undergo transposition reaction and produce chromosome deletion, inversion or duplication (Gray et al. 1996; Parks et al. 2004). This type of transposition reaction, in which two transposon ends on different chromosomes are involved, has not been detected in other organisms. It is tempting to propose that many transposable element systems that transpose via “cut and paste” mechanism can also undergo alternative transposition reactions. Hence, alternative transposition might have played a key role in the genome reorganization process during evolutionary history.

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CHAPTER 2. SPATIAL CONFIGURATION OF AC TERMINI AFFECTS THEIR ABILITY TO INDUCE CHROMOSOMAL BREAKAGE

A paper submitted to the Plant Cell

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Running Head: Chromosome breakage by Ac/Ds transposition
Key Words: transposon, Ac/fAc, sister chromatid, pI, macrotransposon

SYNOPSIS
Chromosome breakage was the first property of transposable elements identified by McClintock. Evidence is presented to show that chromosome breakage can be attributed to alternative transposition reactions which involve the termini of multiple closely-linked elements.

Abstract
Composite or closely linked maize 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 pI 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 directly or reversely oriented Ac/Ds termini can induce chromosome breakage, whereas elements arranged in a macrotransposon configuration do not. 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 in the late 1940s (McClintock, 1946, 1947; McClintock, 1948; McClintock, 1949; McClintock, 1950). McClintock 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) or its derivatives (Doring et al., 1984; Weck et al., 1984; Klein et al., 1988; 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 utilizes 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 bp and 209 bp from the *Ac* 5’ and 3’ ends, respectively, are required for efficient *Ac/Ds* excision (Coupland et al., 1988; Coupland et al., 1989). These sequences contain 11 bp terminal inverted repeats (TIRs) 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 leaves a footprint (minor sequence change) at the donor site, and reinsertions are flanked by 8 bp target site duplications (TSDs) 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 showed that a pair of 5’ and 3’ Ds termini in direct orientation are sufficient to mediate chromosome breakage (English et al., 1993; English et al., 1995); the same configuration is present in chromosome-breaking structures including doubleDs, sesquiDs (Martinez-Ferez and Dooner, 1997), and two Ds elements in opposite orientation in the maize wxl 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 bzl 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 transposon termini can affect chromosome breakage, we isolated and studied 15 maize alleles that contain various configurations of Ac termini in and near the maize p1 locus. 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. 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-rr11* is derived from *p1-vv9D9A* by transposition of *Ac* to a site 13175 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). (Figure 1A and Figure 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 29 new unstable *P1-rr* or *P1-ovov* alleles (with unstable red or orange-variegated pericarp); and from *P1-rr11* (unstable red pericarp) we obtained 4 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 then PCR-amplified using oligonucleotide primers complementary to *Ac/Ds* subterminal and adaptor sequences (Methods). The PCR products produced by each allele were analyzed by polyacrylamide gel electrophoresis; 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-rr11*) or background reference alleles (*p1-ww* and *p1-wr*). Polymorphic bands (one to four bands from each allele; average size approximately 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-rr904, P1-rr459, P1-rr910, P1-rr905, P1-rr908, P1-rr458, ...
and P1-rr460; Figure 1B; Supplemental data 1). 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 (data not shown). 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 (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-rr459, p1-vv577, p1-vv595, p1-vv1171, and p1-vv1172), and in reversed orientation in two alleles (P1-rr910 and P1-ovov454). Additionally, five alleles (P1-ovov455, P1-rr905, P1-rr908, P1-rr458, and P1-rr460) 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 (Dowe et al., 1990; Huang and Dooner, 2008). These five potential macrotransposons 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-rr904 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-rr904 allele contains both reverse- and direct-oriented Ac ends.

A pair of Ac ends 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 pl alleles described here can induce chromosome breakage, pollen from plants homozygous for each pl allele was crossed onto ears of tester plants of genotype dek1/Dek1 C1 R1, and the kernels visually checked for colorless sectors (dek1/Dek1 heterozygous plants were used because homozygous dek1 plants are severely stunted). In addition, control crosses were done using plants of three other genotypes: 1) pl-vv, 2) P1-rr::fAc, and 3) P1-rr::fAc, r1-navajo::Ac. The pl-vv allele contains a single Ac insertion in pl (Emerson, 1917; Xiao and Peterson, 2002). 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 pl-vv. To test whether a single fAc element could induce breakage, we used a stock termed P1-rr::fAc that was derived from pl-vv9D9A by excision of Ac. No evidence of chromosome breaks was observed with fAc alone, or fAc in the presence of an Ac element in trans, provided by the r1-navajo::Ac allele (Dellaporta et al., 1988). Together, these control crosses indicate that neither a single Ac in the pl gene (pl-vv), nor fAc (P1-rr::fAc) alone or in the presence of an unlinked Ac (P1-rr::fAc r1-navajo::Ac), can cause chromosome breaks.

In contrast, frequent colorless sectors were observed on the ears produced in crosses with certain other pl alleles (Figure 2). These include alleles with Ac and fAc present in either direct orientation (P1-rr459, pl-vv577, pl-vv1171, pl-vv1172, pl-vv595, and pl-vv9D9A) and in reversed orientation (P1-rr11, P1-rr910, 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.

Ac macrotransposition does not induce chromosome breakage

Interestingly, five pl alleles (P1-ovov455, P1-rr905, P1-rr908, P1-rr458, and P1-rr460) that contain both a fAc and a nearby 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 fAc and Ac 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 \textit{P1-ovov455} (Figure 3A). The standard \textit{p1-vv} allele, which contains a single \textit{Ac} element between the primer binding sites, was used as a positive control, and the \textit{P1-rr11} allele (which only contains a \textit{fAc} between the primers) was used as a negative control. As shown in Figure 3B, PCR of \textit{P1-ovov455} genomic DNA produced a 0.74 kb band, which matches the size expected following macrotransposon excision. Similarly, we detected bands of 0.36 kb, 1.15 kb and 1.44 kb bands representing the predicted products of excision of the 9.7 kb, 16.5 kb, and 21.7 kb macrotransposons in \textit{P1-rr905}, \textit{P1-rr458} and \textit{P1-rr460} alleles, respectively (Figure 3). A 1.57 kb band expected from macrotransposon excision in the \textit{P1-rr908} allele 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 \textit{P1-rr905} allele. This derivative allele was of \textit{P1-ww} phenotype, as would be expected because macrotransposon excision would result in deletion of the \textit{p1} gene exon 3. Together, these results are consistent with previous reports of excision of \textit{Ac/Ds} macrotransposons of various sizes (Dowe et al., 1990; Huang and Dooner, 2008). Importantly, our results show that macrotransposition \textit{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 \textit{DekI}-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 selected for relative variegation frequency. We employed a similar approach to estimate chromosome breakage frequency among the \( p1 \) alleles tested here. Ears produced by crossing each \( p1 \) allele with the \( dek1/Dek1 \) tester line were compared with a set of five standard ears which represented various grades of \( Dek1 \)-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 \( Ac/fAc \) ends in direct orientation, \( p1-vv9D9A \) exhibits a high relative frequency of colorless sectors (Grade 3.0); it also has the shortest distance between the \( Ac/fAc \) termini (2151 bp). In comparison, allele \( P1-rr459 \) exhibits a low relative frequency of colorless sectors (Grade 1.3), and it has the greatest distance (17840 bp) between the \( Ac/fAc \) ends. The same relationship is observed for alleles with \( Ac/fAc \) ends in reversed orientation (Table 1). Overall, the chromosome breakage frequency is inversely correlated 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 may produce an additive effect on the chromosome breakage frequency (Table 1).

To test whether alternative transposition between unlinked elements 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 \( r-navajo-m1::Ac \) 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 \textit{trans}-active, transposition-defective \( Ac \) element inserted in the \( p1 \) locus. The \( p1-vv5145::Ac \) element has a 1-bp
deletion in the Ac 3’ TIR, 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 \( r-navajo-m1::Ac \) or \( p1-vv5145::Ac \) should generate a deletion of \( P1-rr \) resulting in a colorless pericarp sector. Screens of approximately 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 chromatids as substrates for transposition; excision of these \( Ac \) ends is followed by ligation of the sister chromatids (English et al., 1993; Weil and Wessler, 1993; English et al., 1995; 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 both mitotic and meiotic cells of plants undergoing alternative transposition for evidence of chromatid bridges and fragments. Chromatid bridges were observed in anaphase stages of root-tip cells in seedlings of both \( p1-vv9D9A \) and \( P1-rr11 \) genotypes (data not shown). In addition, 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; Supplemental Figure 1). The proportion of cells containing bridges and fragments ranged from 2.75% to 4.18% (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 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 utilized 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 a 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 which 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 macrotransposon 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. In 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 centromeres 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). In 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 Dekl-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 dekl locus. Depending on the orientation with which the transposon ends reinsert, either a deletion or inversion is generated (Zhang et al., 2006; Zhang et al., 2009). Deletions would produce a Dekl-loss sector that would resemble a chromosome breakage event. In this way, loss of dekl 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. 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-rr11 alleles. However, the highly-condensed mitotic maize chromosomes were somewhat difficult to visualize. In 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-orientated 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 ~50 cM) would generate a bridge which would be apparent at the anaphase I stage. Similarly, reversed-ends 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 utilization 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 which 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 or rice (Messing and Dooner, 2006; Rabinowicz and Bennetzen, 2006). 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 which 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 to identify stocks containing chromosome-breaking “doubleDs” elements inserted on 10 maize chromosome arms (Neuffer, 1995). 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; Zhang et al. 2005, 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

Terminology and maize stocks: The maize pl 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 pl allele suffix, i.e. P1-rr specifies red kernel pericarp and red cob, p1-ww specifies white (colorless) pericarp and white cob, P1-wr specifies white pericarp and red cob, p1-vv specifies variegated pericarp and variegated cob color, P1-ovov specifies orange variegated pericarp and orange variegated cob (Athma et al., 1992). The p1-vv9D9A allele, which is highly unstable, contains a complete Ac element and a terminally-deleted Ac (fractured Ac, fAc) in the second intron of the pl gene (Zhang and Peterson, 1999). Excision of the intact Ac in p1-vv9D9A and reinsertion into sites nearby or within the pl gene leads to the formation of other alleles including P1-rr11, P1-rr910, and P1-ovov454. The r-m3::Ds allele contains a Ds element inserted in the r1 gene which 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, Southern 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 Southern hybridizations were performed according to (Sambrook et al., 1989), except that hybridization buffers contained 250-mM NaHPO$_4$, pH 7.2, 7% SDS, and wash buffers contained 20-mM NaHPO$_4$, pH 7.2, 1% SDS. The $pI$ gene-specific probes 15, 6, and 8B have been described (Lechelt et al., 1989; Zhang and Peterson, 2004).

**Transposon-display:** Ac elements were mapped by a transposon-display approach (Van den Broeck et al., 1998). Genomic DNA (5 µg) was digested with 10 units HinfI or HpyCH4IV (NEB, Beverly, MA) at 37°C for 3 hours. Pre-annealed adaptors (cd-a1 and cd-a2; final concentration 5mM), ATP (final concentration 1 mM) and 1 Weiss Unit ligase (NEB, Beverly, MA) were added to the reaction tubes and incubated for 8 hours at room temperature. The ligation product was used directly for two rounds of PCR amplification (Eppendorf HotMaster Taq, Westbury NY). 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 three minutes to denature the DNA template, followed by 35 cycles of 20 sec at 94°C, 30 sec at 60°C, and 3 min at 65°C, and one cycle of 8 min at 65°C. PCR products (1 µl) were resolved on a denaturing 6% polyacrylamide-7 M urea gel at 1000V for 4 hours, then visualized by a silver stain detection system (Promega, Madison, WI).

Polymorphic bands were excised from the gel, recovered and re-amplified with cd-p2 and Ac27r. The PCR products were purified with the Perfectprep gel cleanup system (Eppendorf HotMaster Taq, Westbury NY) 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 $pI$ gene and are characterized in this paper. Among the remaining 20 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 $pI$ 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 $pI$ gene. However, these latter five cases could not be confirmed by subsequent PCR or genomic Southern blot, and probably represent somatic insertion events.
Oligonucleotide primers and their sequences:

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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 one day, and then maintained at -20°C. The carmine smear technique was applied to developing sporocytes according to (Sharma and Sharma, 1965).

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 testcrosses 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
candidate $pI$ alleles to be tested. Ears produced by test crosses were compared with a set of standard ears and classified into one of five aleurone variegation grades ranging from 0 (no sectors) to 4 (most frequent sectors). This method is based on a description by Emerson (1929) for classification of maize kernel pericarp variegation.

Acknowledgments
We would like to express our appreciation to Dr. Feng Zhang for assistance with PAGE experiments, and to Yang Xu, Huanan Jin, and Peter Howe for laboratory and field assistance. This research was supported by NSF MCB 0450243 to TP and JZ, and NSF MCB 0450215 to DW.
<table>
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<td><em>P1-rr459</em></td>
<td>17,840</td>
<td>2,1,1,1 (4)</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td><em>p1-vv577</em></td>
<td>8,889</td>
<td>2,2 (2)</td>
<td>2.0 (0.0)</td>
</tr>
<tr>
<td><em>p1-vv1171</em></td>
<td>3,621</td>
<td>2,3,3 (3)</td>
<td>2.7 (0.3)</td>
</tr>
<tr>
<td><em>p1-vv1172</em></td>
<td>3,550</td>
<td>2,2,2,3 (4)</td>
<td>2.3 (0.3)</td>
</tr>
<tr>
<td><em>p1-ovov595</em></td>
<td>2,611</td>
<td>3,3 (2)</td>
<td>3.0 (0.0)</td>
</tr>
<tr>
<td><em>p1-ovov9D9A</em></td>
<td>2,151</td>
<td>3,3,3,3,3,3,3,3 (7)</td>
<td>3.0 (0.0)</td>
</tr>
<tr>
<td>Reversely oriented Ac/fAc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P1-rr11</em></td>
<td>13,175</td>
<td>1,1,1,1,1,1 (6)</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td><em>P1-rr910</em></td>
<td>8919</td>
<td>2,2,2,2 (4)</td>
<td>2.0 (0.0)</td>
</tr>
<tr>
<td><em>P1-ovov454</em></td>
<td>823</td>
<td>3,3 (2)</td>
<td>3.0 (0.0)</td>
</tr>
<tr>
<td>Macrotransposon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P1-ovov455</em></td>
<td>6,809</td>
<td>0,0,0,0 (4)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td><em>P1-rr905</em></td>
<td>9,774</td>
<td>0,0,0,0,0,0,0,1 (8)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td><em>P1-rr908</em></td>
<td>16,060</td>
<td>0,0,0,0,0,0,0,0,0 (8)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td><em>P1-rr458</em></td>
<td>16,538</td>
<td>0,0,0,0 (4)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td><em>P1-rr460</em></td>
<td>21,741</td>
<td>0,0,0,0 (4)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>p1-vv</em></td>
<td>N/A</td>
<td>0,0,0,0 (4)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td><em>P1-rr::fAc</em></td>
<td>N/A</td>
<td>0,0 (2)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td><em>P1-rrfAc;r1-nj::Ac</em></td>
<td>N/A</td>
<td>0,0 (2)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P1-rr904</em></td>
<td>9,178/6716/2151*</td>
<td>4,4,4,4 (4)</td>
<td>4.0 (0.0)</td>
</tr>
</tbody>
</table>
*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-vv9D9A. It has three possible pair of Ac/fAc ends. SE, Standard Error.
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># of cells with a B and F/total # of cells</th>
<th>Frequency (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-rr11 (2 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 (2 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 (3 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 (3 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 percent of cells that have a bridge and a fragment. 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 chi-squared statistical tests indicate that there is no significant difference in the frequency of chromosome breakage between any two alleles (p>0.05) (Supplemental Table 1).
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 open and solid red arrowheads represent the 3’ and 5’ termini of \( Ac/fAc \).

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); solid 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 \( p1-vv9D9A \).

FIGURE 2. Five standard ears for evaluating the frequency of chromosome breakage.

The ears are from \( dek1/+ \) tester plants crossed with pollen from plants homozygous for various \( Ac \)-containing \( p1 \) alleles. The functional \( Dek1 \) gene conditions solid purple aleurone color; chromosome breakage at the proximal \( p1 \) locus results in loss of \( Dek1 \) as evidenced by colorless aleurone sectors. The ears were borne on heterozygous \( dek1/+ \) tester plants, hence only half of the kernels can show \( Dek1 \) loss. The photographs show grade 0 (no \( Dek1 \)-loss sectors) to grade 4 (highest frequency \( Dek1 \)-loss sectors) standard ears produced by crossing with pollen from plants homozygous for the following alleles: Grade 0, \( P1-rr458 \); Grade 1, \( P1-rr459 \); Grade 2, \( P1-rr910 \); Grade 3, \( p1-vv9D9A \); Grade 4, \( P1-rr904 \).

FIGURE 3. Detection of somatic macrotransposon excision by PCR.

A. Molecular structures of \( p1 \) alleles containing possible macrotransposons. Five alleles (\( P1-ovov455 \), \( P1-rr905 \), \( P1-rr908 \), \( P1-rr458 \), and \( P1-rr460 \)) have the \( Ac \) 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. The large open and solid arrowheads represent the 3’ and 5’ termini of \( Ac/fAc \). The positions of oligonucleotide primers used for PCR are indicated by the small arrows.
B. Results of PCR analysis of somatic macrotransponon excision. Primers used are shown at top. Alleles tested are shown above each lane: Pvv, p1-vv; 455, P1-ovov455; 11, P1-rr11; 908, P1-rr908; 458, P1-rr458; 905, P1-rr905; 460, P1-rr460. Bands corresponding to expected macrotransposition excision products are indicated. P1-rr11 is used as a negative control. Total genomic DNA from young leaves was used as template.

FIGURE 4. Bridges and fragments at Anaphase I and Telophase I of meiosis in microsporocytes of P1-rr11 plants.
A  Bridge and fragment at Anaphase I of meiosis
B  Bridge and fragment at Telophase I of meiosis

FIGURE 5. Chromosome-breakage models.
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 open and solid red triangles represent the 3’ and 5’ 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.

SUPPLEMENTAL DATA 1. Sequences flanking Ac/fAc in the p1 alleles described here. The Ac/fAc sequences are underlined.
SUPPLEMENTAL FIGURE 1. Cytogenetic detection of chromosome bridges and fragments in maize p1-vv9D9A allele meiotic cells. A and B show the bridges and fragments in Anaphase I of the meiotic cell cycle. C and D show the bridges and fragments in Telophase I of the meiotic cell cycle.
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 open and solid red arrowheads represent the 3’ and 5’ termini of *Ac/fAc*.

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); solid 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*.
FIGURE 2. Five standard ears for evaluating the frequency of chromosome breakage. The ears are from dek1/+ tester plants crossed with pollen from plants homozygous for various Ac-containing p1 alleles. The functional Dek1 gene conditions solid purple aleurone color; chromosome breakage at the proximal p1 locus results in loss of Dek1 as evidenced by colorless aleurone sectors. The ears were borne on heterozygous dek1/+ tester plants, hence only half of the kernels can show Dek1 loss. The photographs show grade 0 (no Dek1-loss sectors) to grade 4 (highest frequency Dek1-loss sectors) standard ears produced by crossing with pollen from plants homozygous for the following alleles: Grade 0, P1-rr458; Grade 1, P1-rr459; Grade 2, P1-rr910; Grade 3, p1-vv9D9A; Grade 4, P1-rr904.
FIGURE 3. Detection of somatic macrotransposon excision by PCR.

A. Molecular structures of p1 alleles containing possible macrotransposons. Five alleles (P1-ovov455, P1-rr905, P1-rr908, P1-rr458, and P1-rr460) have the 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. The large open and solid arrowheads represent the 3’ and 5’ termini of Ac/fAc. The positions of oligonucleotide primers used for PCR are indicated by the small arrows.

B. Results of PCR analysis of somatic macrotransposon excision. Primers used are shown at top. Alleles tested are shown above each lane: Pvv, p1-vv; 455, P1-ovov455; 11, P1-rr11; 908, P1-rr908; 458, P1-rr458; 905, P1-rr905; 460, P1-rr460. Bands corresponding to expected macrotransposition excision products are indicated. P1-rr11 is used as a negative control. Total genomic DNA from young leaves of plants homozygous for the indicated alleles was used as template.
FIGURE 4. Bridges and fragments at Anaphase I and Telophase I of meiosis in microsporocytes of *P1-rr11* plants.

A  Bridge and fragment at Anaphase I of meiosis
B  Bridge and fragment at Telophase I of meiosis.
FIGURE 5. Chromosome-breakage models.

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 open and solid red triangles represent the 3’ and 5’ 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.
SUPPLEMENTAL TABLE 1. Statistical test for frequencies of Telophase I cells with a bridge and a fragment among \textit{P1-rr11}, \textit{P1-rr910}, \textit{P1-rr904} and \textit{P1-rr459} alleles. The numbers of Telophase I Cells with a bridge and a fragment are listed in Table 2. Pairwise comparisons of different alleles are presented below (alleles are identified by \textit{p1} gene suffix number due to space constraints).

