Meiotic Recombination Break Points Resolve at High Rates at the 5' End of a Maize Coding Sequence

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Sequence analysis of recombination break points has defined a 377-bp recombination hot spot within the *anthocyanin1* (*a7*) gene. One-fifth of all recombination events that occurred within the 140-kb *a7-shrunken2* internal resolved within this 377-bp hot spot. In yeast, meiotic double-strand breaks in chromosomal DNA are thought to initiate recombination and are generally located 5' of coding regions, near transcription promoter sequences. Because the *a7* recombination hot spot is located within the 5' transcribed region of the *a7* gene, the sites at which recombination events initiate and resolve appear to be different, but both appear to be regulated in relation to transcribed sequences. Although transposon insertions are known to suppress recombination and alter the ratio of crossovers to apparent gene conversions, the *Mutator1* transposon insertion in the *a1-mum2* allele does not alter the sites at which recombination events resolve.

INTRODUCTION

Meiotic recombination generates chromosomes with novel allelic arrays relative to their progenitor chromosomes. This is one of the processes that yields the genetic diversity on which evolutionary selection can act. However, recombination must be counterbalanced by the advantage of maintaining coevolved blocks of favorable alleles as a unit during meiosis. Indeed, recombination is highly regulated, as evidenced by the control of the frequency (Schuchert et al., 1991; Korol and Iliad, 1994) and the position (Roberts, 1986; Lambie and Roeder, 1986; Tanksley et al., 1992; Moore et al., 1993) of recombination events on chromosomes. Thus, meiotic recombination events do not occur randomly, and certain large intervals (e.g., those surrounding the centromeres) exhibit low rates of recombination per physical length. In addition, some relatively small chromosomal segments in a number of organisms are recombinationally hyperactive, that is, they serve as recombination hot spots (reviewed in Lichten, 1995; also, see Nelson, 1968; Freeling, 1978; Stahl, 1979; Dooner et al., 1985, 1991; Wessler and Varagona, 1985; Dooner, 1986; Sachs et al., 1986; Uematsu et al., 1986; Steinmetz et al., 1987; Ponticelli et al., 1988; Dorer et al., 1989; Brown and Sundaresan, 1991; Janson et al., 1991; Symington et al., 1991; Shiroishi et al., 1993; Civardi et al., 1994). These recombination hot spots exhibit ratios between physical and genetic distances (a ratio that has been termed *p*; Civardi et al., 1994) that are threefold to 100-fold lower than the genome average. In many instances, chromosome regions that exhibit low values of *p* (i.e., recombination hot spots) occur at or in the vicinity of genes.

The favored mechanistic model of recombination (Szostak et al., 1983) predicts that recombination is initiated by the generation of a double-stranded break (DSB) on one chromosome of an allelic pair (Figure 1). After strand invasion, DNA synthesis, and the creation of a Holliday Junction (which can undergo branch migration), the recombination event resolves, leading to an exchange of strands and thus "crossing over." As predicted by this model, in yeast, DSBs are associated with recombination hot spots (Nicolas et al., 1989; Sun et al., 1989; Cao et al., 1990; Schultes et al., 1990; Goldway et al., 1993; Nag et al., 1993). Analyses of the distribution of DSBs along yeast chromosomes suggest that recombination events initiate in the vicinity of transcription promoters (Wu and Lichten, 1994). Much less is known about where recombination events resolve subsequent to their initiation. To determine the physical relationship between the position at which DSBs occur and the final recombination break points, we have used DNA sequence polymorphisms to map recombination break points to high resolution within a maize gene (the *anthocyanin1* (*a7*) locus) that serves as a recombination hot spot (Brown and Sundaresan, 1991; Civardi et al., 1994).

RESULTS

Mutants at the *a7* and *shrunken2* (*sh2*) loci confer visible phenotypes that can be scored on kernels. This feature, in conjunction with the ease with which large numbers of kernels from an F1 generation can be produced in maize, makes these genes and the chromosomal interval defined by them ideal models...
Figure 1. The Double-Strand DNA Break Repair Model Explains Reciprocal Recombination and Gene Conversion.

(A) After premeiotic DNA replication, a cell contains four DNA duplexes carrying markers h, i, and j. In subsequent panels, only the two duplexes that recombine are illustrated.

(B) A double-strand break occurs in one duplex.