<table>
<thead>
<tr>
<th>Alleles:</th>
<th>11 vs. 910</th>
<th>11 vs. 904</th>
<th>11 vs. 459</th>
<th>910 vs. 904</th>
<th>910 vs. 459</th>
<th>904 vs. 459</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi square value</td>
<td>0.164</td>
<td>1.140</td>
<td>0.376</td>
<td>3.132</td>
<td>1.801</td>
<td>0.046</td>
</tr>
<tr>
<td>\textit{P} - value</td>
<td>0.69</td>
<td>0.29</td>
<td>0.54</td>
<td>0.08</td>
<td>0.18</td>
<td>0.83</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL DATA

Sequences flanking Ac/fAc in the p1 alleles used in this study. The Ac/fAc sequences are underlined.

fAc sequence and flanking sequence (common to p1 alleles listed in Table 1, except p1-vv).

P1-rr458 Ac 5’ flanking sequence

P1-rr904 Ac 3’ flanking sequence
TTACCGA\textsubscript{C}CGTTACCGA\textsubscript{C}GTTTTTCATCCC\textsubscript{T}ACTCGGC\textsubscript{G}CGCAAGATCTTTGCGCGGAGTCGTGTTCC
ACGTCA\textsubscript{C}CGC\textsubscript{A}CGTCTGTTGGTGTGCCA\textsubscript{C}A\textsubscript{C}CGA\textsubscript{C}GACCTCGGC\textsubscript{C}CATAGGCTATGCGGCCG
GA\textsubscript{G}C\textsubscript{A}A\textsubscript{A}G\textsubscript{G}G\textsubscript{T}\textsubscript{C}\textsubscript{A}AAACT\textsubscript{G}C\textsubscript{T}ATTTA\textsubscript{A}TTTTTT\textsubscript{T}AG\textsubscript{G}\textsubscript{C}\textsubscript{T}A\textsubscript{A}CG\textsubscript{T}G\textsubscript{TTTTT}\textsubscript{T}G\textsubscript{T}TTTAA
GG\textsubscript{G}G\textsubscript{C}\textsubscript{T}A\textsubscript{A}AA

**P1-rr904** Small additional f\textsubscript{Ac} sequence
TTAGT\textsubscript{A}ATTAAGTAAGCTA\textsubscript{A}ATTAAGCTATGCTTTCA\textsubscript{A}A\textsubscript{A}C\textsubscript{A}G\textsubscript{T}GTTTCA\textsubscript{A}TGGT\textsubscript{A}ATG\textsubscript{G}CTAGGGAAGT\textsubscript{A}TGGTTTCTTCCGATC\textsubscript{A}TT\textsubscript{C}G\textsubscript{A}ACCGGAGG
TAAAAAGCC\textsubscript{G}AAACG\textsubscript{G}G\textsubscript{A}ACG\textsubscript{G}G\textsubscript{A}ACG\textsubscript{G}

**P1-rr459** Ac 3\textsuperscript{'} flanking sequence
TAGAGG\textsubscript{A}TTTTTCCAC\textsubscript{T}C\textsubscript{T}T\textsubscript{C}T\textsubscript{C}TCTG \textsubscript{G}G\textsubscript{C}AC\textsubscript{T}T\textsubscript{G}C\textsubscript{A}C\textsubscript{T}T\textsubscript{G}C\textsubscript{A}C\textsubscript{T}T\textsubscript{G}C\textsubscript{A}C

**P1-rr460** Ac 5\textsuperscript{'} flanking sequence
GGATTTTCCAC\textsubscript{T}C\textsubscript{T}T\textsubscript{C}T\textsubscript{C}TCTG\textsubscript{G}G\textsubscript{C}AC\textsubscript{T}T\textsubscript{G}C\textsubscript{A}C\textsubscript{T}T\textsubscript{G}C\textsubscript{A}C\textsubscript{T}T\textsubscript{G}C\textsubscript{A}C

**P1-rr11** Ac 5\textsuperscript{'} flanking sequence
TT\textsubscript{C}CCC\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C

**P1-rr910** Ac 5\textsuperscript{'} flanking sequence
AT\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C\textsubscript{T}C

**p1-rr9d9q** Ac 5\textsuperscript{'} flanking sequence
TACGGGATTTTTCCATC\textsubscript{T}C\textsubscript{T}TAC\textsubscript{T}TCTCC\textsubscript{G}ACT\textsubscript{A}AACC\textsubscript{A}AC\textsubscript{T}T\textsubscript{G}T\textsubscript{G}T\textsubscript{G}G\textsubscript{A}G\textsubscript{T}\textsubscript{G}\textsubscript{A}G\textsubscript{T}AAGCTG

**p1-vv9d9a** 5\textsuperscript{'} flanking sequence
TACGGGATT TTTCATCCCTG

**p1-vv577** Ac 3\textsuperscript{'} flanking sequence
p1-vv1172 Ac 3’ flanking sequence
NCNACCGTTTTTCTCCCTA

p1-vv1171 Ac 3’ flanking sequence
CCGNNCCNNTTTTCTCCCTA

P1-ovov454 Ac 5’ flanking sequence
ACGGGATTTTCCCATCCTACTTCTCCTCCTCGGCGGCACAACGCTAGGACGAGCGCTAAGCGGGGG

P1-ovov455 Ac 5’ flanking sequence
ACGGGATTTTCCCATCCTACTTCTCCTCCTCGGCGGCACAACGCTAGGACGAGCGCTAAGCGGGGG

P1-rr908 Ac 5’ flanking sequence
GATTTCCTCAATCTTATTTCTTCTCCCTCCTCGGCGGTAGGGAAGGACG

SUPPLEMENTAL FIGURE 1. Cytogenetic detection of chromosome bridges and fragments in maize p1-9D9A allele meiotic cells. A and B show the bridges and fragments in Anaphase I of the meiotic cell cycle. C and D show the bridges and fragments in Telophase I of the meiotic cell cycle.
Literature cited


Martinez-Ferez, I.M., and Dooner, H.K. (1997). Sesqui-Ds, the chromosome-breaking insertion at bz-m1, links double Ds to the original Ds element. Mol Gen Genet 255, 580-586.


CHAPTER 3. ALTERNATIVE AC/DS TRANSPOSITION INDUCES MAJOR CHROMOSOMAL REARRANGEMENTS IN MAIZE

A paper published in *Genes & Development*

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Abstract

Barbara McClintock reported that the Ac/Ds transposable element system can generate major chromosomal rearrangements (MCRs), but the underlying mechanism has not been determined. Here, we identified a series of chromosome rearrangements derived from maize lines containing pairs of closely-linked Ac transposable element termini. Molecular and cytogenetic analyses showed that the MCRs in these lines comprised 17 reciprocal translocations and two large inversions. The breakpoints of all 19 MCRs are delineated by Ac termini and characteristic 8 bp target site duplications, indicating that the MCRs were generated by precise transposition reactions involving the Ac termini of two closely-linked elements. This alternative transposition mechanism may have contributed to chromosome evolution, and may also occur during V(D)J recombination resulting in oncogenic translocations.
Introduction

In the 1940’s, Barbara McClintock reported that the maize Activator (Ac) element could induce transposition of the non-autonomous Dissociation (Ds) element, which she identified as a locus of chromosome breakage. She also showed, using cytogenetic methods, that transposition of Ds was sometimes accompanied by major chromosomal rearrangements including deletions, duplications, inversions, reciprocal translocations, and ring chromosomes (McClintock 1949; McClintock 1950b; McClintock 1950a; McClintock 1951). Because these rearrangements only occurred in the presence of Ac and they retained Ds at their breakpoints, McClintock concluded that Ac/Ds transposition is somehow responsible for their origin. Although several hypotheses have been advanced to explain the origin of McClintock’s MCRs, it is still unclear precisely how these large chromosome rearrangements were generated.

Standard Ac/Ds transposition only changes the position of the transposon in the genome, hence the MCRs isolated by McClintock must have originated by some other mechanism involving transposition. A type of aberrant Ds transposition that results in fusion of sister chromatids, chromosome breakage, and formation of deletions has been described (English et al. 1993; Weil and Wessler 1993; English et al. 1995). Previously, we showed that a pair of Ac termini in tandem orientation produced reciprocal deletion/duplication alleles via a mechanism in which Ac transposase interacts with Ac termini on sister chromatids; thus it was termed sister chromatid transposition (SCT) (Zhang and Peterson 1999). However, SCT cannot fully explain the origin of McClintock’s MCRs because the only heritable products it generates are deletions and inverted duplications.

Recently, we and others showed that a pair of Ac termini in reversed orientation can also undergo transposition (reversed Ac ends transposition) (Zhang and Peterson 2004; Huang and Dooner 2008). In this reaction, Ac transposase acts upon a pair of Ac termini on the same sister chromatid (Fig. 1A). Insertion of the excised Ac termini into nearby sites can produce relatively small rearrangements, including deletions and inversions (Zhang and Peterson 2004; Huang and Dooner 2008). However, the excised Ac ends could also insert into distant sites on the same or different chromosome to generate a variety of MCRs. For
example, insertion into the same chromatid would generate an acentric fragment and a ring chromosome (the upper portion of Fig. 1C and supplemental animation), or an inversion (the lower portion of Fig. 1C and supplemental animation), depending on the orientation of insertion of the transposon ends. In addition, insertion into another chromosome would generate a reciprocal translocation (Fig. 1D and supplemental animation), or a dicentric chromosome and an acentric fragment (supplemental animation), again depending on the orientation of insertion of the transposon ends.

Some MCRs, including acentric fragments (Fig. 1C), acentric rings, and dicentric chromosomes, would likely be highly unstable or result in cell lethality. However, we predicted that certain rearrangements including duplications, inversions, and reciprocal translocations should be transmitted to the next generation. In previous studies, rearrangements generated by reversed-ends transposition were relatively small and could not be directly visualized. We have now confirmed, through cytogenetic methods, the formation of 19 MCRs (17 reciprocal translocations and two large inversions) generated by reversed Ac ends transposition.

Results

Detection of transposon-induced MCRs

The \( p1 \) gene encodes a Myb-homologous transcriptional activator required for the production of red phlobaphene pigments in maize floral tissues including kernel pericarp (the outermost layer of the maize seeds) and cob glumes (Grotewold et al. 1991; Grotewold et al. 1994). The standard \( P1-rr \) allele specifies red pericarp and red cob, and the kernels in a \( P1-rr \) ear are uniformly red. In contrast, three alleles (\( P1-rr11, P1-rr910, \) and \( P1-ovov454 \)) exhibit unstable pigmentation in which kernels are predominantly red or orange, but also exhibit frequent colorless stripe(s) and sectors. All three of these alleles have similar structures, with a fractured \( Ac \) element (\( fAc, 2039 \) bp of 3’ portion of \( Ac \)) inserted in intron 2 of the \( p1 \) gene, and a full length \( Ac \) element inserted nearby, upstream of the \( fAc \) element. In each allele, the full-length \( Ac \) element is oriented with its 5’ terminus closest to the 3’ terminus of \( fAc \); the distances between \( Ac \) and \( fAc \) in \( P1-rr11, P1-rr910, \) and \( P1-ovov454 \) are
13175 bp, 8919 bp and 823 bp, respectively (Fig. 1A). We reasoned that transposition reactions involving reversed Ac ends—i.e., the 5' end of the full-length Ac element and the 3' end of fAc—could be responsible for the colorless kernel pericarp sectors exhibited by these alleles. As shown in Figure 1 and the supplemental animation, most of the predicted products of reversed Ac ends transposition would have suffered loss of p1 gene exons 1 and 2, or separation of exons 1 and 2 from exon 3. Either outcome would prevent p1 gene function and result in colorless sectors. Therefore, we selected seeds with colorless kernel pericarp as candidate carriers of transposon-induced MCRs (see Materials and methods for details).

Plants heterozygous for MCRs including large deletions, inversions, or reciprocal translocations produce a significant proportion of inviable meiotic products. In maize, the resulting gametophytic lethality results in semisterile ears with irregular rows, and defective pollen grains. In some cases, rearrangement events occurred sufficiently early in development to produce large multi-kernel sectors in which the area of colorless pericarp coincides exactly with a region of female semisterility. For example, Figure 2A shows a mature ear from a plant heterozygous for progenitor allele P1-rr11 (red pericarp kernels); the ear has a large sector of kernels with colorless pericarp (source of translocation p1-wwB966). The red pericarp area has normal seed set and regular rows of kernels, while the colorless pericarp sector has semisterile seed set and irregular kernel placement. Most of the rearrangement alleles described here were obtained from smaller colorless-pericarp sectors within which semisterility could not be scored reliably. However, when these colorless pericarp kernels were grown into plants, their ears and pollen were screened for significant levels of sterility. Figure 2B shows pollen grains from a plant heterozygous for a large inversion; approximately 50% of the pollen grains are small, misshapen, and devoid of starch. Plants which produced at least 20% defective pollen and irregular seed set were selected for further characterization. Their progeny were then examined to determine if the pollen abortion and irregular kernel set are inherited. PCR analysis was performed to test whether the candidate plants contain structures indicative of the presence of a MCR; four pairs of oligonucleotide primers were designed to detect the junctions between Ac/fAc and its
flanking sequences (see Fig. 1 for the orientations and approximate positions of the primers). According to the model shown in Figure 1, inversion and reciprocal translocation alleles should produce specific PCR products with primer pairs 1+3 and 8+9, but not with primer pairs 5+6 and 7+3. Based on the combined genetic screening and PCR results, we identified 25 candidate alleles with characteristics consistent with the presence of large inversions or reciprocal translocations.

**Sequence analysis of MCR breakpoints**

The breakpoints flanking Ac in 24 of the 25 MCR candidates were cloned via Ac casting (Singh et al. 2003) (see Materials and methods for details), and the breakpoints flanking fAc in all the MCR candidates were cloned via PCR as described in the Materials and methods section. If the MCR candidates were indeed generated via reversed Ac ends transposition, we expect that the 8 bp sequence flanking the Ac 5’ end should match the 8 bp flanking the fAc 3’ end (target site duplication, TSD). Indeed, 8 bp target site duplications were observed for all of the 19 confirmed MCR alleles (sequence data available in supplemental file MCRsequences.doc). In the case of p1-wwB966, molecular and genetic analyses suggested that the Ac element had excised from the MCR breakpoint and inserted nearby in the genome; its MCR breakpoint sequences were isolated by LM-PCR, and found to share only 7 bp instead of the expected 8 bp common sequence at the junctions of Ac/fAc with their flanking sequences. The base immediately adjacent to the Ac 5’ end is different from the corresponding base flanking the fAc 3’ end; this result is consistent with formation of a TSD by Ac insertion, followed by a 1 bp transversion (G to C) upon Ac excision.

**Mapping chromosomal locations of MCR breakpoints**

To identify the types of structural rearrangements in the MCR lines, we needed to determine the chromosomal locations of the breakpoints. Most breakpoint sequences were mapped to one of the maize chromosomes by PCR using the genomic DNA of a series of oat-maize chromosome addition lines. Each oat-maize line contains all of the oat chromosomes, and one of the ten maize chromosomes (Ananiev et al. 1997; Kynast et al. 2002). Thus,
primers for unique maize sequences will only amplify PCR products from the oat-maize line that contains the corresponding chromosome (Fig. 3). In this way, the breakpoints of 14 MCR candidates were unambiguously mapped to specific chromosomes. In five cases, PCR using oat-maize addition lines gave negative or ambiguous results. These data are summarized in Table 1.

Although the maize genome sequence is not yet complete, many maize BAC clones have been partially sequenced and their chromosome positions tentatively mapped. The sequences of candidate MCR breakpoints obtained here were used in BLAST alignments against the maize high-throughput genomic sequences (HTGS; http://www.ncbi.nlm.nih.gov/blast/). BACs with high homology to eighteen of nineteen MCR candidates were identified, and tentative chromosomal positions of homologous BAC clones were obtained from http://www.maizesequence.org. In most cases, the putative MCR map positions identified by oat-maize PCR agreed with the BAC map positions assigned by the maize genome sequencing project. Three cases gave ambiguous or conflicting results (see Supplemental Table 1). Finally, PCR and Temperature Gradient Capillary Electrophoresis (TGCE) (Fu et al. 2005) were also used to map the MCR breakpoint sequences of some candidate alleles. In all cases in which mapping results were obtained, the map positions were in good agreement with the results obtained by at least one independent method.

**Visualization of MCRs by FISH in somatic cells and at pachytene in meiotic cells**

To confirm the molecular results indicating the presence of chromosomal inversions and translocations, we performed cytogenetic analysis of maize mitotic chromosomes using Fluorescence In Situ Hybridization (FISH). Maize seedlings heterozygous for putative MCR and normal chromosomes were pre-treated with nitrous oxide as a spindle poison, and root tip cell chromosome preparations were hybridized with fluorescently-labeled single gene or gene cluster probes for somatic chromosome identification (Kato et al. 2006). Two probes were used to detect the presence of pericentric inversions involving chromosome 1: one probe was complementary to the *dek1* gene, which is located approximately 700 kb distal to
the *p1* gene on the short arm of maize chromosome 1 (http://www.maizesequence.org). A second probe (“TAG”) hybridizes with TAG- microsatellite locus on chromosomes 1L, 2SL and 4S of most genotypes. The results of FISH using these probes confirm that *p1-wwC30* contains a pericentric inversion of chromosome 1 (Fig. 4A). Similar results were obtained for allele *p1-wwB546* (not shown).

Using the *dek1* and “TAG” probes for chromosome 1 together with probes from other chromosomes, FISH was performed to determine whether the other putative MCRs contain reciprocal translocations. The result from *p1-wwB1023* (a putative T1-5 reciprocal translocation; Fig. 4B) is especially interesting. The probe *serk2-rf2e1* hybridizes to a site on chromosome 5L. In addition to the normal chromosomes 1 and 5 (left side of panel 4B), these cells contain a translocation chromosome in which the *dek1* and *serk2-rf2e1* signals are juxtaposed (inset, Fig. 4B). These results confirm the presence of a translocation involving chromosomes 1S and 5L. Pachytene analysis (see below) also demonstrated the presence of a T1-5 translocation with a breakpoint very close to the end of chromosome 5 (Fig. 4D). This conclusion is consistent with the TGCE mapping data indicating that the translocation breakpoint is only 5 cM from the telomere of 5L; i.e. less than 3% of the total length of chromosome 5 from the telomere. In addition, the frequency of aborted pollen in *p1-wwB1023* heterozygotes is approximately 25%, much less than the 50% abortion frequency expected for translocations that involve chromosome segments containing genes essential for pollen viability. All of these data support the conclusion that *p1-wwB1023* contains a T1-5 translocation whose breakpoint is located very close to the tip of chromosome 5L.

The results of oat-maize PCR indicated that *p1-wwB521* contains a T1-3 translocation; sequencing and BLAST analysis (CytoView, http://www.maizesequence.org) placed the breakpoint on chromosome 3S, bin 3.04 (for a description of maize chromosome bin designations, see http://www.maizegdb.org/cgi-bin/bin_viewer.cgi). In contrast, FISH analysis using probes *myo1* on chromosome 3L and TAG on chromosome 1L showed clearly that the 1S breakpoint is attached to a large segment of 3L (see Supplemental Fig. 1). Together, these results suggest that the breakpoint is located on chromosome 3L, very close to the centromere.
As described above, oat-maize PCR results for *p1-wwB469* were ambiguous, with bands present in multiple addition lines. The best match obtained from BLAST of the breakpoint sequences is located on chromosome 3, bin 3.04 (Table 1). However, no rearrangements were detected by FISH using probes *dek1* (1S), *TAG* (1L) and *rp3* on chromosome 3; whereas, evidence of a T1-4 reciprocal translocation was obtained using probes *dek1*, *TAG*, and *cent4*. The *cent4* probe hybridizes to a site near centromere 4 (Jin et al. 2004). Cells from *p1-wwB469* contain a presumptive translocation chromosome that hybridizes with both the *dek1* (1S) and *cent4* (4S) probes. In addition, *TAG*-microsatellite locus is present on chromosome 4S, and the *TAG* probe hybridized in two positions on a presumptive translocation chromosome containing both the 1L and 4S loci. Overall, these results suggest that *p1-wwB469* contains a T1-4 translocation, with the breakpoint on the short arm of chromosome 4 (Supplemental Fig. 2).

**Cytogenetic analysis of maize pachytene chromosomes**

Although FISH of root tip cells is useful for confirming the identity of chromosomes involved in rearrangements, the resolution is quite low because the metaphase chromosomes are highly condensed. In contrast, maize chromosomes are much less condensed at the meiotic pachytene stage of microsporogenesis, and the homologous chromosomes are synapsed. In favorable preparations, each of the 10 maize chromosomes can be distinguished by their relative lengths, distinctive chromomere patterns, centromere positions, and the presence of deep-staining knobs at specific positions. In reciprocal translocation heterozygotes, pairing of the rearranged chromosome(s) and the corresponding normal chromosome(s) at pachytene forms characteristic cross-shaped configurations. Similarly, pairing of inversion and corresponding normal chromosomes in inversion heterozygote forms an inversion loop. The positions of the breakpoints of translocations and inversions can be determined in such configurations with a much higher degree of resolution than in FISH mitotic preparations. Examples of pachytene figures from a pericentric inversion heterozygote and a reciprocal translocation heterozygote are shown in Figures 4C and 4D, respectively. One breakpoint in each rearrangement is at the *p1* locus on chromosome 1S. In
pericentric inversion \textit{p1-wwC30} (Fig. 4C), the other breakpoint is at approximately 40% of the length of chromosome 1L. In translocation \textit{p1-wwB1023} (Fig. 4D), the other breakpoint is near the telomere on 5L. The results obtained from these and other samples are summarized in Table 1. Overall, the results of pachytene analysis confirm the other mapping data and FISH analysis. The distribution of \textit{Ac/fAc}-induced MCR breakpoints is summarized in Figure 5.

\textbf{Discussion}

We have shown here that certain types of transposition reactions involving the \textit{Ac/Ds} transposable element system can generate major chromosomal rearrangements, including inversions and reciprocal translocations. Sequencing of the rearrangement breakpoints shows that these occur precisely at the termini of either \textit{Ac} or \textit{fAc}, a terminally-deleted derivative element. Moreover, the junctions of both reciprocal translocations and inversions contain complementary 8 bp sequences that most likely represent the 8 bp target site duplications (TSD) generated during \textit{Ac} transposition. Taken together, these results strongly support the hypothesis that MCRs are produced by alternative transposition reactions; i.e., transposition involving the termini of different \textit{Ac/fAc} elements (Fig. 1; see supplemental animation). According to this model, excision of the \textit{Ac/fAc} termini followed by insertion at a chromosomal target site leads directly to a rearrangement of the sequences flanking the transposon termini. Previous studies have identified other products predicted by the alternative transposition model, including 1) local rearrangement, or permutation of the sequences located between the \textit{Ac/fAc} termini that may or may not be accompanied by inversion (Zhang and Peterson 2004), and 2) deletions, some of which have fused the coding sequences of two linked paralogous genes to generate a new chimeric gene (Zhang et al. 2006). Similarly, rearrangements including inversion, deletion and local rearrangement have been generated by transposition reactions of \textit{Ac} and \textit{Ds} at the maize bronze1 locus (Dooner and Weil 2007; Huang and Dooner 2008). Another study has shown that chromosomal rearrangements including deletion, inversion and somatic translocation can also arise through transposition reactions involving \textit{Ds} elements in Arabidopsis (Krishnaswamy et al. 2008). In
all of these cases, the rearrangement junctions have been precise or nearly so. Thus, these results show that MCRs are produced directly by the Ac/Ds transposable element system, and need not involve post-excision repair functions such as Non-Homologous End Joining (NHEJ). An independent study has shown the occurrence of large deletions in Arabidopsis associated with Ds transposition, but in these cases the rearrangement junction sequences were not precise and likely resulted following host repair functions (Page et al. 2004).

**Frequency of MCRs and standard vs. alternative transposition**

We isolated MCRs using a phenotypic screen for losses of maize kernel pericarp pigmentation; the progenitor alleles specify colored pericarp, while generation of an MCR is accompanied by disruption of the p1 gene, leading to a sector of colorless pericarp. Screening is done using one of the progenitor alleles (P1-rr11, P1-rr910, or P1-ovov454) heterozygous with an allele for colorless pericarp (p1-ww or p1-wr), so that a loss of p1 function is immediately apparent as a colorless pericarp sector. Disruption of p1 function by any means could cause a colorless sector; among approximately 100 colorless pericarp mutants derived from P1-rr11, molecular analysis indicates that approximately 90% of these are the result of the alternative Ac transposition mechanism, and the overall frequency of alternative transposition is approximately 0.3% (not shown). Previous studies have shown that excision of a single Ac element from the maize p1 locus occurs at a significantly higher frequency (approximately 5%, depending upon genetic background) (Greenblatt and Brink 1962). Recently, Huang and Dooner reported that a pair of closely-linked Ac/Ds elements at the maize bronze1 locus underwent standard transposition ~5.4 times more frequently than alternative transposition reactions (Huang and Dooner 2008). The observed differences in frequency of standard vs. alternative transposition may be attributed to several factors, including the greater physical distance separating the Ac 5’ and 3’ termini involved in alternative transposition. Consistent with this idea, the number of colorless pericarp sectors we observed in our study was inversely correlated with the distances between the Ac and fAc elements present in each allele; i.e., the P1-ovov454 allele has the least distance between Ac and fAc (823 bp), and produces the highest frequency of colorless pericarp sectors.
(approximately 3 times as many as in P1-rr11). An additional consideration is that the recovery of balanced reciprocal translocations requires joining of the Ac 5’ end to the proximal side of the insertion target site (Fig. 1). Insertion in the opposite orientation (i.e. the Ac 5’ end inserted to the distal side of the target site) is expected to occur half of the time, but this would produce non-transmissable acentric and dicentric chromosomes. Even though alternative transposition may occur somewhat less frequently than standard transposition, and not all products will be viable, it is clear that alternative transposition has the potential to significantly impact chromosome structure over evolutionary time (Huang and Dooner, 2008).

**Alternative transposition and the formation of oncogenic chromosome rearrangements**

In vertebrates, V(D)J recombination produces a vast repertoire of B cell receptor (BCR) and T cell receptor (TCR) proteins. The V(D)J recombination reaction is catalyzed by RAG1 and RAG2, lymphocyte-specific enzymes encoded by two tightly-linked genes, *recombination activator gene 1* (*rag1*) and 2 (*rag2*). RAG1 can bind to recombination signal sequences (RSS) that flank the variable (V) segments, diversity (D) segments, and joining (J) segments (Chatterji et al. 2004; Rhodes et al. 2004; Jung et al. 2006). Kapitonov and Jurka proposed that the RAG1 core and RSSs evolved from the *Transib* transposon family (Kapitonov and Jurka 2005). Interestingly, the excised DNA segments flanked by two RSSs can insert into other DNA sequences *in vitro* and *in vivo*, generating characteristic target site duplications (Agrawal et al. 1998; Hiom et al. 1998; Reddy et al. 2006). These findings suggest that V(D)J recombination is mechanistically related to DNA transposition.

RSSs are composed of highly conserved heptamer and nonamer sequences, separated by a relatively non-conserved spacer of either 12 bp (12 RSS) or 23 bp (23 RSS). V(D)J recombination involving the 12 RSS flanking the 3’ end of the D segment and the 23 RSS flanking the 5’ end of the J segment results in joining of the D and J segments, with concomitant loss of the intervening sequence; this process, termed deletional rearrangement, resembles the excision step of conventional transposition. Recombination involving the 12 RSS flanking the 5’ end of the D segment and the 23 RSS flanking the 5’ end of the J...
segment can also occur, resulting in an inversion of the intervening sequence (inversional rearrangement). This latter reaction resembles the first step of Ac/Ds alternative transposition in which the 5’ and 3’ termini of different Ac/Ds elements are used as substrates. In V(D)J recombination, inversional rearrangement is estimated to occur at a frequency approximately 1/20 that of deletional rearrangement (Shuh and Hixson 2005; Jung et al. 2006). The excised RSSs produced by either deletional or inversional rearrangements can undergo reinsertion into the genome approximately once per 50,000 V(D)J recombination events (Reddy et al. 2006); hence the frequency of reinsertion of RSS following inversional rearrangement would be approximately once per $10^6$ V(D)J recombination events. Reinsertion of the RSS-containing segments produced by deletional rearrangement resembles a standard transposition reaction, and hence would not lead to formation of an MCR. Whereas, RSS reinsertion following inversional rearrangement is analagous to Ac/Ds alternative transposition, and should generate MCRs including inversions and reciprocal translocations. Considering that hundreds of millions of V(D)J recombination events occur daily during development of human lymphocytes, several hundred MCRs are likely to be generated each day in the cells of the immune system as a result of RSS insertion following inversional rearrangement. While most of these MCRs would likely be innocuous, insertions in the vicinity of proto-oncogenes could activate oncogene expression resulting in a potential lymphoid neoplasia (Marculescu et al. 2006). Our model predicts that the MCRs generated by this type of V(D)J recombination can be identified by characteristic sequence features, including a single RSS at each breakpoint, flanked by complementary 4-5 bp sequences representing the TSD formed upon RSS insertion.

**Alternative transposition and chromosomal evolution**

Alternative transposition reactions are not unique to the Ac/Ds system. In the fungus *Fusarium*, transposition involving termini of two adjacent impala elements, a member of *Tc1-mariner* family, can generate deletions and inversions (Hua-Van et al. 2002). In *Drosophila*, transposition involving the termini of different *P* elements can induce a variety of chromosomal rearrangements including deletions and inversions (Gray et al. 1996; Preston...
et al. 1996; Tanaka et al. 1997). In mice, concatemers of transgenes that contain Sleeping Beauty transposons exhibit high frequency chromosome instability and generate chromosomal rearrangements including deletions and inversions (Geurts et al. 2006); these results are consistent with the generation of rearrangements via alternative transposition reactions, although the actual mechanism is not yet known. In each of these systems, alternative transposition reactions occur when two or more transposon ends are present in a “non-standard” configuration; i.e., in either reversed or tandem orientation. It is well-known that Ac/Ds and other transposable elements exhibit a preference for local transposition (Dooner et al. 1994); the resulting clusters of elements would have a high probability of undergoing subsequent alternative transposition reactions. Thus, the MCRs generated by alternative transposition events could represent an important evolutionary mechanism for genome evolution (Huang and Dooner, 2008).

In general, closely related species exhibit karyotypic differences that can be accounted for by specific MCRs including inversions and translocations. Chromosomal evolution involves considerable rearrangements within chromosomes and between nonhomologues (Devos 2005; Tang et al. 2008). These rearrangements are particularly clear among the grasses for which many comparative maps are available (Devos 2005). In addition, rearrangements following allopolyploidization events, such as with maize, scramble the gene order from the contributing genomes. These rearrangements contribute to chromosomal evolution and likely result in part from MCRs. Also, we recently demonstrated that alternative transposition reactions can produce deletions that generate new chimeric genes and alter the expression of genes near the breakpoint (Zhang et al. 2006); similar effects on gene coding and/or expression could be expected from the translocations and inversions reported here. These genetic changes are a direct and immediate outcome of the alternative transposition reaction, and thus could promote the fixation of chromosomal rearrangements during evolution.

Materials and methods

Genetic stocks:

Alleles of the p1 gene are identified by a two-letter suffix that indicates their expression
pattern in pericarp and cob: e.g., *P1-rr* (red pericarp and red cob); *P1-wr* (white pericarp, red cob); and *P1-ww* (white pericarp and white cob). The standard *p1-vv* (variegated pericarp and variegated cob) allele described by Emerson (Emerson 1917) contains an *Ac* insertion in the second intron of a *P1-rr* gene. From *p1-vv*, we obtained a spontaneous derivative termed *P1-ovov1114* (orange-variegated pericarp and orange-variegated cob), in which the *Ac* element had undergone an intragenic transposition to a site 153 bp upstream in *p1* gene intron 2 and inserted in the opposite orientation (Peterson 1990). From *P1-ovov1114*, we obtained a spontaneous derivative termed *p1-vv9D9A* (Zhang and Peterson 1999); this allele contains an *Ac* element, a 112-bp rearranged *p1* gene fragment, and the terminally deleted *Ac* element *fAc*. From *p1-vv9D9A*, we obtained the alleles *P1-rr11, P1-rr910*, and *P1-ovov454* described here; these alleles were generated from *p1-vv9D9A* by transposition of the full-length *Ac* element from its location in intron 2 of *p1* to sites 13175 bp, 8919 bp and 823 bp, upstream of *fAc*, respectively (Fig. 1A).

**Screening candidate transposition-induced MCRs**

Most products of reversed *Ac* ends transposition are predicted to have a deletion of *p1* gene exon(s) 1 and 2, or separation of exons 1 and 2 from exon 3, resulting in a loss of *p1* gene function and the appearance of colorless sectors. The size of sector generated reflects the time of development at which the particular event occurred; early events give rise to large multi-kernel sectors, whereas later events give rise to single-kernel sectors and colorless stripes. To identify putative MCRs, we screened the mature ears produced by plants carrying one of the parental alleles (*P1-rr11, P1-rr910*, or *P1-ovov454*) for large colorless pericarp sectors (Fig. 2A) and single colorless kernel sectors. Reversed *Ac* ends transposition is predicted to generate a wide variety of possible products (see supplemental animation). In this study, we focused on the detection and characterization of large inversions (Fig. 1C) and reciprocal translocations (Fig. 1D), both of which are expected to carry an active *Ac* element. *Ac* can be detected by its ability to induce excision of *Ds* from the *r1-m3::Ds* allele of the maize *r1* gene, which is required for kernel aleurone pigmentation. Hence we selected kernels with colorless pericarp and spotted aleurone as containing candidate rearrangement
PCR amplifications:
PCR amplifications were performed as described (Saiki 1989) using the oligonucleotide primers shown in Table 2. HotMaster Taq polymerase from Eppendorf (Hamburg, Germany) was used in the PCR reaction. Reactions were heated at 94° for 3 min and then cycled 35 times at 94° for 20 sec, 60° for 30 sec, and 65° for 1 min/1 kb length of expected PCR product and then at 65° for 8 min. In most of the PCR reactions, 1 M betaine and 4%–8% DMSO were added. The band amplified was purified from an agarose gel and sequenced directly. Sequencing was done by the DNA Synthesis and Sequencing Facility, Iowa State University.

Ligation medicated PCR (LM-PCR)
2.5µg Genomic DNA is digested with 20 units of HpyCH4IV (New England Biolabs, Ipswich, MA) in total 20µl volume at 37°C for 3 hours, then a mix containing the corresponding adaptor, HpyCH4IV buffer, ATP, and T4 DNA ligase (New England Biolabs, Ipswich, MA) was added to the restriction digestion mix. The total volume is 40µl; the final concentration of the restriction digestion buffer is 1X, the final concentration of the adaptor is 0.5 pmol/µl, the final concentration of ATP is 0.5 mM, and the final concentration of T4 DNA ligase is 20 cohesive end units/µl. Ligation is carried out at 22°C overnight; 2µl of the ligation mix is used as template in the first round of the nested PCR reaction, and 1µl of the mix of the first round PCR is used as template in the second round of PCR. PCR is performed as described above.

HpyCH4IV Adaptor: GTA TCA CCA CCA GAG GAG CAA GCG AGT TCA CAG AAT CAC ACG AGT AGA GT and C TCA TCT CA GC
Primer in the first round PCR: CCA CCA GAG GAG CAA GCG AGT TCA CAG AAT
Primer in the second round PCR: G CAA GCG AGT TCA CAG AAT CAC ACG AG
The above two primers are complementary to the adaptor sequence. Other primers used in this paper are summarized in table 2. Note: Primers 1, 2, and 6 differ between alleles due to
the distinct Ac insertion sites; primers 10-15 are specific for each rearrangement allele; their sequences are not shown here

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<td>2</td>
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<td></td>
<td>6</td>
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**Cloning the breakpoints of MCR candidates**

Ac casting was performed as described by Singh *et al.* (Singh *et al.* 2003) to clone the breakpoint sequences flanking Ac. As shown in Figure 6, the sequences upstream of Ac (black line) are known, whereas the sequences downstream of Ac (green line) are unknown. Because Ac tends to transpose locally, Ac may excise and reinsert at a site close to the breakpoint in opposite orientation. Such short-range transposition events can be detected in DNA prepared from somatic tissues by amplification with nested PCR using primer 1/4 and 2/5 (Fig. 6). To avoid the negative Ac dosage effect (the higher Ac dose, the less frequent Ac transposition), DNA templates were made from rearrangement heterozygous plants carrying
only a single copy of Ac in the genome. The first round of PCR was performed as described above, and 1 µl of the product of the first round PCR was used as template for the second round PCR. To obtain the precise MCR breakpoint junctions, we performed additional PCR using primer 5 paired with a primer (primer 11 or 14, Fig. 1) complementary to the flanking sequence (obtained via Ac casting). In some cases, the Ac element had undergone a germinal excision from the rearrangement breakpoint; these breakpoints were cloned by LM-PCR using primers 1 and 2 (Fig. 1) as described above.

The model for generation of MCRs predicts that the breakpoint sequences flanking the Ac/fAc termini were derived from the Ac/fAc insertion target site, and thus should be present as contiguous sequences in the progenitor genome (a/b or c/d in Fig. 1). Therefore, we used BLAST to compare the sequences adjacent to the 5’ Ac termini in MCRs (obtained from Ac casting or LM-PCR, above) with the maize sequence database (http://www.plantgdb.org) in order to identify the sequences predicted to flank the 3’ fAc element. These sequences were used to design a new primer (primer 12 or 13, Fig. 1) which was used in PCR with primer 3 to amplify the breakpoint sequence flanking the fAc element (Fig. 1). If the BLAST analysis of the sequence flanking Ac did not produce a highly similar match, then two additional primers (primers 10 and 11 or 14 and 15, Fig. 1) complementary to the breakpoint sequence flanking Ac were used in ligation-mediated PCR (LM-PCR) to amplify the target site sequence from the progenitor alleles (P1-rr11, P1-rr910 or P1-ovov454). Then, a primer (primer 12 or 13, Fig. 1) was designed based on this new sequence and paired with primer 3 to amplify the breakpoint sequence flanking the fAc element.

**Chromosome preparation and FISH:**

Somatic chromosome spreads were produced as previously described (Kato et al. 2004) except the concentration of cellulase was increased from 2 to 4% in the enzymatic mixture for root-tip digestion and slides were used within 4 hr of preparation. Probe hybridization was carried out at 55° for 12–24 hr and washed in 2x SSC for 20 min (Kato et al. 2004). Chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI) containing Vectashield mounting media (Vector Laboratories, Burlingame, CA). Signals were captured

<table>
<thead>
<tr>
<th>Probe</th>
<th>dek1</th>
<th>TAG</th>
<th>5S rDNA</th>
<th>myo1, rp3</th>
<th>Cent4</th>
<th>Exp1</th>
<th>serk2, rf2e1</th>
<th>NOR</th>
<th>BAC8L</th>
<th>BAC9S</th>
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<td>2L</td>
<td>3L</td>
<td>4</td>
<td>5L</td>
<td>5L</td>
<td>6S</td>
<td>8L</td>
<td>9S</td>
</tr>
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*In addition to 1L, “TAG” microsatellite can hybridize with chromosome 2 and the short arm of chromosome 4; the signal patterns are recognizably distinct in each chromosome.

**Cytological analysis of male inflorescences**

Cytological analysis of male inflorescences undergoing meiosis was carried out in plants heterozygous for the rearrangements to determine the breakpoints in the rearrangements. The immature tassels were fixed in a 3:1 mixture of 95% ethanol : propionic acid (v : v) at room temperature for one day and then maintained at -20C. Cells at the pachytene stage of prophase I were stained with a propio-carmine solution (Sharma and Sharma 1965).

**Acknowledgments**

We thank Ronald L. Phillips (University of Minnesota) and members of his lab for providing genomic DNA from oat-maize addition lines; Pat Schnable (Iowa State University) and members of his lab for the TGCE genetic mapping of MCR breakpoints (http://magi.plantgenomics.iastate.edu/index.html); and Tim Bruihler, Lisa Coffey, Chris Cosgrove, Ryan Dietz, Peter Howe, Klint Kersten, Amanda Kopp, and Aaron Newell for field and laboratory assistance. This research was supported by NSF MCB 0450243 to TP and JZ, NSF MCB 0450215 to DW, and NSF DBI 0423898 to JB.

**References**


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**Figure 1** Model for generation of Major Chromosome Rearrangements by Reversed Ac ends Transposition. The lines depict maize chromosomes, with centromeres indicated by black and green circles. The red arrows indicate Ac (double-headed arrow) and fAc, a ~2.0 kb fragment containing the 3’ end of Ac (single arrow head). The open and solid arrowheads indicate the 3’ and 5’ ends, respectively, of Ac/fAc. The fAc element is inserted into the
second intron of the maize *p1* gene, whose exons are indicated by solid boxes. The small vertical arrows indicate the Ac transposase cleavage sites.

(A) *Ac* transposase cleaves at the 5' end of *Ac* and the 3' end of *fAc*.

(B) Following transposase cleavage at the junctions of *Ac/p1* and *fAc/p1*, the internal *p1* genomic sequences are joined to form a circle. The "×" on the circle indicates the site where joining occurred, marked by a transposon footprint. The *Ac* 5' and *fAc* 3' ends are competent for insertion anywhere in the genome. *C* and *D* depict the outcomes of insertion into two possible target sites (short vertical lines).

(C) The *Ac/fAc* termini insert into a site on the opposite arm of the same sister chromatid; in the upper figure, the *Ac* 5' end joins to the proximal side of the target site to form a ring chromosome, and the *fAc* 3' end joins to the distal side of the target site to form an acentric fragment. Alternatively, ligation of the *Ac* 5' end to the distal side of the target site and the *fAc* 3' end to the proximal side of the target site would generate a pericentric inversion (lower).

(D) The transposon ends insert into a site in another chromosome; the *Ac* 5' end joins to the distal side of the target site, and the *fAc* 3' end joins to the proximal side of the target site to generate a reciprocal translocation.

The short horizontal arrows indicate the orientations and approximate positions of PCR primers. Primers are identified by numbers above or below the arrows. The structures of *P1-rr11* and *P1-rr910* are similar to that of the *p1* allele in *IA*; i.e., the *Ac* element is located upstream of the *p1* gene; in *P1-ovov454*, however, the *Ac* element is inserted in intron 2 of *p1*. Primers 1, 2, and 6 differ for alleles *P1-rr11*, *P1-rr910*, and *P1-ovov454* due to differences in the *Ac* insertion sites; see Materials and methods for details.