(C) A 5’ to 3’ exonuclease attacks the exposed 5’ ends.

(D) Strand invasion. The free 3’ ends are used as primers for DNA synthesis. The uncut homolog (black) serves as the template in these synthesis reactions.

(E) DNA synthesis (dashed lines) results in a four-strand intermediate with two Holliday Junctions. This structure can be resolved by cutting and religating at two sites involving the inner strands (resulting in F) or at one site involving the inner strands and another site involving the outer strands (resulting in G).

(F) Gene conversion in the absence of reciprocal recombination occurs if cuts and religation occur at positions 1 and 2 as depicted in (E). In this example, marker i from the gray duplex has been converted to I from the black duplex. Markers h and j, which flank the conversion tract, have not exchanged. The maximum extent of the gene conversion tract is indicated by the positions of the vertical arrows. The actual extent of the conversion tract depends on the results of mismatch repair.

(G) Gene conversion in combination with reciprocal recombination. Molecules recombinant for markers h and j would result if cuts and religation occur at positions 2 and 3, as depicted in (E). The recombination break point is adjacent to a gene conversion tract. This conversion tract would usually not be detectable in plants. Instead, the apparent position of the recombination break point would be shifted from the position indicated by the right arrow to that indicated by the left arrow. If the Holliday Junctions migrate away from the DSB site after DNA synthesis (E), the apparent recombination break point would be further removed from the DSB site at which this recombination event initiated.

Figure 2. Isolation of Intragenic Recombinant A′ Alleles from Cross 1.

(A) Genetic and physical distances on the long arm of chromosome 3. CEN 3 indicates the centromere.

(B) Isolation of intragenic recombinants from cross 1.

for the study of meiotic recombination. Another important feature, the a1-sh2 interval, has been cloned as a yeast artificial chromosome (Civardi et al., 1994).

Isolation of Recombinants

As depicted in Figure 2B, intragenic recombinants at the a1 locus could be selected directly from the progeny of cross 1 (see Methods) on the basis of their phenotype. The a1-mum2 and a1::rdt mutant alleles contain transposon insertions in the a1 sequence that disrupt gene function. a1-mum2 and a1::rdt behave as stable, recessive alleles when carried by kernels with the genotypes used in this study (see Methods). Thus, kernels that carry both of these mutant alleles are colorless. Most of the gametes generated from plants derived from such kernels carried one of these two mutant alleles. However, rare recombination events between the two transposons (which are 1.2 kb apart) generated chimeric dominant alleles (A′1) that condition colored aleurones, as shown in Figure 3. These ker-
nels that carry $A_1^*$ alleles were shrunken because the 5' end of the $a_1$ gene is closest to the $sh2$ gene (Civardi et al., 1994). Twenty-four such recombinants were isolated from a total population of 742,100 kernels generated over two pollinating seasons (Table 1). Hence, as shown in Figure 4B, the genetic distance associated with this 1.2-kb interval is 0.0065 ± 0.0009 centimorgans (cM) (see Methods). Of the 24 recombinants, 17 were analyzed. The validity of each of these 17 recombinants was confirmed via genetic crosses and DNA gel blotting experiments using a closely linked restriction fragment length polymorphisms (RFLP) marker (see Methods).

Because certain transposons can suppress rates of recombination (Dooner, 1986) or alter the ratio of crossovers to putative gene conversions (Dooner and Kermicle, 1986; but see Dooner and Raistin, 1990), we were concerned that the Mutator1 (Mu1) transposon in the $a_1$-mum2 allele might affect the distribution of recombination break points in the $a_1$ locus. To control for any effects that the Mu1 transposon might have on recombination, intragenic recombinants at the $a_1$ locus also were isolated from another heterozygous genotype (cross 2), as described in Figure 5A. Cross 2 is identical to cross 1, except that in the former cross, the $a_1$-mum2 allele has been replaced by the wild-type $A1-LC$ allele. The $A1-LC$ allele is identical in sequence to $a_1$-mum2, except that the former allele lacks a Mu1 insertion (see Methods). Because the $A1-LC$ allele conditions a colored aleurone, it was not possible to identify intragenic recombinants from cross 2 directly. Instead, the recombination break points associated with 21 $A1$ $sh2$ (and 35 $a_1$ $Sh2$) recombinant chromosomes isolated from 67,000 gametes produced by $A1-LC$ $Sh2$ $a1::rdt$ $sh2$ heterozygotes were mapped relative to the $a_1$ locus. These recombinants should carry recombination break points somewhere within the 140-kb interval defined by the $a_1$ and $sh2$ loci (Figure 5A). To identify those chromosomes carrying recombination break points within or near the $a_1$ locus, plants carrying the 21 independently derived $A1$ $sh2$ recombinant chromosomes (and the 35 $a_1$ $Sh2$ chromosomes) were analyzed via DNA gel blots. Those $A1$ $sh2$ chromosomes that arose via crossovers within the 6-kb interval defined by the $rdt$ insertion in the $a1::rdt$ allele and the first EcoRI site distal to the $a_1$ locus (indicated with an asterisk in Figure 5A) would be expected to display a novel RFLP pattern (Figure 5A; see Methods).