**Figure 2** Phenotypic effects of reciprocal translocations and large inversions.

(A) Mature ear of progenitor allele *P1-rr11* (red pericarp kernels), with origination sector of translocation *p1-wwB966* (large sector of kernels with colorless pericarp). Note that kernels in the colorless pericarp sector are larger and irregularly spaced because ~50% of the eggs have aborted (compare with smaller, evenly-spaced kernels in red pericarp portion of ear).
Kernels with purple-spotted aleurone indicate Ac-induced excision of Ds from r1-m3::Ds allele; see Materials and methods for details.

(B) Pollen from a large inversion heterozygote exhibiting ~50% semi-sterility. The larger, starch-filled pollen grains (dark) carry a complete haploid genome, whereas the smaller, irregular, translucent pollen grains carry an imbalanced chromosome set following meiosis in the inversion heterozygote.

**Figure 3** MCR breakpoint mapping by PCR using oat-maize addition lines as templates. PCR was performed using primers complementary to the sequences at the predicted MCR breakpoints. Sources of template genomic DNA are indicated at the top of each lane. Lanes marked 1 to 10 indicate DNA from oat-maize addition lines containing maize chromosomes 1 to 10, respectively. Lane marked Progenitor contains DNA from the maize allele which was the progenitor of the MCR (P1-rr11, P1-rr910, and P1-ovov454).

(A) p1-wwB966.

(B) p1-wwB1023.

(C) p1-wwC30.

**Figure 4** Cytogenetic analysis of MCR alleles. Cells of plants heterozygous for a rearrangement and corresponding normal chromosomes were characterized by FISH of mitotic metaphase chromosomes (A and B), and by propiocarmine staining of meiotic pachytene chromosomes (C and D). Arrows indicate sites of characteristic features (C and D).

(A) Chromosome 1 pericentric inversion heterozygote (p1-wwC30) hybridized with dekl (red, detects 1S) and TAG (green, detects 1L; also 2S, 2L and 4S). Inversion chromosome is enlarged in inset.

(B) Translocation chromosome T1-5 heterozygote (p1-wwB1023) hybridized with dekl (red, detects 1S), TAG (white, detects 1L; also 2S, 2L and 4S), and serk2-rf2e1 (green; detects 5L). T5-1 translocation chromosome is enlarged in inset.

(C) Chromosome 1 pericentric inversion heterozygote (p1-wwC30).
(D) Translocation chromosome T1-5 heterozygote (p1-wwB1023).

**Figure 5** Distribution of chromosomal rearrangement breakpoints.
Schematic diagram of maize chromosomes 1 to 10 (left to right) divided into genetic bins by blue lines. In each rearrangement, one breakpoint is in bin 1.03, at the site of Ac/fAc insertions in or near the p1 and dek1 genes (yellow); positions of the other breakpoint in each rearrangement allele are shown schematically in white. Notations in red, below chromosomes, indicate those rearrangements that map to particular chromosomes, but whose position within the chromosome is unknown. Evidence supporting the map positions shown here is summarized in Table 1.

**Figure 6** Local somatic Ac transposition as the basis for Ac casting (Singh et al. 2003). All the symbols have the same meaning as those in Figure 1.

**Supplemental Figure 1** FISH analysis of p1-wwB521. Translocation chromosome heterozygote (p1-wwB521) was hybridized with myo1 (red, detects 3L) and TAG (green, detects 1L; also 2S, 2L and 4S). Hybridization signals on translocation chromosome indicate that the breakpoint is located on chromosome 3L, very close to the centromere.

**Supplemental Figure 2** FISH analysis of p1-wwB469. Translocation chromosome heterozygote (p1-wwB469) was hybridized with dek1 (red, detects 1S), cent4 (green)(Jin et al. 2004), and TAG (white, detects 1L; also 2S, 2L and 4S). The results indicate that p1-wwB469 contains a presumptive translocation chromosome that hybridizes with both the dek1 (1S) and cent4 (4) probes; in addition, TAG probe hybridized in two positions on the same presumptive translocation chromosome. Overall, these results suggest that p1-wwB469 contains a T1-4 translocation, with the breakpoint on the short arm of chromosome 4.

**Table 1. MCR breakpoint mapping and cytogenetic characterization.**
The results in each column indicate the chromosome and/or map position of the MCR breakpoints identified by the indicated techniques. For alleles \textit{p1-wwB47}, \textit{p1-wwC22}, and \textit{p1-wwB531}, \textit{NS (No Signal)} indicates that no PCR products were observed from oat-maize addition line templates using primers complementary to the candidate MCR breakpoint sequences. For \textit{p1-wwB469}, multiple bands in more than one oat-maize addition line were observed, but only the addition line containing maize chromosome 4 yields a band of the expected size. For \textit{p1-wwD41}, PCR products of similar size were amplified from the oat-maize addition lines containing either maize chromosome 2 or maize chromosome 7; however, the PCR product from the addition line containing maize chromosome 7 has much greater similarity to the breakpoint sequence than that from the chromosome 2 line (98% vs 81%). In column marked TGCE, “Non-polymorphic” indicates that the sequence was not polymorphic in the mapping population used. For allele \textit{p1-wwB576}, both oat-maize PCR and sequence results indicate the presence of a T1-5S translocation, while FISH analysis indicates a T1-9L translocation. Further genetic analysis (not shown) has confirmed the presence of a T1-5S translocation. The anomalous FISH results may be due to seed misidentification, or structural heterogeneity among the different maize lines used in each technique.

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Somatic Ac transposition
Zhang_Supplemental Figure 2
CHAPTER 4. CHARACTERIZATION OF CIS-REARRANGEMENTS INDUCED BY ALTERNATIVE TRANSPOSION OF REVERSED AC/DS TERMINI IN MAIZE.

A paper to be submitted to Genetics

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Running Head: Transposon induces flanking deletion by insertion to sister chromatid

Key Words: transposon, chromosome breakage, Ac/fAc, sister chromatid, pl

Abstract

Alternative transposition can induce genome rearrangements, including deletions, inverted duplications, inversions and translocations. To investigate the types and frequency of the genome rearrangements caused by a pair of reversed Ac/Ds termini, we screened 100 new mutant alleles induced by an intact Ac and a fractured Ac (fAc) structure at the maize pl locus. The rearrangement types were characterized by PCR pattern analysis and/or by direct sequencing. The results show that approximately half of the mutant alleles (52/100) comprise various inversions, translocations or rearrangement of the inter-transposon sequence. Among 37/100 deletions, 20 have deletions extending into the external flanking sequence, while 17 have deletions within the inter-transposon sequence. Interestingly one deletion allele, with only one nucleotide distance between Ac and fAc, is not competent for alternative transposition, evaluated by chromosome breakage. Finally, 11/100 alleles appear to have arisen by simple Ac excision or other unknown mechanism. We propose a new model for the formation of inter-transposon deletions through insertion of reversed transposon termini into sister chromatid sequences. These results document the types and frequencies of genome rearrangements induced by alternative transposition of reversed Ac/Ds termini in maize.
Introduction

Transposable elements and repeat sequences comprise the majority of many eukaryotic genomes. In maize, it is estimated that approximately 78% of the maize genome is composed of transposable elements and other repeated sequences (Walbot 2008). Transposable elements play important roles in plant gene evolution and genome reorganization (Bennetzen et al. 2005; Dooner and Weil 2007). Transposable elements are broadly classified as either retroelement (Class I), or DNA transposon (Class II) (Wicker et al. 2007). Retroelement transposition occurs via a “copy and paste” mechanism, which can lead to massive amplification and is thought to be a major contributor to genome size differences. In contrast, most DNA elements move by a “cut and paste” mechanism. Although DNA elements are less abundant compared with retroelements, they can lead to a variety of genome rearrangements (Gray 2000; Huang and Dooner 2008; Ross et al. 1979; Watanabe et al. 2007) including gross chromosome rearrangement in eukaryotes which may contribute to genome evolution (Lister et al. 1993; McClintock 1951; McClintock 1978; Zhang et al. 2009).

The well-known Ac/Ds elements in maize were initially identified by Barbara McClintock through their ability to induce chromosome breakage. In addition, McClintock identified a number of major chromosome rearrangements that were apparently induced by the chromosome-breaking Ac/Ds system (McClintock 1951; McClintock 1978). Subsequent genomic cloning and sequence analysis suggested that the original chromosome-breaking Ds element has a “double Ds” structure; i.e. one Ds element is inserted within a second identical Ds, in the opposite orientation (Doring et al. 1984; Klein et al. 1988; Martinez-Ferez and Dooner 1997). Chromosome breakage is thought to occur when the Ac transposase attempts to transpose a pair of directly-oriented Ds 5’ and 3’ termini located on different sister chromatids (English et al. 1993). This reaction, termed sister-chromatid transposition, can induce not only chromosome breakage but also can generate deficiencies and inverted duplications (Zhang and Peterson 1999) (Zhang et al. 2006). A second type of alternative transposition involves reverse-oriented 5’ and 3’ Ac/Ds termini; for example, two directly-oriented Ac or Ds elements will have their apposed 5’ and 3’ termini in reversed
orientation. Previously our lab isolated and characterized the \textit{P1-rr11} allele which contains a full-length \textit{Ac} element and a terminally deleted \textit{Ac (fAc)} inserted in the maize \textit{p1} locus. The 5’ end of \textit{Ac} and the 3’ end of \textit{fAc} are in reversed orientation, separated by a ca. 13 kb intertransposon segment. In this configuration, reversed-ends transposition can induce a variety of chromosome rearrangements, including deletions, inversions, and translocations (Zhang and Peterson 2004; Zhang et al. 2009). In addition, this reversed-ends configuration can also induce chromosome breakage (Huang and Dooner 2008; Yu et al. submitted).

In this paper, we investigated the genome rearrangement types and frequency produced by reversed ends transposition at \textit{p1} locus. The results show a high relative frequency of deletions and inversions, in agreement with previous reports (Huang and Dooner 2008; Zhang and Peterson 2004). In addition, we identified a substantial class (17%) of previously uncharacterized new mutant alleles which contain deletions of various segments of the DNA between the reversed \textit{Ac/Ds} termini. Based on the structures of these deletions and the known propensity of \textit{Ac/Ds} elements to transpose during DNA replication (Greenblatt 1968; Greenblatt 1974; Greenblatt 1984; Greenblatt and Brink 1962), we propose a model for their formation. In addition, we investigated the frequency of chromosome breakage induced by the resulting alleles which contain various lengths of DNA separating the reversed \textit{Ac/Ds} termini. Interestingly, the chromosome breakage frequency (and hence the alternative transposition frequency) declines precipitously for alleles in which the reversed \textit{Ac/Ds} termini are in very close proximity (91 bp and 1 bp).

Materials and Methods

Maize stocks and crosses: The maize alleles \textit{P1-rr11}, \textit{P1-rr910} and \textit{p1-ovov454} were isolated and described previously (Yu et al. submitted; Zhang and Peterson 2004; Zhang et al. 2009). Plants in which the \textit{P1-rr11}, \textit{P1-rr910} or \textit{p1-ovov454} alleles were heterozygous with an allele for colorless kernel pericarp (\textit{p1-ww} or \textit{p1-wr}) were crossed by pollen from plants of genotype \textit{p1, r1-m3::Ds}. The \textit{r1-m3::Ds} allele contains a \textit{Ds} element inserted in the \textit{r1} gene required for kernel aleurone pigmentation; \textit{Ac}-induced excision of \textit{Ds} from \textit{r-m3::Ds...
results in purple aleurone sectors (Kermicle 1980). The mature ears were screened for kernels with colorless pericarp and spotted aleurone, as these were predicted to have undergone a loss of $p1$ function, but to have retained Ac activity. Selected kernels were sown, and pollen from mature plants was checked using a hand microscope to estimate pollen abortion frequency. Plants were self-pollinated to homozygose the new mutant alleles for molecular analyses.

**DNA extraction and PCR analyses:** 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). HotMaster Taq polymerase from Eppendorf (Hamburg, Germany) was used in the PCR reaction. The PCR mix was heated at 94°C for three minutes to denature the DNA template, followed by 35 cycles of 20 sec at 94°C, 30 sec at 60°C, and 1 kb/1 min at 65°C, and one cycle of 8 min at 65°C. Primers used for PCR analyses are listed in Table 1; primer locations are shown in Figure 2. Candidate local rearrangement alleles were analyzed using the following primer pairs: P1-2915r and P1-15588f (for alleles derived from P1-rr11), or P1-7061r and P1-15588f (for alleles derived from P1-rr910). Candidate internal deletions flanking the Ac 5’ terminus were analyzed using the following primers: Ac120r plus one of Ac4436f, P1-12046r or P1-7061r (for alleles derived from P1-rr11); and Ac120r plus one of Ac4436f or P1-12046r (for alleles derived from P1-rr910). Candidate internal deletions flanking the fAc 3’ terminus were analyzed using the following primers: Ac4436f plus one of Ac120r, 9D9A5537f, P1-7869f or 9D9A33680f (for alleles derived from P1-rr11); and Ac4436f plus one of Ac120r, P1-7869f, or 9D9A33680f (for alleles derived from P1-rr910). PCR products were excised from agarose gels, purified using the Perfectprep gel cleanup system (Eppendorf HotMaster Taq, Westbury NY), and sequenced by the DNA Synthesis and Sequencing Facility, Iowa State University.

**Evaluation of chromosome-breakage frequency:** The maize Dekl gene is required for differentiation of kernel aleurone cells; endosperm cells lacking Dekl function fail to differentiate into aleurone cells and thus cannot synthesize anthocyanin pigments (Lid et al. 2002). Wild-type Dekl 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. The *dek1/Dek1* plants were then crossed as ear parents by pollen from plants homozygous for the candidate *p1* alleles to be tested. Kernels of mature ears were examined and the numbers of colorless sectors were used as an index to the chromosome-breakage frequency. The chromosome-breakage frequency grading is evaluated according to (YU et al. 2009).

**Results**

**Inversion and deletion are the major products of reversed-ends transposition.**

The *P1-rr11* and *P1-rr910* alleles condition predominantly red kernel pericarp pigmentation with white (colorless) sectors of variable size (ZHANG and PETERSON 2004; ZHANG et al. 2009). Both alleles contain an identical *fAc* element in the second intron of the *Pl* gene and a full-length *Ac* element upstream of the *Pl* transcription start site. The distances between the *Ac* 5’ terminus and *fAc* 3’ terminus in *P1-rr11* and *P1-rr910* are approximately 13 kb and 9 kb, respectively. In both alleles, *p1* exons 1 and 2 are located between the *Ac/fAc* termini and are predicted to be deleted by reversed-ends transposition (ZHANG and PETERSON 2004; ZHANG et al. 2009). Therefore, we reasoned that new alleles arising from reversed-ends transposition could be selected based on loss of *p1* function (colorless kernel pericarp) and retention of *Ac* activity (purple aleurone sectors) (Materials and Methods). In this way we selected and characterized a total of 100 putative mutant alleles (42 and 58 derived from *P1-rr11* and *P1-rr910*, respectively).

To determine what types of structural changes had occurred in the mutant alleles, we performed PCR using four pairs of primers specific for the four junctions of *Ac/fAc* and the flanking genomic DNA: (1) the *Ac* 3’ terminus; (2) the *Ac* 5’ terminus; (3) the *fAc* 3’ terminus; and (4) the *fAc* 5’ end junction (Figure 1A and Table 2). Representative gel analysis results are shown in Figure 1B. According to the alternative transposition model proposed previously (HUANG and DOONER 2008; KRISHNASWAMY et al. 2008; ZHANG and PETERSON 2004), we analyzed and interpreted the data as shown in Table 3.
The largest class (52%) was composed of alleles in which PCR primer pairs 1 and 4 were both positive while pairs 2 and 3 were negative (pattern $+ - - +$). This pattern could be produced by one of several different types of rearrangements including inversions, translocations, and local rearrangements (classes a, b and c).

a. Translocations and large inversions (5 cases). Chromosome translocations and very large inversions would be expected to exhibit a higher than normal percentage of pollen abortion (50% for translocations, and up to 50% for inversions depending on size). By screening for pollen abortion followed by further molecular characterization we identified four translocations ($p1$-wwC15, $p1$-wwB47, $P1$-wwB33, $p1$-wwB1023) and one pericentric inversion ($p1$-wwC30). These cases have been described elsewhere (ZHANG et al. 2009).

b. Small inversions (46 cases). Alleles with a $+ - - +$ pattern and normal pollen abortion frequency were classified as putative inversions of relatively small size. Large inversions would be expected to exhibit significant levels of pollen sterility resulting from crossovers within the inverted segment.

c. Local rearrangements (1 case). Local rearrangements are defined as those cases in which the intertransposon sequences (ITS) have been circularly permuted; i.e., the sequences have remained between the transposon termini, but they have changed their relative position and, in some cases, their orientation. These permutations result from reversed-ends transposition followed by insertion into the ITS of the same chromatid (HUANG and DOONER 2008; ZHANG and PETERSON 2004). Candidate local rearrangement alleles were subject to one additional PCR test designed to detect the junction formed by excision of the $Ac/fAc$ termini (MATERIALS and METHODS). Only one allele (derived from $P1$-rr11) was confirmed as a local rearrangement.

d. External deletion (20 cases). Alleles in which only PCR primer pair 1 or pair 4 give a positive result (pattern $- - - +$ or $+ - - -$) were classified as probable $p1$ left- or right-side deletions.

e. Internal deletions (17 cases). PCR pattern is $+ - + +$ or $+ + - +$. Characterization of these alleles is described further below.
f. No Ac/fAc structure (6 cases). This class produced no positive reaction with any of the four primer pairs (− − − − pattern). These probably represent segregation of the wild-type allele together with a transposed Ac. They were not investigated further.

g. Simple Ac excision (2 cases). The PCR pattern (− − + + pattern) from each of P1-rr11 and P1-rr910 derived alleles. It can be explained by Ac excision from the original locus.

h. Unknown (2 cases). Two cases show PCR patterns (+ + − − and + + + − ) that are not expected from any known single standard or alternative transposition event. The origin of these cases is unknown and will not be considered further here.

Previous research has identified some cases of “fused ends”; i.e., cases in which the ITS is deleted and the 5’ and 3’ Ac/Ds termini are ligated together (GORBUNOVA and LEVY 1997; KRISHNASWAMY et al. 2008). The presence of fused ends among the mutant alleles tested here could be detected by PCR with Ac 5’ end primer (Ac120r) and Ac 3’ end primer (Ac4436f) (Table 2). Three alleles (p1-wwB42, P1-wwB54, p1-wwB59) from P1-rr11 showed a single strong band, but subsequent sequence analysis showed that each of these cases was in reality an internal deletion (+ + − + pattern). In summary none of the alleles we identified here contains fused Ac/fAc ends.

**Sequence analysis of the flanking Ac 5’ end or fAc 3’ end deletion lines.**

The breakpoints of 12 of the other 14 internal deletion alleles were isolated by PCR and sequencing (MATERIALS AND METHODS). DNA sequence results show that in each case, the Ac/fAc structure is intact, and the deletions are confined to the inter-transposon sequences flanking either Ac or fAc (Figure 2). Each deletion extends precisely from either the Ac 5’ end or the fAc 3’ end to a site in the intervening DNA. No single allele has a deletion of sequences flanking both the Ac and fAc termini. Because the deletions were selected on the basis of colorless kernel pericarp (i.e., lack of p1 function), it is expected that each deletion should remove some sequences essential for p1 expression. Indeed, all deletions but 1 (p1-wwB3) have removed part or all of p1 gene exons 1 and 2, which encode the Myb-homologous DNA binding domain of the p1 transcriptional activator protein. The
The results described above show that deletions occur within the inter-transposon segment at a significant frequency. In order to gain more insight into the mechanism of deletion formation, we wanted to determine the frequency of deletions flanking the *Ac* 3’ end; i.e., the *Ac* terminus which is adjacent to the external flanking sequence (Figure 2). However, we could not readily detect deletions from this terminus in the *P1-rr11* and *P1-rr910* alleles because such deletions would not remove sequences essential for *p1* function. Therefore we screened for deletions using a third allele termed *P1-ovov454* which conditions orange variegated pericarp and cob; i.e., orange tissue with red and colorless sectors. In the *P1-ovov454* allele, both *Ac* and *fAc* are located in the *p1* gene intron 2 separated by a distance of approximately 0.8 kb (Figure 2) (Zhang et al. 2009). Importantly, deletions extending from the *Ac* 3’ end into the upstream flanking sequence could remove *p1* exons 1 and or 2, both of which are essential for *p1* function. To screen for such deletions, we selected 133 kernels with colorless pericarp and spotted aleurone from *P1-ovov454* heterozygotes. These kernels should carry a non-functional *p1* gene, and retain *Ac* activity. Plants grown from these kernels were screened by PCR using primers Ac4436f and Ac120r to identify those plants which retained the *Ac/fAc* structure of the progenitor allele. Only three of the 133 plants tested positive for this band. The other 130 plants most likely contain deletions of the ITS as a consequence of reversed-ends transposition involving the *Ac* 5’ end and *fAc* 3’ end. The three plants which retained the *Ac/fAc* structure were then tested using an *Ac* 3’ end primer (Ac4436f) together with a flanking sequence primer (PA-A11) in order to detect
possible one-sided deletions. Two of the plants were positive for this product; i.e., they retained an intact Ac 3’ junction and hence did not contain flanking deletions. One of the three tested plants was negative for the Ac 3’ flanking sequence product, and therefore likely represents a Ac 3’ flanking deletion. These results indicate that one-sided deletions flanking the Ac 3’ end are rare (1/133) compared with inter-transposon deletions flanking the fAc 3’ end (8/100) (Table 4). Due to this approximately 7-fold difference in deletion frequency, we propose a specific mechanism for the formation of inter-transposon deletions (See Discussion).

Reversed Ds ends in very close proximity are not competent for alternative transposition in maize.

Previously we reported that alleles containing pairs of reverse-oriented Ac/Ds ends can cause chromosome breakage, and chromosome breakage frequency is inversely proportional to the distance between the element termini (Yu et al. 2009). The previous study examined pairs of Ac/Ds elements separated by distances ranging from 0.8 kb to 13 kb. To examine the effects of shorter inter-transposon distances on chromosome breakage, we tested the intertransposon deletion alleles described here for the frequency of loss of a distal visible marker gene (Dek1; MATERIALS AND METHODS). Most of the inter-transposon deletion alleles derived from P1-rr11 or P1-rr910 show increased chromosome breakage as the distance between the reversed Ac/Ds ends decreases (Table 5 and Figure 3). For example, allele P1-wwB54 has an Ac/fAc separation distance of 331 bp and exhibits a relatively high chromosome breakage frequency (Grade 2.9). However, two alleles with shorter element separation distances have drastically reduced chromosome breakage: p1-wwB59 (91 bp between Ac/fAc) exhibits a relatively low chromosome breakage frequency (Grade 1.0). This is the same frequency as that of the progenitor allele P1-rr11, in which the Ac/fAc termini are separated by 13 kb. Even more striking, p1-wwB42, which has only one nucleotide separating the Ac/fAc termini, exhibits no detectable chromosome breakage (Grade 0). To determine whether these alleles have mutation(s) in the Ac/fAc termini that would render them immobile, approximately 120 bp of the 5’ Ac and 3’ fAc termini and subterminal
regions were sequenced, but no changes were found. We conclude that reversed Ac/Ds ends in very close proximity (<100 bp) have reduced frequencies of alternative transposition.

**Discussion**

The aim of this study is to investigate the types and frequency of genome rearrangements induced by reversed Ac/Ds termini at the maize P1 locus. The results indicate that about half of rearrangements are chromosome inversion, translocation or local rearrangement (ITS rearrangement); 20% are chromosome external deletions; and 17% are internal deletions (ITS deletion). Although the alternative transposition frequency increases as the reversed Ac/fAc distance decreases, one internal deletion allele, with only one nucleotide distance between Ac and fAc, is not competent for alternative transposition as evaluated by chromosome breakage. Here, we propose a mechanism for the generation of small internal deletions, and we discuss the implications of our results for the development of transposon-based genome rearrangement tools.

**Models for generation of transposon-induced flanking deletions.**

The high frequency of inter-transposon deletions flanking Ac/fAc termini in P1-rr11 and P1-rr910 prompted us to consider how these alleles were generated. Previous studies (DOONER *et al.* 1988; HUANG and DOONER 2008) proposed that deletions flanking Ac or Ds termini could be explained by abortive transposition of a single transposon end. A similar model was also proposed for deletions flanking the bacterial Tn5 transposon (JILK *et al.* 1993) (Figure 4). If this model is correct, then one may expect that the frequency of “one-sided” deletions flanking the Ac 3’ end and the fAc 3’ end should be similar. This is based on the premise that transposition of the Ac 3’ end should occur at least as frequently as that of the fAc 3’ end. However, we detected significantly more “one-sided” deletions flanking the fAc 3’ end than the Ac 3’ end (8/100 vs. 1/133, respectively).

In an alternative model, Page et al proposed a hybrid transposition mechanism to explain the formation of deletions flanking Ds elements in Arabidopsis (PAGE *et al.* 2004). Two transposition steps are involved: First, a newly-replicated Ds element inserts into a nearby unreplicated region and forms a pair of Ds elements in reversed orientation. In a
subsequent transposition reaction, the 5' end of one Ds and the 3' end of the other Ds excise, leading to deletion of the internal sequence. The chromosome arm is repaired by ligation of the two broken ends. This model does not seem to apply to the deletions we isolated for at least three reasons: 1) The fAc in the P1-rr11 and P1-rr910 alleles is immobile and hence cannot participate in the first transposition reaction of the Page et al. model. 2) The deletion junctions we isolated are precise and exhibit no evidence of the involvement of NHEJ-type repair. 3) This model does not explain the difference in frequency of deletions flanking the external Ac 3' side vs. the inter-transposon fAc 3' end.

The Drosophila P element can undergo a type of alternative transposition involving the termini of P elements located on homologous chromosomes (Hybrid element insertion model) (PARKS et al. 2004; PRESTON et al. 1996). If such a hybrid element inserted into nearby DNA, a “one-sided” flanking deletion could be generated (Figure 5). However, no evidence for a similar hybrid element mechanism was detected in two different experiments in maize (YU et al. 2009). Previous work has shown that pairs of Ac/Ds elements on the same chromosome can induce chromosome breakage by alternative transposition mechanism (HUANG and DOONER 2008; WEIL and WESSLER 1993; YU et al. submitted). When the distance between the two interacting Ac/Ds/fAc termini is increased to >200-kb, the chromosome breakage frequency is highly reduced (HUANG and DOONER 2008). Therefore, even if alternative transposition reactions involving hybrid elements were to occur, the frequency is expected to be too low to explain the relatively high frequency of inter-transposon segment deletions observed for the P1-rr11 and P1-rr910 alleles.

We have previously presented evidence that pairs of reversed Ac/fAc termini on the same chromatid are competent for transposition (ZHANG and PETERSON 2004). To explain the high frequency of inter-transposon segment deletions, we propose that, during reversed-ends transposition, the active Ac/fAc termini can insert into the region between Ac and fAc on the sister chromatid; this scenario would produce two daughter chromatids containing reciprocal deletions of the inter-transposon segment. Each resulting chromatid would have a deletion flanking either the 5' Ac or 3' fAc ends (Figure 6). Insertion of the Ac/fAc termini in the opposite orientation would lead to formation of a chromatid bridge and chromosome
breakage, as previously proposed (Huang and Dooner 2008; Yu et al. 2009). This model fits previous observations, including: (1) Ac/Ds elements exhibit a pronounced preference for local transposition; (2) the Ac element from the standard p1-vv allele often inserts into the sister chromatid (Greenblatt and Brink 1962); (3) the maize p1 gene promoter and exon 1 sequences are preferred sites for Ac insertion (Athma et al. 1992), and this region is located within the Ac/fAc inter-transposon segment in the P1-rr11 and P1-rr910 alleles.

**Generation of inversions vs. deletions by reversed ends transposition.**

The Ac/Ds transposon system is well-known for having a preference for transposition to closely-linked sites (Greenblatt 1984). In contrast, the orientation of insertion of the Ds termini appears to be random in several large collections produced for gene tagging (Kolesnik et al. 2004; Nishal et al. 2005; Pan et al. 2005). If the insertion orientation of reversed-ends transposition is also random, then one would expect that inversions and deletions would be generated at a similar frequency. However, in the experiments described here, inversions were recovered at a two-fold greater frequency than deletions. There are several factors that can account for this difference. First, deletions which extend from the 3’ fAc upstream beyond the Ac element would be selected against, because our initial selection was for spotted kernels, i.e. kernels which retain Ac activity. Second, very large deletions are known to have reduced transmission frequency (Birchler and Levin 1991; Lin et al. 1997), presumably due to the loss of essential genes; whereas, inversions have a normal gene balance and should transmit normally. Consistent with this point, a higher frequency of inversions than deletions was reported at the maize Bz locus (Huang and Dooner 2008).

**Reversed Ds ends distance and chromosome rearrangement tool development**

This and earlier reports indicate that reversed Ac/Ds termini can produce chromosome rearrangements such as inversions, deletions and translocations (Huang and Dooner 2008; Zhang and Peterson 2004; Zhang et al. 2009). The ability to generate chromosome rearrangements may be valuable for plant chromosome engineering and/or functional genomics analysis, and it will be important to determine the parameters affecting the
frequency of alternative transposition events. Previous reports have shown that chromosome breakage frequency is inversely related to the distance between the interacting \textit{Ac/Ds} termini (Dooner and Belachew 1991; Yu et al. 2009). Here, we have shown that alleles in which the \textit{Ac/fAc} termini are separated by 0.3 kb to 3 kb segments have the highest chromosome breakage frequency (Table 5). However, alleles with a separation distance of less than 100 bp exhibit much reduced chromosome breakage frequency (Table 5, alleles \textit{p1-wwB59} and \textit{p1-wwB42}). Chromosome breakage in these alleles can be fully explained as a consequence of reversed \textit{Ac/Ds} ends transposition, and hence should be a good indicator of alternative transposition frequency. Interestingly, somatic extrachromosomal circular \textit{Ds} molecules were previously reported in maize and transgenic tobacco tissues; these circular \textit{Ds} elements appeared to be incapable of transposition and were considered likely to be abortive transposition products (Gorbunova and Levy 1997; Gorbunova and Levy 2000). Consistent with this idea is the fact that the \textit{p1-wwB42} allele, which has only a single nucleotide separating the \textit{Ac} and \textit{fAc} termini, exhibits no detectable chromosome breakage. Together these observations support the conclusion that structures in which the inter-transposon separation distance is less than 0.1 kb are not competent for transposition. Although other factors, such as genome location, host factors, and local sequence context may affect transposition frequency, our results provide new information regarding optimal separation distance.

\textbf{Acknowledgments:}

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Table 1. PCR primers and their sequences used in this study.

Table 2. Combinations of PCR primers used to determine allele structures.

Table 3. PCR pattern and explanations. “+” represents positive. “−” represents negative. Deletions are classified as “flanking external deletion” if the deletion removes sequences outside of the Ac 3’ end or the fAc 5’ junction with flanking DNA. Deletions within the inter-transposon segment (i.e., between the Ac 5’ end and the fAc 3’ end) are termed “small internal deletion”.

Table 4. Statistical test for frequencies of comparisons of flanking Ac 3’ end deletion from P1-ovov454 (1/133) and flanking fAc 3’ end deletion alleles from P1-rr910 or P1-rr11 (8/100).

Table 5. Chromosome-breakage frequency exhibited by different alleles.
<table>
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<tr>
<th>Primer ID</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
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<td>P1-2415f</td>
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<td>9D9A5537f</td>
<td>CGCCACCTGATGATCGAAGC</td>
</tr>
<tr>
<td>P1-2915r</td>
<td>GACAGGTCGCAGTTGGGTTGGG</td>
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<td>P1_7061r</td>
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<td>pp1'</td>
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<td>P1-7869f</td>
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Table 2

<table>
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<tr>
<th></th>
<th>$P1$-$rr11$</th>
<th>$P1$-$rr910$</th>
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<tr>
<td><strong>PCR pairs 1</strong></td>
<td>P1-2415f and Ac4436f</td>
<td>9D9A5537f and Ac4436f</td>
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<td><strong>PCP pairs 2</strong></td>
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<td>Ac120r and P1-7061r</td>
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<td><strong>PCR pairs 3</strong></td>
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<td><strong>PCR pairs 4</strong></td>
<td>Ac2680f and pp1’</td>
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<td><strong>Fused $Ds$ ends</strong></td>
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<tr>
<td><strong>Local rearrangement</strong></td>
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<td>P1-7061r and P1-15588f</td>
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Table 3

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<th>P1-rr910</th>
<th>Inferred rearrangement</th>
<th>Frequency (%)</th>
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*Three alleles (p1-wwB42, P1-wwB54 and p1-wwB59) are with + − − + pattern based on initial PCR analysis. Further sequence analysis corrected them to + + − + group. Local rearrangements alleles were isolated by an additional primer pair (Table 2). Major rearrangement alleles were identified by high frequency pollen abortion.
Table 4

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<th>Test</th>
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<th>P-value</th>
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Table 5

<table>
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<th>Alleles</th>
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<th>Ear BFB grade (total ear number)</th>
<th>Average BFB grades</th>
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<tr>
<td><strong>P1-rr11</strong> and its derived flanking Ac/fAc deletion alleles**</td>
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<td><em>P1-rr11</em></td>
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<td><em>p1-wwC7</em></td>
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BFB, Chromosome break-fusion-bridge; SE, ND, Not determined.
FIGURE 1. PCR reaction analyses by four pairs of primers. (A) Structure of the parental alleles, showing the location of the four PCR primer pair. (B) 20 sample of PCR pattern result. Primers names and sequences are shown in Table 1 and Table 2.

FIGURE 2. The P1-rr11 or P1-rr910 derived flanking Ac/fAc adjacent small internal deletion alleles’ structure and p1-ovov454 structure. The solid black boxes are the p1 gene exons 1, 2, and 3 (left to right). The open and solid red triangles represent the 3’ and 5’ termini of Ac/fAc. The dashed lines with arrows show the deleted region for each allele named at the end of the arrows. The positions of within the p1 gene sequence are indicated.

FIGURE 3. Representative ears for evaluating the frequency of chromosome breakage. The ears were from dek1/+ tester plants crossed with pollen from plants homozygous for various Ac-containing p1 alleles. The functional Dek1 gene conditions solid purple aleurone color; chromosome breakage at the proximal p1 locus results in loss of Dek1 as evidenced by colorless aleurone sectors. The ears were borne on heterozygous dek1/+ tester plants, hence only half of the kernels can show Dek1 loss. The number and size of colorless sectors indicate the chromosome break frequency. The photographs show grade 0 to grade 3 represent ears produced by crossing with pollen from plants homozygous for the following alleles: Grade 0, p1-wwB42; Grade 1, p1-wwB59; Grade 2, p1-wwB8; Grade 3, p1-wwB34.

FIGURE 4. Single end abortive transposition model. The model was modified from (DOONER et al. 1988; JILK et al. 1993). One single Ac 5’ end is excised and inserted to a nearby site on the Ac/fAc internal region. The insertion results in a small internal deletion flanking the Ac 5’ end. Similarly, a small internal deletion flanking the fAc 3’ end could be obtained by a single fAc 3’ end transposition.

FIGURE 5. P elements induce flanking deletion models. Model is modified from (PARKS et al. 2004). Two P elements are present at different sites on homologous chromosomes. Alternative transposition in which the two P elements are involved can induce a deletion flanking one P element.

FIGURE 6. The reversed Ac/fAc termini sister chromatid insertion model. The ovals represent centromeres; other symbols as in Figure 1. The chromosome containing the Ac/fAc locus is partially replicated. Ac transposase (black circles) recognize the reversely oriented 3’ and 5’ termini of Ac/fAc on the same chromatid; cleavage by transposase occurs at Ac or fAc termini. Excised 5’ and 3’ termini of Ac/fAc insert into a site between Ac/fAc in the sister chromatid. The reciprocal twin deletions alleles form following insertion and completion of chromosome DNA replication.
**Figure 1**

### A

![Diagram showing the positions of primer pairs relative to Ac and fAc](image)

### B

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Figure 2
Figure 3

$p1$-wwB42  p1-wwB59  p1-wwB8  p1-wwB34
Figure 4

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4
Figure 5
Figure 6
References


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CHAPTER 5. AC/DS TRANSPOSON-INDUCED CHROMOSOME REARRANGEMENTS IN TRANSGENIC RICE

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Running head: alternative transposition and chromosome modification

Key words: alternative transposition, transposon, chromosome deletion, inversion, Ac/Ds

Abstract

Traditional Ac/Ds transposition has been used for gene isolation and functional analysis in a variety of plants, including rice. We showed previously that alternative transposition, referring to transposition events involving the termini of different elements, can induce a variety of genome rearrangements, including deletion, duplication, inversion or translocation. To assess the ability of Ac/Ds alternative transposition to induce genome rearrangements in rice, we transformed a reverse-orientated Ac/Ds ends construct together with an Ac transposase gene in cis into rice (Oryza sativa ssp. japonica cv. Nipponbare). A green fluorescence protein (GFP) marker between the reversed Ds termini was used for efficient screening of rearrangement events. Molecular analyses indicate that a total of 25 independent rearrangements were obtained from three different chromosome loci. The rearrangements include chromosome deletions and inversions, and one translocation. Most of the deletions occurred within the T-DNA region, but two cases removed 72.5 kilobase pairs (kb) and 79 kb of rice genomic DNA flanking the transgene. The 79 kb deletion can be maintained as a heterozygote, but appears to be homozygous lethal as no homozygous deletion plants could be recovered. In addition to deletions, a variety of inversions were obtained from one transgene locus. The inversions range from very small (within the transgene DNA) to over 1 million base pairs (Mb) in size. For 11 inversions both breakpoints were sequenced, and all of these contained the typical 8 base pairs (bp) Ac/Ds targeted site duplication (TSD),
confirming their origin as transposition products. Together, our results indicate that alternative Ac/Ds transposition can be an effective tool for functional genomics and chromosome manipulation in rice.

**Introduction**

With the completion of multiple plant genome sequencing projects (2000, 2005), a major goal in plant genome biology research is the determination of the functions of individual genes and gene families. Two mutagenesis methods have been widely used for generation of loss of function alleles. One method employs chemical mutagens, such as ethyl methanesulphonate (EMS) (Hirochika et al., 2004). One disadvantage of chemical mutagenesis is that multiple independent mutations are commonly generated, and several generations of backcrossing may be needed to separate the desired mutation from others in the background. In addition, the mapping and molecular isolation of genes containing EMS-induced mutations is often laborious and time-consuming. A second method employs T-DNA or transposable elements for gene tagging (Miyao et al., 2003; Sallaud et al., 2003). T-DNA and transposon insertion sites can be easily mapped and isolated, but the generation of mutant collections containing sufficient insertions in large, low gene density genomes is often challenging. Chemical and insertional mutagenesis methods are used primarily to target individual genes. Ionizing radiation can induce chromosome deletions, inversions, translocations and other kinds of genome rearrangements (Cecchini et al., 1998). However, this method has not been widely used in recent years because the random nature of the breakpoints can render the products somewhat difficult to analyze. Another chromosome rearrangement tool uses the Ac/Ds transposable elements combined with the Cre/Lox site-specific recombination system (Medberry et al., 1995; Osborne et al., 1995; Stuurman et al., 1996). The approach involves a number of steps: first, plants are transformed with a construct containing a mobile Ds element harboring a lox locus; second, the transformed plants are crossed with an Ac transposase source line to induce Ds transposition; third, plants containing transposed Ds are crossed with a line expressing Cre transposase which will induce deletion or inversion of the chromosome segment between the transposed Ds element and the original transgene insertion. This approach has the disadvantage that several plant
generations are required before the desired rearrangements can be detected. Additionally, one must map a potentially large number of individual $Ds$ insertion sites to identify lines containing $Ds$ insertions at the desired locus which are to be crossed with the Cre recombinase.

Alternative transposition refers to transposition events in which the termini of different transposon are involved (Zhang et al., 2006). Previously we have demonstrated that a pair of directly-orientated $Ac/Ds$ ends can undergo alternative transposition leading to the formation of chromosome deletions and inverted duplications (Zhang and Peterson, 1999). A series of nested deletions flanking the $p1$ gene on maize chromosome 1 was isolated and characterized. The deletions ranged in size up to 4.6 cM (Zhang and Peterson, 2005). Another type of alternative transposition reaction involving reverse-oriented $Ac/Ds$ termini can generate deletions, inversions and translocations (Zhang and Peterson, 2004; Huang and Dooner, 2008; Zhang et al., 2009). These results arising from natural configurations of $Ac/Ds$ elements in maize prompted us to test whether alternative transposition could be reproduced in transgenic systems for functional genomics purposes. The potential advantages of $Ac/Ds$-induced alternative transposition as a mutagenic tool include: 1) deletions can remove multiple copies of clustered genes, thereby simplifying the identification of gene functions; 2) it is relatively easy to clone the rearrangement breakpoint sequences; 3) a single locus capable of undergoing alternative transposition reactions can generate a broad spectrum of possible products; 4) $Ac/Ds$ exhibits a preference for local transposition, thereby enriching for rearrangements in the desired genome regions; 5) rearrangements such as inversions and translocations may be useful for manipulating chromosome structure, and for detection and analysis of chromosome-level influences on gene expression, e.g. position effect. In previous work, an alternative transposition-based system was introduced to Arabidopsis and a variety of rearrangements were detected, thus validating the principal. However, most of the rearrangement events obtained appeared to be somatic, apparently due to inefficiency of the selection markers (Krishnaswamy et al., 2008).

Here, we describe the development of an alternative transposition-based system for generating genome rearrangements in rice. The system utilizes a pair of $Ds$ termini in
reversed orientation, together with suitable marker genes for detection of rearrangements. A variety of chromosome rearrangements were isolated, and these have the hallmarks of transposition-induced events. We conclude that alternative transposition can be a useful mechanism for genome manipulation in rice.

Results

Transgene construct and transgenic rice start lines

A transgenic construct (pRAc) designed to undergo alternative transposition is diagrammed in Figure 1. The construct includes a hygromycin-resistance gene (HPTII) driven by CaMV 35S promoter for positive selection of transformants and alternative transposition events. In addition, a synthetic green fluorescent protein gene (GFP) driven by the maize ubiquitin 1 (UBI) promoter was used as a negative screening marker (Jeon et al., 2000; Jeong et al., 2002; Kolesnik et al., 2004; Kumar et al., 2005). To eliminate the need for crossing with an Ac transposase source, the construct contains a 5’-truncated Ac element driven by the 35S CaMV promoter (Qu et al., 2008). The 3’ end of the truncated Ac element is in reversed orientation with the 5’ end of a 3’-truncated Ds. The GFP marker is located between the Ac 3’ end and the Ds 5’ end. In this configuration, alternative transposition events involving the reversed Ac/Ds termini will result in loss of GFP. In addition, downstream of the 5’ Ds end is a RFP gene that can be used as a positive screening marker (Figure 1).