All of the progeny from cross 2 carry a nonrecombinant $a1::rdt$ $sh2$ chromosome from their paternal parent. Within the $a1$-$sh2$ interval, this chromosome is identical to the $a1::rdt$ $sh2$ parental chromosome shown in Figure 5A. Recombinant progeny also carry a chimeric chromosome derived from their maternal parent. Chimeric chromosomes that arose via recombination break points occurring within the 134-kb interval defined by the indicated EcoRI site and the $sh2$ locus have the genotype $a1::rdt$ $Sh2$ or $A1-LC$ $sh2$ and carry parental $a_1$ alleles (Figure 5B, lanes 3 and 5, respectively). In contrast, those chimeric chromosomes that arose via recombination break points within the 6-kb interval can be identified because they carry novel $a1$-hybridizing EcoRI restriction fragments (Figure 5B, lanes

<table>
<thead>
<tr>
<th>Year</th>
<th>No. Colored Shrunken Kernels</th>
<th>Population Size</th>
<th>cM$^a$</th>
</tr>
</thead>
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<tr>
<td>1991$^b$</td>
<td>9</td>
<td>215,300</td>
<td>0.0084</td>
</tr>
<tr>
<td>1993</td>
<td>15</td>
<td>526,800</td>
<td>0.0057</td>
</tr>
<tr>
<td>Pooled$^c$</td>
<td>24$^a$</td>
<td>742,100</td>
<td>0.0065 ± 0.0009</td>
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$^a$ Intragenic recombination was assayed using the scheme outlined in Figure 2B. Because only one of the two possible classes of recombination events could be identified (see Figure 2B), the genetic distance was calculated by doubling the rate at which colored, shrunken recombinants were recovered. This calculation assumes that two events occur at equal frequencies. $^b$ Data from Civardi et al. (1994). Subsequent genetic tests established that one of the 10 colored, shrunken kernels originally reported by Civardi et al. (1994) did not carry a legitimate intragenic recombinant. $^c$ The homogeneity $x^2$ value (0.839, with one degree of freedom) indicates that there is no significant difference between the rate of intragenic recombination in 1991 and 1993. The data for these 2 years were therefore pooled.

Figure 3. Phenotype Confounded by Chimeric $A1^*$ Alleles.

The single, colored kernel on this ear derived from cross 1 has the genotype $A1^* sh2$ $a1::rdt$ $sh2$. 

Table 1. Rate of Intragenic Recombination at the $a_1$ Locus
4 and 6, respectively). These novel \( aT \) alleles are designated \( A1^* \) and \( aT^* \). The novel restriction fragments associated with \( A1^* \) alleles are not present in nonrecombinant siblings (i.e., plants grown from colorless, shrunken, or colored round kernels from cross 2) of several \( A1^* \) \( sh2 \) recombinants (data not shown). By this RFLP assay, eight of the 21 \( Al \) \( sh2 \) (and eight of 35 \( aT \) \( Sh2 \)) chromosomes were established to have arisen via crossovers within a 6-kb interval 5' of position +1083 (the \( rdt \) insertion site in the \( aT \) locus). The genetic distance associated with this 6-kb interval is therefore 0.026 cM (see Methods and Figure 4A).