The strategy to produce rearrangements in the rice genome is shown in Figure 2. The pRAc construct containing the Ac transposase gene and a pair of reversed Ac/Ds termini will be introduced into rice lines through Agrobacterium-mediated T-DNA transformation. The expressed Ac transposase can excise the reversed Ac/Ds termini, leading to a loss of the DNA fragment containing the GFP gene. The reversed Ac/Ds ends form an active transposon and integrate elsewhere in the genome, with a preference for linked insertion sites. Integration into the flanking DNA will generate either a deletion or an inversion, depending on the orientation with which the Ac/Ds termini integrate.
The pRAc construct was transformed into *Oryza sativa* ssp. *Japonica* cv. *Nipponbare* by Agrobacterium-mediated transformation. To facilitate downstream analysis, we screened 17 independent T-DNA lines by Southern blot hybridization to estimate transgene copy number. Three lines (Numbers 6, 7, and 9) contained a single-copy T-DNA insertion and were selected for starter lines. The rice genomic sequences flanking the T-DNA insertions in these three lines were cloned by Inverse-PCR; their origins were confirmed by re-amplification of the expected fragment using primers specific for the T-DNA and flanking DNA. Transgene insertions No.6, No.7, No.9 are located on rice chromosomes 1, 5, and 3 respectively. Minor sequence changes were detected at the T-DNA insertion junctions: No.6 (19 bp deletion), No.7 (915 bp deletion and 25 bp insertion) and No.9 (17 bp deletion). (Table 1). These sequence changes were assumed to have occurred upon T-DNA integration.

**Frequent somatic alternative transposition events**

The alternative Ac/Ds transposition model predicts that the inter-transposon segment (ITS) located between the reverse-oriented Ac/Ds 5’ and 3’ termini will join together and form a 3.1 kb circle DNA. The predicted circular DNA would likely be a transient product and has not been detected directly. However, indirect evidence for circle formation stems from detection of alleles containing permutations of a 13 kb ITS in maize (Zhang and Petersen, 2004). Here, a PCR assay was devised to check the circle junctions using the genomic DNA of the three starter lines. A PCR primer pair (primer 1 and primer 2) flanking the Ds ends can prime a PCR reaction (Figure 3 and Supplementary data 1) only from a circularized DNA molecule. We did detect the expected size bands in all three transgenic lines (Data not shown). The bands were excised from the gel and directly sequenced using primer 1. Representative DNA sequence traces are shown in Figure 3. The first 20 nucleotides (nt) match the sequence flanking the Ds 5’ end. Exactly after the 20nt, multiple peaks arise on the upper three sequence traces. These results suggest that the PCR products contain a mixture of multiple somatic excision “footprints”. In contrast, the two lower sequence traces give clear sequences, each containing a distinct presumptive transposon “footprint”. In each case, the first 19 nt match the sequence flanking the Ds 5’ end, followed by two non-matching bp, followed by 19 nt matching the sequence flanking the Ds 3’ end. These latter
two clear sequences most likely represent PCR products containing a single predominant Ac/Ds excision clone. These results support the hypothesis that excision of reversed Ac/Ds termini generates a circular molecule from the ITS. We also conclude that alternative transposition events occur frequently in these rice vegetative tissues.

**Multiple putative germinal rearrangement lines obtained by marker-assisted screening and PCR analysis**

The process for screening for transposition-induced rearrangements is shown in Figure 4. Seeds harvested from the three starter transgenic lines (T1 plants) were germinated on ½ MS plates containing hygromycin. The Hygromycin-resistant (HPH+) seedlings (T2) were classified according to the results of screening for GFP expression: GFP- seedlings (from 0% to 25% in this class) were considered as putative rearrangement lines and kept for further analysis; the GFP+ seedlings were growing for harvesting. The harvested seeds (T3) were used to continue screening GFP- and HPH+ plants. A few homozygous GFP+ and HPH+ T2 plants from No.7 were identified by PCR analysis using flanking T-DNA primers. Seeds harvested from the homozygous T2 plants were directly screened for GFP- plants without HPH screening. Genomic DNA was extracted from seedlings identified as containing putative alternative transposition events. Three specific PCR primers pairs on the T-DNA region were used to test for rearrangements induced by the reversed Ds ends structure (Figure 1). PCR pair A detects the 35S promoter/Ac transposase junction; PCR pair B detects the Ds 5’ end/GFP junction; PCR pair C detects the HPH gene. Based on the PCR patterns obtained, putative rearrangements could be classified as inversion or translocation (+ - + pattern); or deletion (- - + or + - - patterns). The (+ - +) pattern could also be generated by fusion of the reversed Ac/Ds termini; i.e. deletion of the ITS without transposition of the Ac/Ds termini. Most rearrangements events appeared to be chromosome inversion or fused reversed Ds ends judged by positive for primer pairs A and C, and negative for primer pair B. Approximately 10 plants appeared to contain deletions. Several plants from starter line No.9 yield approximately 10% GFP- and HPH+ progenies; however, in these cases all three PCR primer pairs yield positive results, and their progenies exhibit restored GFP expression in the
next generation. We conclude that the GFP gene in those plants was probably transiently silenced.

**Molecular analysis of the rearrangement lines**

An important feature of the alternative transposition system is that one fixed breakpoint is at the T-DNA insertion site and the other variable breakpoint sequence is joined with the reversed Ds ends (see Figure 2). All the breakpoint sequences of the rearrangements described here were cloned by Inverse-PCR using Ds specific primers (Supplementary data 1). A total of 25 independent events were identified from the three starter lines. According to the cloned breakpoint sequence and original T-DNA insertion sites, we classified them as deletions, inversions or translocations (Table 2).

The No.9 T-DNA, located on rice chromosome 3, gave rise to mutants M9-1, M9-2 and M9-3. Cloned breakpoint sequences analysis from both Ds ends of M9-1 shows that the Ds 5’ end joined with the sequence 87 kb upstream of the left border and the Ds 3’ end joined to the sequence immediately downstream of that site. Both junctions contain an identical 8 bp Target Site Duplication (TSD), thus confirming that this inversion was generated by a single transposition event. Sequence analysis of M9-3 shows that the Ds 5’ end joined with a site 79 kb downstream of the right border, indicating that M9-3 is a 79 kb deletion allele. To further confirm the result, we did Southern blot analysis on the M9-3 line (Figure 5A and B). Genomic DNA from a heterozygous plant carrying the M9-3 allele was cut with Sac I or SpeI restriction enzyme. The blot was first hybridized with a probe (M9-3P) near the breakpoint site, about 80 kb to the T-DNA insertion site. One additional band (8.4 kb for Sac I and 12 kb for Spe I) was found in the M9-3 line compared with DNA from wild type or parental No.9. The same blot was rehybridized with a T-DNA probe (UBIP); both the 10 kb and 5.1 kb bands in parental No.9 disappeared and are replaced by a new 8.4 kb SacI band in M9-3 lane. This result is consistent with the expectation that the UBI:GFP fragment was circularized and lost. Bands of the same size (8.4 kb for Sac I and 12 kb for Spe I) are detected with both the UBIP and M9-3P probes in the M9-3 DNA; this result confirms that the Ds 5’ end is ligated with the M9-3P-containing fragment in the deletion. Together, both the sequence data and Southern blot results support the conclusion that M9-3 contains a 79
119

kb deletion generated by the alternative transposition model. Although the deletion structure was successfully transmitted to the next generation, we did not detect any plants homozygous for the deletion among 26 plants tested by PCR.

To further test whether the internal 79 kb region on the M9-3 allele is deleted, we performed fluorescence in situ hybridization (FISH) analysis on rice mitotic chromosomes. FISH probe 9D is composed of two DNA fragments (3.7 kb and 3.8 kb) located within the 79 kb region. FISH probe 9F is a positive control composed of two DNA fragments (4.7 kb and 3.3 kb) located nearby the deleted region. The FISH results are shown in Figure 7. In addition to a normal chromosome 3 with both 9D and 9F signals, one chromosome (presumptive deletion) contains only the 9F signal. These results confirm the presence of the deletion in the M9-3 allele. This 79 kb region deleted contains approximately 16 predicted genes, according to the Plant Genome Database (http://www.plantgdb.org). Except for two genes encoding putative transposon proteins and three genes encoding glutathione S-transferase, the other genes are of unknown function. Possibly one or more of these genes are essential for plant viability.

One plant of the M9 line gave rise to 19 GFP negative seedlings. Sequence analysis of two of these indicate that they contain identical structures in which the 5’ and 3’ Ds ends are joined together. The remaining 17 GFP negative seedlings were analyzed by PCR using Ds 5’ and 3’ end-specific primers; the results suggest that all contain the same fused Ds end structure. Most likely all of these 19 seedlings were generated in a single pre-meiotic event.

Seven independent rearrangement mutant alleles were obtained from parental line No.6, which is located on chromosome 1 (Table 2). Both flanking Ds 5’ and 3’ ends sequence were obtained from the M6-1 alleles. The M6-1 breakpoint sequence was located on rice chromosome 7. Judging from the breakpoint sequence and the TSD generated by Ds insertion, M6-1 represents a T1-7 reciprocal translocation. Recently we reported the isolation of 19 major chromosome rearrangements lines induced by reversed Ac/Ds ends transposition in maize (Zhang et al., 2009). The translocation observed in M6-1 indicates that major chromosome rearrangements can be induced by Ac/Ds alternative transposition in rice. Unfortunately the M6-1 line failed to produce any seed, preventing further analysis.
From the parental No.7 line, we obtained multiple chromosome inversion events. The inversions range in size from less than 5 kb (i.e., contained within the T-DNA region) to approximately 1.5 Mb (Table 2 and Figure 8). These results support the idea that reversed \textit{Ds} ends exhibit local insertion preference, as for standard \textit{Ac/Ds} transposition. Although a few deletions within the T-DNA were identified, none extended into the flanking genomic DNA. Deletions beyond the right T-DNA border are expected to escape our screening due to loss of the Hygromycin-resistant gene. In addition, it is possible that one or more gametophyte-essential genes are located near the right border. A subset of the inversion lines were analyzed by genomic Southern blot. The results from line M7-2, which carries a nearly 1 Mb inversion, are shown in Figure 6A and B. The heterozygous M7-2 genomic DNA was cut with \textit{Spe I}, and the blot was hybridized with flanking T-DNA insertion site probe 7P3P. One additional 12 kb band was observed in the M7-2 sample compared with the wild type plant. Because the T-DNA region does not have a \textit{SpeI} restriction site, the homozygote parent line No.7 shows a 24 kb size band. The same blot was rehybridized with probe (69P), which originates from a site near the break point, approximately 1 MB from the T-DNA locus. The same 12 kb band is detected with both 7P3P and 69P probes, indicating that these sequences are now conjoined as a result of the 1 Mb inversion. In addition, results from an \textit{NcoI} digest probed with 69P also show an extra band in the M7-2 sample, again supporting the presence of a 1 MB chromosome inversion.

**Discussion**

We demonstrate here a method for generating chromosome rearrangements using alternative transposition in transgenic rice plants. The reversed \textit{Ac/Ds} ends structure can efficiently induce a variety of chromosome rearrangements including inversions, deletions, and translocation in three different loci tested. This method may be suitable for gene mutagenesis or chromosome engineering.

**Frequency of alternative transposition**

Previously, researchers have reported variable \textit{Ac/Ds} transposition frequencies in rice transposon tagging projects. Transposition frequencies range from 0.1% of F2 seeds
containing germinal Ds excisions (Kolesnik et al., 2004), to greater than 70% transposition in a system using callus-derived regenerated plants (Kim et al., 2004). Our Ac/Ds construct is modified from a vector provided by the Sundaresan group and used for activation tagging in rice (Qu et al., 2008). In this activation tagging system, Qu et al. (2008) reported transposition frequencies of 43.4% in T2 seeds. In later generations, the germinal transposition frequency varied depending on transgenic line: one-third of the single T-DNA-locus transformants showed high transposition frequencies (20%–83.3% of plants having at least one transposition per plant). In our material, screening of T2 plants for GFP- and HPH+ plants yielded transposition frequencies ranging from 0-31%; results were variable in different plants. Over all, the frequency of GFP HPH+ seedlings was approximately 10% among all seedlings tested from heterozygous parent plants. This frequency is much higher than that reported for non-tissue cultured Ac-Ds transposon tagging system (0.1%)(Kolesnik et al., 2004). Possibly, the low frequency reported by Kolesnik et al. (2004) may be due in large part to selection for unlinked transposition events, which will reduce the total number of recovered events due to the local transposition preference of Ac/Ds (Dooner and Belachew, 1989; Athma et al., 1992). In our hands, we observed that transposition frequency was reduced as more advanced generations were tested. Alternative transposition events could be detected in all of the three original T1 lines. In the T2 generation, about 60% plants still had detectable germinal transposition events. Somatic transposition was detectable in T3 and T4 plants, based on the results of PCR analysis of ITS circle formation: all plants tested showed the presence of the somatic circle junction, but the band intensity was significantly weaker than for T1 plants (data not shown). Together, these results support the previous conclusion that callus regeneration enhances Ac/Ds transposition (Ki et al., 2002; Greco et al., 2003; Kim et al., 2004).

**Fused Ds ends structure produced by abortive transposition**

The maize Ac/Ds transposable elements are thought to transpose via a cut-and-paste mechanism. Previously Levi’s group detected extrachromosomal Ds circles in maize and transgenic tobacco somatic cells. Because they failed to detect integration of the circular Ds into the plant genome, they concluded that the circular Ds is likely an abortive transposition
product (Gorbunova and Levy, 1997). Small extrachromosomal circular DNA molecules would not be stable in the plant genome. In our study which uses reversed Ac/Ds ends, abortive transposition will result in lost of the GFP gene and fusion of the Ds ends. We obtained a number of fused ends events, the sequences of which are shown in Table 3. Sequence 1 and 2 shows 5 and 3 nt insertion between the intact Ds ends. Sequence 3 shows that in addition to the 3 nt insertion there is a 3 nt deletion in the 3’ end of Ds structure. The inserted nucleotides are different from the original flanking Ds 5’ or 3’ ends sequence. These results suggest that the fused Ds ends are produced by the non-homologous end-joining (NHEJ) pathway.

Use of alternative transposition for genome-wide gene mutagenesis or chromosome structure analysis

Loss of gene function is a powerful tool for functional analysis. Targeted gene replacement by homologous recombination is highly efficient in yeast (Winzeler et al., 1999). Site-specific gene disruption has been developed for mice, but is difficult or unavailable for most other vertebrates, including rats (Koller and Smithies, 1992; Rossant, 2003). In plants, gene targeting methods are still inefficient (Townsend et al., 2009), and hence most functional genomics approaches rely on collections of knockout mutations. Arabidopsis, rice and maize are the three most widely used model plants. Because of its smaller genome size and relatively simple genome composition, Arabidopsis has been widely targeted resulting in collections containing approximately 379,674 independent insertion events targeting 91% of predicted genes (http://signal.salk.edu/Source/AtTOME_Data_Source.html). According to a recent report, only about 200,000 T-DNA or transposon insertion lines, which together knockout about 50% of predicted genes, are available in rice (Krishnan et al., 2009). For maize, it is estimated that 1,800,000 independent insertions would be required to tag every gene in maize with a 95% probability (assuming completely random insertions, a 2400 MB genome size, and 4 kb average gene size) (Krysan et al., 1999; Haberer et al., 2005). As an alternative to single-gene insertions, alternative transposition could produce large deletions, thereby removing 10 or more genes in a single transposition event as in the rice M9-2 deletion reported here and the 4.6 cM deletion flanking the maize P1 locus (Zhang and
Peterson, 2005). In addition, deleted regions may also contain gene regulatory elements, which may be otherwise difficult to detect by simple insertion mutations. Finally, structural rearrangements such as chromosome inversions and translocations may be a valuable resource. Inversions or translocation have been used for genetic research for many years (McClintock, 1931; Maguire, 1972). Compared to the natural translocation or inversion lines, the large rearrangement lines generated by alternative transposition contain defined endpoints and can be used to manipulate copy number of defined chromosome segments (Birchler, 1980; Birchler and Levin, 1991; Zhang et al., 2009).

Although alternative transposition has been reported in a subset of transposable element systems, it seems reasonable to propose that many “cut and past” transposons may undergo alternative transposition. For a number of reasons, alternative transposition may actually be more useful in animals than in plants. First, due to differences in gametogenesis, large deletions are more likely to be transmitted in animals. Development of the gametophyte stage of the plant life cycle involves several post-meiotic haploidic mitotic cell divisions (Candela and Hake, 2008). Multiple, widely-distributed genes are expected to be essential for completion of these mitotic divisions and survival of the gametophyte. Gametes containing large deletions will likely have severely reduced transmission frequency. In contrast, animals do not have such mitotic cell division processes; the products of meiosis develop directly into gametes. Thus, the sperm and egg cells containing chromosome fragment deletion or even chromosome arm losses are still functional in fertilization in animals including human (Massa et al., 1992; Maranda et al., 2006). Second, the chromosome aberrations including deletions, duplications, inversions and translocations are frequently associated with human congenital diseases and cancer (Rabbitts, 1994; Albertson et al., 2003). Thus, we propose that alternative transposition may be used as both a functional genomics research tool, and for the development of model disease systems for medical research.

Methods

\textit{pRAc vector}
The 6.8 kb *Ds* elements from activation-tagging Ac-*Ds* vector *pSQ5* was amplified with 50 bp deletion at the *Ds* 3’ ends (Qu et al., 2008). The amplified defective 6.8 kb *Ds* element replaces the original *Ds* element of *pSQ5* in revered orientation. The 5’ end-truncated *Ac* element’s intact 3’ end with the *Ds* 5’ ends constitute the only reversed *Ds* ends structure. The *pRAc* was partially sequenced to confirm junctions of fragments and sequence of the *Ds* ends.

**Plant transformation**

The *pRAc* plasmid is transformed to the Agrobacterium strain *EHA105*. The callus introduction and rice transformation are performed by Plant Transformation Facility at Iowa State University.

**Genomic DNA extractions, Southern blot hybridization**

Young leaves of individual plants were ground in liquid nitrogen, and genomic DNA was extracted with CTAB (cetyltrimethylammonium bromide) reagent (Saghai-Marroof et al., 1984). Agarose gel electrophoresis and Southern hybridizations were performed according to (Sambrook et al., 1989), except that hybridization buffers contained 250-mM NaHPO$_4$, pH 7.2, 7% SDS, and wash buffers contained 20-mM NaHPO$_4$, pH 7.2, 1% SDS. The probes (7P3P, 69P and UBIP) for southern blot were amplified directly by PCR reaction, a 0.5 kb size fragment between *BamH1* and *HinP1I* from a PCR amplification band is used as M9-3P probe (primers see Supplementary data 1). The PCR products or restriction enzyme cutting products were purified by Qiagen PCR purification kit (Hilden, Germany).

**PCR reaction analysis**

About 20 ng of the ligated DNA or genomic DNA was used for template. HotMaster Taq polymerase from Eppendorf (Hamburg, Germany) was used in the PCR reaction. PCR was performed with an initial 3 min denaturation at 94°C, followed by 35 cycles (each cycle: 94°C, 20 sec; 58°C, 30 sec; and 65°C for 1 min per 1 kb length of expected PCR product, then a final 10 min at 65°C.).

**Isolation of the flanking T-DNA or rearrangemental breakpoint sequences**

Both the T-DNA flanking and the rearrangement breakpoint sequences were isolated by inverse-PCR (Ochman et al., 1988). About 1 µg of the genomic DNA was digested with
*HpyCH4IV* or *MSP I* (NEB, Beverly, MA). After overnight digestion, the samples were ethanol precipitated and dissolved in water. Self-ligation of the digested DNA was performed in a 400 µL volume containing 10 Weiss Unit ligase (NEB, Beverly, MA) at 4°C for 12 hours. The primers for inverse-PCR are listed on Supplementary data 1.

**Hygromycin and GFP screening**

Mature seeds of transgenic plants were germinated at 25°C for 3-5 days on ½ MS medium with or without 50 mg/L hygromycin. The emerging seedlings were screened for GFP fluorescence using a dissection microscope (SHZ10, Olympus Co., Japan).

**FISH and immunostaining**

FISH probes were amplified by directly PCR amplification (the primers see Supplementary data 1). Chromosome preparation, FISH, image capturing, and image processing were performed as described (Kato *et al.*, 2004; Han *et al.*, 2006).