**Mapping Recombination Break Points**

By virtue of their origin, the chimeric \( A1^* \) and \( A1 \) alleles generated via crosses 1 and 2 include portions of the \( aT::rdt \) and \( aT-mum2 \) (or \( A1-LC \)) alleles. As shown in Figure 6, the \( aT::rdt \) allele contains a centrally located PstI recognition site (indicated with an asterisk) that is absent from the corresponding interval of the \( aT-mum2 \) and \( A1-LC \) alleles. Fifteen of the \( A1^* \) and all eight of the \( A1 \) alleles were polymerase chain reaction (PCR) amplified, gel purified, and subjected to PstI digestion. As shown in Figures 7A and 7B, these analyses demonstrated that in all 23 cases, the recombination break points associated with the \( A1^* \) and \( A1 \) alleles resolved 5' of the diagnostic PstI site. Because the recombination break points associated with the 15 \( A1^* \) alleles must have resolved 3' of the \( Mu1 \) insertion (position -97; Figure 2B), this experiment mapped these break points to a 643-bp interval. Similarly, the average value of this ratio (\( p \)) in the maize genome is 1456 kb/cM (Civardi et al., 1994).

(A) The physical distances associated with the indicated intervals of parental chromosomes from cross 2 are shown in the top three lines. The fourth line indicates the extent of the \( aT \) probe. Lines five and six show the positions of relevant restriction enzyme recognition sites on the parental chromosomes relative to the \( aT \) and \( sh2 \) loci. The position of the 700-bp \( rdt \) transposon insertion is indicated. (The X indicates a crossover that would generate a progeny chromosome that has a novel \( aT \)-hybridizing RFLP.) Lines seven and eight represent schematics of recombinant chromosomes derived from cross 2 via a crossover in the position indicated by the X between lines five and six. (B) DNA samples from plants homozygous for \( Al-LC \) or \( aT::rdt \) (lanes 1 and 2) or from plants grown from colorless, round (lanes 3 and 4) or colored, shrunken (lanes 5 and 6) kernels carrying recombinant chromosomes derived from cross 2 were digested with EcoRI, transferred to nylon membrane, and hybridized with the \( aT \) probe indicated in (A). Numbers at left indicate the positions of molecular size standards in kilobases.
the eight A7* alleles must have resolved within this 643-bp interval or 5' of position -97 but 3' of the centromere distal EcoRI site that is indicated with an asterisk in Figure 5A.

Because a7-mum2 (and its apparent progenitor, A1-LC) exhibits abundant DNA sequence polymorphisms relative to al::rdf, it was possible to map more precisely the positions of recombination break points by sequencing diagnostic portions of the 643-bp interval from each of the 15 A7' alleles and eight A7* alleles. These analyses demonstrate that recombination break points do not resolve uniformly across the a7 locus (Figure 6); rather, they cluster within a 377-bp interval of the a7 gene. Fourteen of the 15 recombination break points associated with A7' alleles and four of the eight recombination break points associated with A7* alleles resolved within this 377-bp interval (the remaining four recombination break points associated with A7' alleles resolved at undetermined locations 5' of position -97). Hence, this 377-bp interval exhibits values of p equal to 62 kb/cM and 29 kb/cM in the two experiments (see Methods and Figure 4B). In contrast, the overall value of p in the maize genome is 1456 kb/cM (Civardi et al., 1994). Hence, as measured by crosses 1 and 2, this 377-bp recombination hot spot is 23 and 50 times more recombinant than the genome as a whole.

**Figure 6. Locations of Recombination Break Points Associated with Chimeric A7' and A7* Alleles.**

The A1-LC allele is identical in sequence to a7-mum2, except for the presence of the Mu7 insertion (indicated by dashes) in the latter. The vertical bars on the schematic diagrams of the a7 alleles represent sequence polymorphisms between the a7-mum2 (or A1-LC) and al::rdt alleles that were used to define the intervals to which recombination break points of A7' and A7* alleles were mapped. The bar widths are proportional to the number of base pairs associated with the indicated sequence polymorphisms. The locations and sequence coordinates of PstI sites are shown. The PstI site that is present in a7::rdt but absent in a7-mum2, and A1-LC is indicated by an asterisk. As depicted here, the centromere and the sh2 locus lie to the left and right of the a7 locus, respectively (see Figure 2A). The number of break points from crosses 1 and 2 associated with A7' and A7* alleles that map to the various intervals are indicated on the horizontal bars labeled cross 1 and cross 2.

**Distinguishing between Reciprocal Recombination and Gene Conversion**

Recombination events can resolve via reciprocal recombination or the related process of gene conversion. Gene conversion occurs via the nonreciprocal transfer of DNA sequences from one nonsister chromatid to another (Figure 1). Therefore, some of the A7' alleles isolated in this study could have arisen via the nonreciprocal transfer of sequence information from one a7 allele to the other so that the region containing a transposon was replaced. Such converted alleles would confer a colored kernel phenotype. However, because in these experiments only A7' alleles that were in coupling with the closely linked sh2 mutant were selected (Figure 2B), only those gene conversions that removed the rdt transposon from the a7::rdt sh2 chromosome would be expected to be recovered.

The genetic markers php10080 and sh2, which are located ~2 and 0.1 cM proximal and distal of the a7 locus, respectively (Figure 2A), were used to distinguish between reciprocal recombination events (i.e., single crossovers) and putative gene conversion events. The former would be expected to exhibit an exchange of flanking markers, whereas the latter would not. The a7::rdt sh2 stock used in crosses 1 and 2 (Figures 2 and 5)
Figure 7. Mapping Recombination Break Points within A1' and A1" Alleles Using the Diagnostic PstI Site.

(A) Recombinant alleles were PCR amplified (using primers XX026 and XX025; Figure 6) from genomic DNA isolated from plants with the genotype A1'at::rdt or A1"at::rdt. Agarose gel electrophoresis revealed one to three PCR products from each reaction. The expected 1.3-kb a7-hybridizing product, derived from A1' or A1" alleles, was detectable in all reactions (represented by alleles A1'-276, A1'-102, and A1"-275). The 2.0-kb a7-hybridizing product, derived from a7::rdt, was detectable in most reactions (represented by allele A1"-276). Some reactions yielded an 0.8-kb nonspecific PCR product that did not cross-hybridize with an a7-specific probe (represented by alleles A1'-27A and A1'-102). The DNA gel blots involving the a7-specific hybridization probe are not shown. Numbers at right indicate the size of each individual fragment in kilobases.

(B) The 1.3-kb PCR products derived from each of the recombinant alleles were gel purified and subjected to PstI digestion and electrophoresis. In each of the 23 A1' and A1" alleles, the diagnostic 631-bp fragment was recovered (see Methods).

Figure 8. Distinguishing between Reciprocal Recombination and Gene Conversion Events via RFLP Analysis.

Dna samples from plants with the indicated genotypes or stocks were digested with HindIII, transferred to nylon membrane, and hybridized with a php10080-specific probe. Similar results were obtained in DNA gel blots after digestion with EcoRI. Numbers at right indicate the size of each individual fragment in kilobases.
were identified by virtue of the fact that recombination within the 134-kb interval that composes the remainder of the a7-sh2 interval has a value of \( \frac{0.026}{1456} \) kb/cM (Civardi et al., 1994). In contrast, the 134-kb interval that is substantially less recombination-active. Subsequent mapping using DNA sequence polymorphisms established that recombination break points isolated via the two approaches mapped to the same 377-bp interval, it can be concluded that the Mu1 transposon insertion in the a1-mum2 allele suppresses recombination rates, it does not alter the distribution of recombination break points within the a1 gene. Because all crosses in which recombination was measured involved the a1::rdt allele, we cannot draw conclusions regarding the effects of the rdt transposon insertion may have on recombination.

In yeast, it has been shown that DNA synthesis associated with recombination results in mutations (Strathern, 1995). However, all sequenced regions of the At' and At* alleles were identical to either a1-mum2 (or A1-LC) or a1::rdt, that is, recombination was precise (data not shown). However, given the low rate at which unrepaired recombination-related nucleotide misincorporation errors occur in yeast (between \( 10^{-5} \) and \( 10^{-6} \) per base; Strathern, 1995), the failure to uncover any novel sequence alterations in the vicinity of the 19 maize recombination break points analyzed in this study may be the result of inadequate sampling.

The results from crosses 1 and 2 resolved within a 377-bp interval, the value of \( p \) was measured as 62 and 29 kb/cM in crosses 1 and 2, respectively (Figure 4B). This interval is therefore 23 and 50 times more recombinant than the genome as a whole (as measured via crosses 1 and 2, respectively).