**Acknowledgements:**

We thank Dr. Venkatesan Sundaresan’s lab providing the *pSQ5* plasmids. We thank Dr. Bing Yang for providing the Agrobacterium *EHA105* strain and for suggestions on rice plant culture. We thank the ISU Plant Transformation Facility for rice transformation, and the laboratories of Drs. Jo Ann Powell-Coffman and Jeff Essner for assistance with fluorescence microscopy. This work was supported by the National Science Foundation Grants No. 0110170 and 0450243.
Table 1 T-DNA insertion sites of the starter lines

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>T-DNA flanking sequence and chromosome location</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 6</td>
<td>TTATAACAAAGTATGCTTTAT---ATACTAGGTACTGGTACTCC</td>
</tr>
<tr>
<td></td>
<td>Chr01:20709036...20709017---20708998...20708979</td>
</tr>
<tr>
<td></td>
<td>CGCTGCAAGGTGCAGGTAGTTTGGTTACACCACAATAATTCCAGT---</td>
</tr>
<tr>
<td></td>
<td>TAGTACCAGGGTTGTATTGA</td>
</tr>
<tr>
<td>No. 7</td>
<td>Chr05: 25358982...25358963---25358047...25358028</td>
</tr>
<tr>
<td></td>
<td>GCGGTCTTCTCCCGCCGC---CACAAGTGCACAACATACACG</td>
</tr>
<tr>
<td>No. 9</td>
<td>Chr03:1911750...1911731---1911713...1911694</td>
</tr>
</tbody>
</table>

20 nt flanking T-DNA insertion site sequence and chromosome location is shown. The dash line represents the T-DNA inserted loci in a right border to left border direction. The underline sequence represents the inserted unknown sequence. The chromosome location is referred to TIGR rice genome release 5.0.
Table 2 Rearrangements lines generated by alternative transposition

<table>
<thead>
<tr>
<th>Parental line</th>
<th>Rearrangement line</th>
<th>Rearrangement type</th>
<th>Size (kb)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.9</td>
<td>M9-1</td>
<td>Inversion</td>
<td>87</td>
<td>TSD confirmed</td>
</tr>
<tr>
<td></td>
<td>M9-2</td>
<td>Fused Ds 5’ and 3’ end (on construct)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M9-3</td>
<td>Deletion</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>No.6</td>
<td>M6-1</td>
<td>T6-7 Translocation</td>
<td></td>
<td>TSD confirmed</td>
</tr>
<tr>
<td></td>
<td>M6-2</td>
<td>Deletion</td>
<td>0.34(on construct)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M6-3</td>
<td>Deletion</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M6-4</td>
<td>Inversion</td>
<td>100</td>
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<tr>
<td></td>
<td>M6-5</td>
<td>Inversion</td>
<td>2.5(on construct)</td>
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</tr>
<tr>
<td></td>
<td>M6-6</td>
<td>Inversion</td>
<td>800</td>
<td>TSD confirmed</td>
</tr>
<tr>
<td></td>
<td>M6-7</td>
<td>Fused Ds 5’ and 3’ end (on construct)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.7</td>
<td>M7-1</td>
<td>Deletion</td>
<td>4.0(on construct)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M7-2</td>
<td>Inversion</td>
<td>940</td>
<td>TSD confirmed</td>
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<tr>
<td></td>
<td>M7-3</td>
<td>Inversion</td>
<td>3.5(on construct)</td>
<td>TSD confirmed</td>
</tr>
<tr>
<td></td>
<td>M7-4</td>
<td>Inversion</td>
<td>53</td>
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</tr>
<tr>
<td></td>
<td>M7-5</td>
<td>Inversion</td>
<td>1000</td>
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</tr>
<tr>
<td></td>
<td>M7-6</td>
<td>Inversion</td>
<td>190</td>
<td>TSD confirmed</td>
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<td>M7-7</td>
<td>Inversion</td>
<td>27</td>
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<td></td>
<td>M7-8</td>
<td>Inversion</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M7-9</td>
<td>Inversion</td>
<td>1500</td>
<td>TSD confirmed</td>
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<tr>
<td></td>
<td>M7-10</td>
<td>Inversion</td>
<td>820</td>
<td>TSD confirmed</td>
</tr>
<tr>
<td></td>
<td>M7-11</td>
<td>Deletion</td>
<td>5.9 (on construct)</td>
<td></td>
</tr>
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<td></td>
<td>M7-12</td>
<td>Inversion</td>
<td>5.6 (on construct)</td>
<td>TSD confirmed</td>
</tr>
<tr>
<td></td>
<td>M7-13</td>
<td>Deletion</td>
<td>0.34(on construct)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M7-14</td>
<td>Fused Ds 5’ and 3’ end (on construct)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M7-15</td>
<td>Deletion</td>
<td>5.6(on construct)</td>
<td></td>
</tr>
</tbody>
</table>

If the cloned sequence from Ds 5’ and Ds 3’ contain the 8 bp Target Site Duplication for the inversion/translocation lines, we marked “TSD confirmed” in the note row. Empty cell in the note row represents that only one flanking Ds end sequence is cloned and the other Ds end sequence is not available because of inverse-PCR failure or not try. The T-DNA region is not taken into counting the deletion/inversion sizes unless “on construct” is marked.
### Table 3 Junction sequence of fused *Ds* ends

<table>
<thead>
<tr>
<th>Conceptual fused <em>Ds</em> ends</th>
<th>Sequences of the PCR clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>−CATCCTACTTTTCATCCCTG</td>
<td>TAGGGATGAAAAACGGTC−</td>
</tr>
<tr>
<td>−CATCCTACTTTTCATCCCTG</td>
<td>TAGGGATGAAAAACGGTC−</td>
</tr>
<tr>
<td>−CATCCTACTTTTCATCCCTG</td>
<td>TAGGGATGAAAAACGGTC−</td>
</tr>
</tbody>
</table>

1  
2  
3  

1 −CATCCTACTTTTCATCCCTG AGAGC TAGGGATGAAAAACGGTC−
2 −CATCCTACTTTTCATCCCTG ATG TAGGGATGAAAAACGGTC−
3 −CATCCTACTTTTCATCCCTG GAT GGATGAAAAACGGTC−
Figure Legends

**Figure 1 T-DNA region of the transgene vector pRAc** RB and LB, Right and left borders of the T-DNA; 35S, CaMV 35S promoter; Ubi, maize ubiquitin 1 promoter; GFP, green fluorescence protein gene as a negative selection marker; RFP (DsRed), red fluoresce gene as a positive screening marker for presence of transgene construct; HPH, hygromycin phosphotransferase gene as a plant transformation selection marker and a positive screening marker for transgene construct. All genes shown are in the same transcriptional orientation (left to right). The T-DNA is with the pCAMBIA-1300 backbone. The Ds 3' end and Ds 5' ends are shown in a reversed orientation; the other Ds ends are deleted and not functional. PCR primers are located on the construct.

**Figure 2 The inversion or deletion models induced by reversed Ds ends transposition** The expressed Ac-transposase recognizes the reverse oriented Ds ends. The internal fragment (UBI::GFP) between the reversed Ds ends forms a circle, which will be lost at the next cell cycle. The excised Ds ends, bond by the Ac-transposase, will integrate into the chromosome. Depending on the integration orientation, the transposition will induce the fragment between the Ds ends and integration site inversion/deletion. The integration site can be located on the T-DNA region or flanking genomic DNA, or even different chromosome (resulting in chromosome translocation or dicentric/acentric chromosomes; not shown).

**Figure 3 Detecting the repaired junction of predicted somatic circles** At the top is diagrammed the 20 bp sequence flanking the reversed Ds 5' and 3' ends. The PCR products were obtained by primer 1 and primer 2 on the transgenic genomic DNA extracts. Below are the partial electropherogram sequence figures by primer 1. The transposition “footprint” sequences are read from the two lower electropherogram figures. The transposition footprint is shown in small letters.

**Figure 4 Selection procedure of the rearrangement lines** Seeds harvested from T1 or T2 plants are germinated and screened by GFP or Hygromycin resistant markers. The ⊗ indicates self-pollination. The T2 plants homozygous for the transgene insertion were identified by PCR analysis using flanking T-DNA primers.

**Figure 5 Southern blot analysis of M9-3**
(A) M9-3 structure and restriction map. S, Sac I; Sp, Spe I. Lines below the map indicate the restriction fragments produced by digestion with Sac I or Spe I and hybridizing with the indicated probes marked with black bars. The dashed line was used because the 79 kb genomic region is expected to be deleted in M9-3. The Sac I-8.4 kb and SpeI-12 kb bands are expected by probes M9-3P and UBIP. (B) Southern blot analysis of M9-3. Genomic DNA was prepared from wild type plant (Wt), heterozygous parental No.9 (9) and heterozygous M9-3, and cut with Sac I and Spe I. The Blot was hybridized with M9-3P probe first and then rehybridized with UBIP. The common 8.4 kb with Sac I and 12 kb bands with Spe I are overlapped in the left and right figures.

**Figure 6 Southern blot analysis of M7-2**

(A) M7-2 structure and restriction map. Sp, Spe I; N, Nco I. Lines below the map indicate the restriction fragments produced by digestion with Spe I or Nco I and hybridizing with the indicated probes marked with black bars. The dashed lines represent the two fragments are joined together by the nearly 940 kb chromosome inversion event in M7-2. The T-DNA region is marked as Figure1. (B) Southern blot analysis of M7-2. Genomic DNA was prepared from wild type plant (Wt), homozygous parental No. 7 (7) and heterozygous M7-2, cut with SpeI and NcoI, and the Spe I-blot was hybridized with probes M7P3 firstly and then rehybridized with 69. The 12 kb bands were overlapped in the left and middle figures.

**Figure 7 FISH analysis of M9-3 allele** Cells of plants heterozygous for a rearrangement and corresponding normal chromosomes were characterized by FISH of mitotic metaphase chromosome using probes 9D and 9F. The probe 9D (green) is located within the 79 kb deleted region. The probe 9F (red) is located on flanking region. Arrows indicate the chromosome with the 79 kb deletion. (A) The 9D and 9F signals are shown together; (B) only DAPI; (C) only 9D; (D) only 9F.

**Figure 8 Map of inversion and deletion alleles derived from transgene line No.7** Upper, map showing a portion of rice chromosome 5. The site of insertion of the transgene is indicated by the dashed lines. Vertical arrows indicate the breakpoints of each inversion; sizes are given in parentheses. Before the inversion alleles name an “I” letter is added. Lower, map of the transgene insertion. Lines below show the extent of deletion found in the
alleles. Before the deletion alleles name a “D” letter is added. The sizes of the inversion are calculated not taking account of the T-DNA sequence unless the breakpoint is on the T-DNA region.

Figure 1
Figure 3

Transposition footprints

d: GGGGGGCCCCTTGACCGAGCTGACGTGACCGG

e: GGGGGGGCCCTTGACCGAGCTGACGTGACCGG

Figure 4

```
<table>
<thead>
<tr>
<th>Homozygous T2 plants</th>
<th>GFP⁺ HPH⁺ T2 plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td></td>
<td>GFP⁺ T3 plants</td>
</tr>
<tr>
<td></td>
<td>GFP⁺ HPH⁺ T3 plants</td>
</tr>
<tr>
<td></td>
<td>GFP⁺ HPH⁺ T2 plants</td>
</tr>
<tr>
<td>Putative rearrangement lines</td>
<td></td>
</tr>
</tbody>
</table>

identified single copy T1 plants
```
Figure 5

(A)  

(B)
Figure 6

(A)

(B)
Figure 7
Figure 8
Supplementary data 1

PCR primer sequences:

Detecting somatic circle primers:
Primer1: 5′-CTGGAGTTCTGACCCCTG-3′.
Primer2: 5′-AGAGAGGGGCACGACC-3′.

Primers used to make Southern blot probes:
7P3P probe: 5′-GCAACCCTGTTCTGACCCCT-3′ and 5′-CGATGGGGAGGTCAGGGG-3′.
69P probe: 5′-CCAGGTGTGGGCAATGGA-3′ and 5′-CGATGGGGAGGTCAGGGG-3′.
UBIP probe: 5′-CCCGTTTCTCGTTTCT-3′.
UBIP probe: 5′-CGATGGGGAGGTCAGGGG-3′.
M9-3P probe: 5′-GGCCGGCGCGCGCGACG-3′ and 5′-TGTCGCAGCCGCCGCC-3′.

PCR checking primers:
Ar: 5′-GGTTGTGGCTGTGGTTGTGGTTCTGGT-3′.
Af: 5′-AGATAGGAAAGGGAAGGTGGC-3′.
Bf: 5′-CTGGAGTTCTGACCCCTG-3′.
Br: 5′-CCCGTTTCTCGTTTCTGTT-3′.
Cf: 5′-GCAAGAAATCTGCTGC-3′.
Cr: 5′-CTGGGGACACTACGACATTATTG-3′.

Cloning T-DNA insertion sites or rearrangement breakpoint sequence primers:
LB1: 5′-GCATATAAGAAACCCTTAGTATTTGT-3′.
LB2: 5′-TCACACATGAGCGAAACCCT-3′.
LB3: 5′-GTACTAAAATCCAGATCCCCCG-3′.
LB4: 5′-CTGGAGTTCTGACCCCTG-3′.
RB1: 5′-TGCCGAAAGGGGATGTG-3′.
RB2: 5′-TGTCGCAGCCGCCGCC-3′.
RB3: 5′-CGCCAGGGTTTCCCAGTGAC-3′.
RB4: 5′-ACCCGCGCAATATATCTTCTGAAAC-3′.
Ds5′f: 5′-TTCCGCTGTTCCACCCG-3′.
Ds5′r1: 5′-GCTCTACCGTTTCCGATTACCG-3′.
Ds5′r2: 5′-CCCGTTTCCGTTTCCG-3′.
Ds3′r: 5′-TGCCGAAAGGGGATGTG-3′.
Ds3′f1: 5′-ATGAAAATGAAAACGGTAGAGG-3′.
Ds3′f2: 5′-GTAGATGTATTTTACCGACCGT-3′.

FISH probes:
9D-3.7kb: 5′-ATCGTCATCTTCTCCTCCCAT-3′ and 5′-GCTTTGGTCTCCGCTGCGGTA-3′.
9D-3.8kb: 5’-GTAGAGGAGGATAAATGTTGTGGT-3’ and 5’-TGAGGGAAGGGGAAGGGAC-3’;
5’-GTCCCTTCCCCCTCCCTCA-3’ and 5’-CCCTACTCGTCCAAGGTATCTCTACTCT-3’.

9F-4.7kb: 5’-CGTTTTTCACCCCTACTCAATGG-3’ and 5’-TTTTGTCCGCTTCCCTTTTTC-3’;
5’-GAAAAAAGGGAAGCGGACAAAA-3’ and 5’-TAGCATAACAAAAGAAAAGGGTGGT-3’;
9F-3.3kb: 5’-CTGTGGCAGGGGTAGTGATGATTTAG-3’ and 5’-TTTTGGGAGAATAAGGGGAGATAGG-3’;
5’-GGTGGCAGTGAGTCTTGGTG-3’ and 5’-GCAGGAGAGCGAGGGGC-3’.
Supplemental data 2  
Cloned breakpoint sequence  
Note: The highlighted sequences are confirmed target site duplication and the underlined nucleotides are the breakpoint sites for Ds 5' (at the beginning) or 3' (at the end) ends.  

M9-1  
TTATCTGAAGATCATAACAGATGAAAGGACTGATTTCGCTTTCGGCGACAG  
TGACCGGTGTCCGACTTCTTGTGTTTCTCTTTGGCAAACCCGAAGCTTAGTACATTAT  
ACTCTGCTACTACTTTAAGCACTGAGACAATGCTATAGTTAAATGTCTATCTGTC  
CTTTGAGTATATTAGGTATGCTAAAAATACGGAAAGACTACATAG  
ATGCAAGCCGCAAAGCTGCTCAAAGGACCTAAACGCCAGCTTAAATAGGAA  
ACAATTTGGTAGATGATCACCAGCAATCTCCTTGACCCACCTTCA  
TGATGACCAAACCCGAACGAGGCTTACGCGATTTAACAAAGATTCATCTTTG  
CGAGTTGAACATGTGAATAAGAGAGACAGGCTCAGCTTACCTTTGAGCAG  
GTTATCTTTGAGCCAGCCACCCCTCGATGGCTGTGAAAAT  
GAGAGGAAGATGAAAGGACTGATTTCGCTTTCGGCGACAG  

M9-2(fused ends on construct)  
TAACAAATCCGGTTATACGATAACCGGTGTACCGGGAATTTTNCAANNTACTTTT  
CATCCCTGAGAGCTAGGGATGAAAACGGTCGGTAACGGTCGGAAAAATCTCTT  
ACCGTTAAAAACCCACCATATTCCATATTCTGGAAAGCATACCTACATGCTG  
ACGCAACCTACAAAAACTAGAAGAAATGTCAAGAACATACACCGCATCATGCT  
GATCGACTACATCTCCAGGAATTTCCCTCAGAGAGATACAAAGCAACGGCGTCAG  
GTTAGTGAACGACTCCCTCGGTTCACGAGCGCCCGG  

M9-3  
CAAGGAAGGTGAGGAGGAAGCCGAGCAAGCTGACGGCGCCGCCGCAGCGC  

M6-1  
TTGAAAATACCAACAAAAAGGGGAAGCAGAATTGGCAATTTTTTCTGACCATCTC  
TCTGCAAGGCTGGAGCATCTGGGGAATGCTATGAGCAGCGTACCTCGGC  

M6-2(on construct)  
CTTTGCTAACAAACAAACACATTTGGGAACTTTTCTGACCATCTC  
TCGTAAGGCTGGAGCATCTGGGGAATGCTATGAGCAGCGTACCTCGGC  

M6-3  
ATTATTCGACTCTCATGTGATATCCTGCTAATGGGATCACTTTTTATCATATACAC  
AATCTCTCCACGGTCATGGCCCTTTCTATTTCCATATTTGAGTATGACTAGTGGT
CAGACTACAGTTACACCATGAACACATAACACACACAAAAATATTATAGGG
CCTCTAAGAGGTCGGGGGATATTATTAGGAGATAGGCTCAACCGAGATGTT
ATTATACCGGGAATTACATATAAACCACAACTCCCATACAAAATATTAGT
AAAATGAAAGCATTAAAAATAGCTCAAGAGGTTAAAGTGAGAAAAATAATTTTAGG
GTTACGTTGCTATAGTCAAATATGTGTTATGGAAGATGTTATATGGTTAATTCTT
TCAACATATATATTTCTCAATATCTCAATAGATTTAGCTACTATTCTGTAATAAAC
TGAAGATACATAAGACCTAAAAATATTGTACATGTCTGACTGCACTAG
CTCTGCCATGTGCCCATGCTGGTCCCATCCAGAGAAACTACCGTCTGCTAG
CTCATAAACACATTACACATAGGGAATACATTGACCGTAAAAGTTCTCTGTATA
TGAATAACACGGTGGATGCTAGGGTGGTTCAATAAATAGTAAAGCTGAACAA
TAATGGACACGAACGCG

M6-4
TTCGCTAGAATAAAGATTACGATACACAGCAGGCTAGGAATTAATATAATTGTATA
GGCGTCAATTAAATCCCCAGGTTGTTTTGCCGGTGTTAATGCTTTTCTATA
AGCTACATAGGTGGAGTAAAATGTGGTACAGGATTGAGTCAAAGGAAAGTGGGT
AACATCAAGGTGCAATCAGCATATAGTAAAGGAATCTCTACCAGAAGTGAATT
GCGAAGGGCTTGGTCCCTCTCTGATGAGACTTGGAGTGAAGACGAGC
TCAGTCTCCACTGTCTTTTGGTCGCTGCTTTCTCTGAGACCGACATTGCTCTTTA
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GAATGAGAGATCGTGGAGTATGTTCACTAGCGACAGGATGTTAAGG
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GAGTCAGAGGATAAGGATGTCAGAGGATGTAAGG
GATGAAATTACCAAACTGGAAATATATTATGATATTGGTATAAAGTTATG
TAAATTGTTAACAAGACTGGAAATAATTATATGATATTCTGTAATAAAC
TA
M6-5 (on construct)
ATCTCTGTCCGTACCTGCTCCAGGACACCATCCTCGAGATTCCGCTCCACCGCTGGAC
TTGCTCAGGCTGTCGCACTCAGAAGAATTTGCTGCGCGAGCAGACGTGAGCC
CGGCGCGCGCGCGCTCTCCCTCTCTCTCTCTCTCTCCAGCGCAAGCTACGG
GGATTCCTTTCCCACCGCTCCTTTCAGTCTTCTCTCCCTTCTCCCGCGCTAAATAATA
GACACCCCTCCACACCCCTCTTCCTCCTCCTCCCGACACCTCGTGGTGTGGAGAGGC
M6-6
GCACCAATCGAGATGGCTCCCTCCACGGAGGAGGAGGCTGACCCACCTT
GGCCACTGTGACTAGTGTTTTGCCTGGAATCTCGGGTGCGTGGCAGGATG
ATTATACCGCAAAACTTACTCTTTACCATGTTTTCTGCAAATATGGACACGATG
CTTCAAAGTGCATACATTACAATTTCCAAACGCGGATGTTATTTATTTCC
AGGCCAGAAAAAGAAACAGCAAGTAATATGAGTGACAGAGACTGCTAGCTCCT
CATTGGTTATATTACCAAGAATTTGATAAAATTTCAATTAAAAATGAGAGGT
ATGAAACTATACATACATACATACGTGCACTTTTTTTTGACTAGCAACAAA
CATTTTCACAAATACACAGGAGATGGTTTCTCTGCTTTCTCTCCTCAAAAACGAG
AGGAACAAGACCTGAAAATGCCAACAATTGGCAGCACGTGTTTACTGCACGATG
CACAATTTGGAATACAGGATATTTAAGGATCGCAACACAAACAGAGGATGCTATAGT
GAAACCTATAGTCCACCTGTTTCCTGGTTAATCATCCAGGTTATGCAACTGCAACCAACAAA
AGATATGAAATAAACAGAGGGGAAGCAAGAGCAAGGAGCATGCGTAGGGGCTAAATCCA
TGTACATTATTCAAAACATGATTTGTTTATTTTATTTTAAACAATAATATAATGCTACTGTG
ATCTATATCATATTTGATTTAATCTGCTGAAATAATATAATGCTACTGTGATCTATATA
TCATATTGCAGTTTTGTCTCGGTGAATTTGTCGAACCCTAATATCAGCAGAAA
CAGTTTAAAGCACAGTTTCTGGAGGCCTAGAAATTACCACATTAGGCGGAGAAAA
AAAACTAAAATATTA
M6-7 (fused ends on construct)
AAACTAACAACATCGGTTATACGATAAACGGTGTCGGTACGGGATTTTCCCATCCTA
CTTTCATCCCTGATGAGGGATGAAACCGTGTCGAGGAGTTAAATACC
TCTACGTTTTTCTATTTTACTTAAAACTACATTAGGTTGGAATAAAAC
ACTACCTCAGACCTTACAGGAGATGAAATTTAAACCCCTTCAACACTTTTAC
TCTGCTTCTCCGCGCTTAAAATATATAATAATATTTTTAAAAATAATATAATATTAAG
AAAGTATAT
M7-2
GAAACGTGTTTAGATTACCTTTACTGGTTTTAAAGGTGTTNCAACGACCAGCTTGGTCAC
TGATGAACATAAAGAATCTTTTATAAATATATGATTTTTTTAAACCAACTTT
GTTTCTGCAAAATACAGCAGACGTGAAAGGAAAACGTCTTGGGAAAGGAGAC
TGGAGGAATTAGAAAAAGTACAGTGTAACGGGAAAACAGCCTAGGTTTCCNNAAT
CTTATCCCTTCTCCGTAATCCGTAGCCTTTAGAATCAATGGACTTTTTTTAAACCAAGAAAGGAG
GTTTCTCTCCCACAACTGGGAAACAAAGTCCCGTACCAGGAGATTGCGTG
TCACGCTATAATACCCAGACTCAAAGACATTGCCCTTCTCCCATCCATCCAGCTACA
CCACGTACACTCCGTTGCCCACACTCTGCAACCCAAAGCTGAAAGGGGCTCACC
TCGACAAAACCAAGCAAGCTGTCGACGGCTACAGCTCGAGCTCAGGAGGAGAC
GAAAAAAAAGATTTGAATACTAGTGAAGAACAATGGTTGCGGGATGAGCAG
GTCGAGACCCCG
M7-3 (on construct)
CTTCTACCTCGAAAAACAAATTCCGTATTCTCTGCAATGTCTCAAG
GAAAAACGTGCAATACATACATTGACATTACAGCATATAATTGGTTGATGTTGGGAAAGCA
TCATATTAGACTGTAATAATACAGAAAAATCTGTGTTATAATACAGGGTTTGAAGAAAGAA
AAGCTGAAGCCCTTCTTCTAGTCCGGATCTAGAACTAGTACGTGCACTGCACTG
M7-4
AGACAAACCCAAATCTGCGCNAGCAATGACATTCTAAATAGGACCTTCAAGCCTAG
CAGCATACAGTGCGCTGATGAAACACTTGCCACTTCTAGGATGACCTTTCGGCGAGCAG
CATCCCGTGCGGTGAAAACACAGCTGGCCCTCCCGTTATGTGTTTCTCCCTCGACGCTCAA
CCTGAACCCGACTGTAGCAGAAATTTCTCTGCGGCATTGCGTTTCCCGCTTCCG
TGAACACTGCGCAGCC
M7-5
TATCAGCCAAACAAACAGGAGTGCTCCCAGACGCCATGAGGAGACAGAGCTGT
TCAGAGGACACTACCTGCACGCTTCCAGACACCGGCGTGTACCTTCCAG
AAAGAGGAAGGAAAGAGTAGTAACAGAGGGAAGTCGAGGATAGTACGTCGAGGACTTCCTACT
GCTCAATCGCTGCCAAGCGAATCCGTTTCTCTCGTGCTAGAACTAGTAGCTTTCA
GGATTGGAGTCCATGATGTTACGTGCTGGAAGACGCCATTGCTGACACAAAGG
AGTTGATCGGTTTTCTGATGAGCTGCCCTCCGG

M7-6
GGTTGGTCTACGACCGGCACGCGAGGAGACGTTCCGGGAAAGCTGG
GATGGCGCTGCAGCGCCGATCGAGCTGTTGTTGGAGCGGGGGTTGGTGAGTTGCTGATT
CATGAGGTTCTCTCATGAAACTAGACCTTGTTGTGGTATGCAAGAACATCTGTG
CTTGGATTATATCCTGAAAATTTCAGCGAAAAGCGCAGCACGAGCACTAGCG
AAATGTTAGTAACCCGTGATCTCGGAGTAGTATGAGTCAATCGACATCAG
TGCACTTTTATGGATCTTAGTGAATTGCCAAGAAAGACTCCCCCATGT
GTATAAAGTGTAACCAGTTTGAAGATTATATGTTGAGTAAAATGAAGGGCC
AAACTTTGCAAAATTGCATAAAAAGAAAAGAAGATATAGTACCTAGGGGGAG
TAGCTGTGAAAAAGAGAGATCAGCTACTCATCAGCAGTTAATGTCCACC
TTAGCCTATTTGAACTTGGTCTGCAAGACAGATCAGCTACTCATCAGCAG
TGTCGCTGCTGGCCAGGTACGTGCTATTTGAGCTGGGCTGATT

M7-7
CGAGAGCGCATCCTGATGGCTGTTTTTTCTNGCGTCACGCAACATTTCTCTTTT
CCCTATCATCTCTCTTGGCAATGGACGCAAATTGCGGATGAACATGGCAAAC
AAGTCAGCTTTCAAGAAGGTATCTCTCTTACGTAACCATTGCAGTCAATCATCAG
TCAGATAGATTTCATATTTCTATTTAATCTAGTTGTGACTGAGATGTTTTCTCTAC
TTTCATCCATTAGCTGTGCAAGAATCAGTCTGACAAAGTGGCCAGCACTGGG
CTGACAGGGGGGCTCATTGTGGCACAGAACAATCCAAGCAGCTGCATGGG
GGAGTAATTCTCTTACTGAGTTAGGCATCAATGTTGCTGATT

M7-8
CGGGGATTGGGAACCCGATATATAGCATTTTCTCTTAGGTTTTATCCTGATTAATC
ACTGAGGGAAGGATTGGAGGATTGTGNNGGAA
TTATTCTCCTCTCACTCTCCTGCAAATCTCTTTGAACTTGGTOGCCGGAAATGGCC
CCTTACAACATTAGTTTGCCAGCTCTGAAGAGATTATCTCTGAGCTGGAAATGGCGC
CATTAATCTCTACTTATTGAGTCTCTATTACAGCTACGCTACCCACCTGGCGGA
TTAGTACCATGGCTGAATTGGCCAGTTNNAANNGTACCTCCTCGCCTCTGAGC
AGGCACTTCTGGCTACCTGACAGCTAC

M7-9
CGCCTTCGCCCTCAGCAGACGAGANAAAATCTCCAAATTGGTTATGAGATG
AACTAGTGCTCTGAAACAATGCGAAAAGTCTCAGAGTATACGATGC
TTTGCAAGTGAATATTGCTAGTTGGGTAGCTTGAGTATGCTG
ACAGTAGATCTGAGTATGAGGTGAGGATTTGGAGAGCTCTCATCAGATGC
CTGGAACCTCAGGTTGTGACAGTACCCATCTGACTNAGCACAAGGT
GACTTTTTCTGATATTCTCTCTTTTCTGAATAGATTGTTCTTGTGCAAGACCA
AGAATCTCAGAGTTTTTTCTCCTACTCAGTGTGTTGTAATTTCGAGAG
TGTAAGTGCTCCTGCAACTAACGTCTTTGACCTGACCGTTGGTTGT
TTTGCAGCAAGCGAGTTGTCACCCTCAGTTCTCTATCTCTGACTGTTGGC

M7-10
ATATGGCAATACAGCTGANGTTCTTTTGCTGTANAANAATTTCAGTATTTTTTT
TCCTGAAAAACGTGTTCCAACAATATTGAGTCACCTAGAAAGTTGAGCGGTCTTT
CAGGCTAGGCGGCTAGCACCAGCTAGATCAGCAAACTTTTGGCCG
GGCTCACTATTCTTAAATATTCGAGATGAGGACCCTCCTCCTCCTCCTGATCCAGT
GTTTTGTTTAATATTCGAGCTCAGCAAAACATCAGCAAACTTTGAAACTTTGGAAC
CATACAAATGAAATTTGTGTACCTCCTGGATCTCTCTCTTTTTGGCCCAT
TTGGCTCAGTTTGGAGGACCAGCAAGGCTGTGGCTTTTGCGAATCATTTTTC
AGGGAAAGCCTGGTTCTTTTCCAAACGGGTTGGAAGGGGAGAGGAGGTGGTGGCG
CCATTCTCCTCCTCCTCCTTGATGAGTGGACTGTTTGCTGATTTTGAT
M7-11 (on construct)
AGAGAGACTGGTGATTTCAGCGTGTCCTCTCCNAATGNNNTGAACTTCTTTATA
TAGAGGAAGGGTCTTGGCAGAGATAGTGGGATTGTGCGTCATCCCTTACGCT
GTGGAGATATCACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCT
TTTTCCACGATGCTCCTGCGTGTTGGGTGGGAGTCATCCTTTTGGACCACAGTGC
AG
M7-12 (on construct)
GCCCGGGAGAGGCGGTTTGCGTATTGGCTAGACAGCTTGGCCAAACATGGTG
GAGCAGACACTCTCCTGCTACTCCTCAAGAAGATATCAAGGCGTCAAGACAGAC
CAAGGCGGTATTCTCAAAGGGGTATTATCGGCCGAAAATCCCTCTCCTGG
ATTCCATCTGCCAGTACTCTGACTCTCTTCATGAAAAAGGACAGTAGAAAGGACAG
TGCGTGTGAACATTGCGTATATGGCGAAATACCAATCAGTACTCTGCT
GAAAAGAAGAGCCTGCAAGCAGGCTTTCAACAGCAGCTCTTTAAGAAGATGGTGA
AGTGGAGACAGCATCAGTCTCGCTACTCCAAGAAGGGGATCTGGTTCAGTGC
AGAAGACCAAGGGCTATTGAGACCTTTTCACAAAGGGGTAAATACGGGAAAACCTTC
TCGGATTCCCTGGCAAGCTATCTCATGACTGTTAAGGAGCTAAAGAGAG
AAGTGTCGCCACACTCAAGGCTCATCTGGCACTATCTTTGACAGTATAGC
GATGCCTGCTGCTCCTTTTGGCAAAAGGAAAGAGGGACAGTAGTACAGTGC
GATTCTCCTCTTTGGCAGGTGCACTCATCCTCTTCTTCTCGCAAGA
CCCTCCTGCTATATAGGAGAAGTTAGTTCTTCTTTGGAGAGACAGCTGAAAT
CCAGTCTCTCTCAAAATCTCATCTCTGCTGAGCTTTTGCAGATCAGGCCC
AATGGAGATAGGAAGGCCAACATCGCAAGCAGCAGCTCTGTGCAAGAGTTCTTG
ATCGGAAAAGTTTCGAGACCTCCTCGCCAGTATGAGCCTGAGGGCAG
AAATCTCGTGCTTTCCAGCTTATGGAGGGCGTGGATAGTTCTCTCGCGGTA
AATAGGAGC
M7-13 (on construct)
CTTTGGGCTAACAATAGAAGCAGGACATATAAGTCTATGCGACACATGACACAATATAA
AGTTAAAAAACATATTCCATAATCCTGCTACATTTGAGATCAGCATACA
TAAACTATTCAAAACAGGAGCTCTCTTGCTGCAAAACATAGACAACTTTG
GCAGGAGGAGGCGATTGTGCAATTACCTTGGAGAAATATCCTCTGCGCCTTT
CATCCTGTTAATACACAAATTAAGTCAAGTTTTGGGATAAAATACATATAAGA
GAACATGAATTTGATATGCGAGGGAGTATAAATAATACATATAGGAGAACATGAA
TCTGTAAGCTAACACGGCCTGAGCTAGGCAGCTAGCAGCTAGCGC
M7-14 (fused ends on construct)
AATCGGTTTACGATAACCGTACGGGATTTTCCCATCTACTTTTCTCCCC
TGGATGGATGAAACCGGCTCGGTAACCGGCTCGGTAATAATACCTCTACGTTTTTCAT
TTCCATATTAAACTTGCAGGCACGGAAACGAAAACGGGATATAACCGGCTGAAACGC
GTCGG
M7-15 (on construct)
GCGCAGCTATTACCCCGCACGACATATCCACGCGCCTCCTACATCGAAGCTGAA
AGCAGCGAGATTCTTCCCTCCCGAGACGCTGACGATCGCATTGCACTGCGAGACGCTGCG
AATCTCTGATCAGAAACTTTCTCGACAGACGTCGCGTGATGCTGAGTTCCAGGCTTTTCAT
ATATCTCATTGCCCCCCCGATCGCAGAAAGCTCGAGAGATAGGATTTGATA
AGAGACTGTTGATTTTACGCTGCTGTCTCTCAATGAAATGAACTATTTTATAT
AGAGAGAGGGTCTTTCGCAAGGATAGTGAGATTGTGCTGATTCTCCCTTAGAGTCAG
TGAGATATCACATCAATCCACTTTGCTTTGAAAGACGTTGTTTGGAAACGTCTTCTT
TTCCACAGATGCTCTCGTGGGTGGGCTGACATTTTGGGAGCCACTGTCGGCA
GCGACGCGAGGACATCTTTGAAACGATAGCCTTCTTTTATCGCAATGATGGC
TTTTAGTGGTGCACCTCTTTCTACTGTCTCTTTTGATGAAAGTGCAGATAGC
TGGGCAATCAGAGTCCCGAGAGGTTTCCCGATATTACCTTTTGGTAAAGTCTC
AATACCCCTTTGGTCTTCTGAGACTGTATCTTTGATATTCTGGAGTAGACGGAG
AGTGTCTGCTCCACCATGTTCCACATCAATCCACTTTGCTTTGAAAGACGTTGTTG
GAACGTCTTTTTTCCACGATGCTCTCTCGGGGTGGGGGCTCATTCTTGGGAC
References


CHAPTER 6. GENERAL CONCLUSION

In the 1940’s, Barbara McClintock identified Ds as an element of genome instability. The Ds element can induce frequent chromosome breakage at its chromosome location and also can induce rearrangements including chromosome duplication, inversion and translocation (McClintock 1950; McClintock 1956). In addition to inducing genome instability, the Ds element is also mobile in the presence of the Ac element. Subsequently, various researchers discovered mobile elements in many different kinds of organism. As molecular techniques developed, the Ac/Ds elements were sequenced in the 1980s. McClintock’s original Ds element contains a “double Ds” structure. Later, some other Ac/Ds-type chromosome breaking structures were sequenced; they all contain at least one pair of directly oriented Ds ends (Doring et al. 1984; Fedoroff et al. 1983; Martinez-Ferez and Dooner 1997). Functional tests confirmed that one pair of directly orientated Ds ends is sufficient to induce chromosome breakage (English et al. 1993). The directly orientated Ds ends induce chromosome breakage by a sister chromatid transposition mechanism (Weil and Wessler 1993). Sister chromatid transposition can also produce chromosome deletion or inverted duplication in addition to chromosome breakage (Zhang and Peterson 1999). Furthermore, analyses from the Dooner lab demonstrated that two closely linked Ac/Ds elements can induce chromosome breakage; however, the orientation of the Ac/Ds elements was not known (Dooner and Belachew 1991; Ralston et al. 1989). The P1-rr11 allele used in the current study does not contain directly-oriented Ds termini, but instead contains a pair of reversed Ac/Ds ends. Multiple p1-ww alleles from P1-rr11 were analyzed and found to contain a variety of rearrangements including inversion, deletion or local rearrangement structure. It was concluded that these rearrangements were generated by a reversed ends transposition mechanism (Zhang and Peterson 2004).

In Chapter 2, we systematically tested all possible chromosome breakage structures by isolating 14 alleles with Ac/fAc structure. A pair of directly oriented Ds ends structure can efficiently induce chromosome breakage as previously reported. The reversely oriented structure can also induce chromosome breakage presumably by a reversed Ds ends transposition mechanism. Macrotransposon structure, which is proposed to be able to induce
chromosome breakage, can not lead to any chromosome breakage but can drive the internal sequence to other location (HUANG and DOONER 2008; RALSTON et al. 1989). The Ac/fAc distance is negatively correlated to chromosome breakage frequency. A pair of Ac/Ds ends at a long distance, such as two Ds ends in different chromosomes, is not a suitable substrate for alternative transposition and does not lead to detectable chromosome breakage. Although transposable element may play a key role in genome dynamic, genome instability is constrained in a relatively stable status in nature.

Interestingly, a chromosome-breaking locus on maize chromosome 9 controlled by the En/Spm system was identified genetically (CORMACK and PETERSON 1994). Although the structure of the locus is yet unknown, it is conceivable that it contains direct-or reverse-oriented En/Spm termini which can undergo alternative transposition reactions of the type. If true, this would partially support the hypothesis that other “cut and paste” transposons also can undergo alternative transposition.

In additional to chromosome breakage, Barbara McClintock also reported that the Ac/Ds transposable element can induce major chromosome rearrangements. What is the mechanism for this kind of rearrangements? In Chapter 3 we tried to answer the question. Based on the rearrangement model by the alternative transposition, we propose that the reversed Ds ends transposition generates large chromosome inversion or translocation. By efficient genetic screening, we obtain 17 reciprocal translocation and 2 large inversion alleles. Further cytogenetic anlaysis or FISH analysis confirmed the results. The breakpoint junctions of all 19 alleles are mediated by the Ac or fAc. The standard 8 bp target site duplication flanking the Ac/fAc ends suggest that all the rearrangements come from the Ac/Ds transposition events. In additional to response the fundamental biology question, the study also suggests the transposable element may play a role in chromosome evolution by transposition reaction. One character of our large rearrangements lines is with defined breakpoint sequences. Although there are nearly thousands of translocation/large inversion lines in maize, no one is with cloned breakpoint sequence. According to our knowledge, no chromosome translocation line with a defined breakpoint sequence in plants is available now.
The 19 large maize rearrangemental lines provide a good resource for further gene positional effect analysis or chromosome fragmental dosage analysis.

In Chapter 4, we investigated the types and frequency of the genome rearrangements caused by a pair of reversed Ac/Ds termini. We screened 100 mutant alleles induced by an intact Ac and a fractured Ac (fAc) structure at the maize p1 locus from two alleles P1-rr11 and P1-rr910. The rearrangement types were characterized by PCR pattern analysis and/or by direct sequencing. The results show that about 50% of the mutant alleles show inversion pattern and about 20% show deletion pattern. Sequence analysis results show the majority of another set of 17/100 is flanking Ac or fAc small deletion. Interestingly one deletion allele, with only one nucleotide distance between Ac and fAc, is not competent for alternative transposition, evaluated by chromosome breakage. The flanking Ac/fAc deletion alleles were generated from their parental lines by the reversed Ds ends inserting to sister chromatid model. This model further reinforces the idea that alternative transposition reactions can widely reorganize the genome.

The first three parts demonstrate that alternative transposition can induce chromosome breakage and genome rearrangements in maize. In Chapter 5, we transform the reversed Ds structure into rice plant to check whether it can work in a similar way in non-maize plant and also to check whether it can be used for rice functional genomic analysis and chromosome engineering. We totally isolated 25 independent rearrangement alleles from three different genomic loci. The majority of the rearrangements are chromosome inversions; the inversion size varies from within the T-DNA sequence region to 1.5 Mb on the rice genomic region. In addition to the inversion, two deletion alleles were obtained. One deletion is 79 kb in size. Only the heterozygote seeds can be obtained among the 24 progenies. Similarly, the typical 8 base pairs (bp) Ac/Ds transposon targeted site duplication (TSD) from some inversion events confirmed the events truly come from the transposition products. Our results suggest that alternative transposition may be used for functional genomics and chromosome operation in rice.

Barbara McClintock proposed that transposable elements are latent genome reorganization factors which can respond to environmental changes or stress conditions.
(McClintock 1984). However, how transposable elements can sense and respond to environmental changes is still not fully understood. One possible connection is through DNA methylation. Stress environments can affect plant DNA methylation (Martienssen and Colot 2001; Steward et al. 2002), which can in turn affect transposable element activity (Becker and Kunze 1996; Ros and Kunze 2001; Wang et al. 1996; Wang and Kunze 1998). Thus, it is conceivable that stress conditions can reorganize the host genome through enhanced transposon activity. However, standard transposition does not lead to major changes in genome structure. Alternative transposition can produce large genome rearrangements and sometimes can generate novel genes. Thus, the host organism may benefit more from alternative transposition than from standard transposition. It is tempting to speculate that stress conditions, by affecting methylation and/or chromatin structure, may regulate the competence of transposon termini to undergo alternative transposition events. Some transposon termini, such as those of widely-separated elements, may then undergo alternative transposition reactions and greatly reorganize the genome. This hypothesis, though speculative, may provide one mechanism by which alternative transposition may facilitate adaption to different environments.

References


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