This 377-bp recombination hot spot is located near the 5' end of the transcribed region of the a1 gene. While this article was under review, Patterson et al. (1995) and Eggleson et al. (1995) published restriction mapping data that demonstrate that recombination events resolve at high rates in the 5' and 3' ends of the booster (b1) and r1 loci of maize, respectively. In yeast, the meiotic DSBs that are thought to initiate recombination are generally located 5' of genes, near transcription promoter sequences (Wu and Lichten, 1994). If this is true in plants, it therefore appears that although the sites at which recombination events initiate and resolve may be different, both appear to be spatially regulated in relation to transcribed sequences.

The Mu1 Transposon Does Not Alter the Spatial Distribution of Recombination Break Points

These results extend to Mu1 the finding that the Dissociation (Ds) transposons (Dooner, 1986) have an inhibitory effect on recombination rates. In the present experiment, the 1.4-kb Mu1 insertion present in a1-mum2 (used in cross 1), reduced recombination rates in a nearby 377-bp interval by \( \approx 50\% \). The results from crosses 1 and 2 are directly comparable, because the A1-LC and a1-mum2 alleles are identical in sequence except for the presence of the Mu1 insertion in the latter allele. However, because most recombination break points isolated via the two approaches mapped to the same 377-bp interval, it can be concluded that although the Mu1 transposon insertion in the a1-mum2 allele suppresses recombination rates, it does not alter the distribution of recombination break points within the a1 gene. Because all crosses in which recombination was measured involved the a1::rdt allele, we cannot draw conclusions regarding the effects of the rdt transposon insertion may have on recombination.

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The 377-bp Hot Spot May Block Holliday Junction-Associated Branch Migration or 5' to 3' Exonuclease Activity

Based on the current models of recombination that explain gene conversion (Szostak et al., 1983), the DSBs that initiated these two gene conversion events must have occurred within the region of the a1 locus that was converted, that is, 3' of interval V (Figure 6). In contrast, data from yeast indicate that the bulk of the DSBs that initiate recombination events occur 5' of gene coding regions (Wu and Lichten, 1994). If the DSBs that initiate maize recombination events also occur 5' of gene coding regions, some of the DSBs that led to the reciprocal recombination events reported in this study probably occurred 5' of the a1 recombination hot spot. Hence, because the two
conversion events initiated via DSBs 3' of the recombination hot spot in the a7 gene, these data suggest that DSBs that occur in spatially distinct intervals can resolve within the same recombination hot spot. In addition, these results suggest that the recombination hot spot identified in this study has some feature that terminates the process of branch migration or 5' to 3' exonuclease activity (Figure 1) from both directions and thereby constrains the sites at which Holliday Junctions resolve.

METHODS

Gene Symbols, Allele Descriptions, and Genetic Stocks

The a7 locus encodes the enzyme dihydroquercetin reductase (EC 1.1.1.219), which is required for the biosynthesis of anthocyanin pigments that color the aleurone layer of maize kernels (Reddy et al., 1987). The closely linked shrunken2 (sh2) locus (Figure 2A) encodes ADP-glucose pyrophosphorylase (EC 2.7.7.27), which is involved in starch biosynthesis (Tsai and Nelson, 1966). As shown in Figure 3, kernels that do not carry wild-type alleles at these loci exhibit colorless and shrunken phenotypes, respectively.

The recessive a7-mum2 allele contains a 1.4-kb Mutator1 (M1) transposon insertion at nucleotide -97, and the recessive a7::rdt allele contains a 0.7-kb rdt transposon insertion at nucleotide +1083 (O'Reilly et al., 1985; Shepherd et al., 1988; Brown et al., 1989; positions are relative to the start of transcription in the A1-LC allele). The recessive a7::rdt and a7-mum2 alleles both condition a colorless phenotype in the absence of trans-acting regulatory transposons (Dotted [D] and MuDR, respectively). The stocks used in this study do not carry Dt or MuDR. The a7-mum2 stock without MuDR is derived from that described by Schnable and Peterson (1989). The a7::rdt and sh2 marker alleles used in this study were extracted from a commercially available Asgrow sweet corn line (Civardi et al., 1994) and the F1 hybrid Sweet Belle. This hybrid and its two parent inbreds, Ac1068 and Ac1069, were gifts from the Asgrow Seed Company (Kalamaio, MI). Ac1068 and Ac1069 carry distinguishable alleles at the php70080 locus (php70080-Ac1068 and php70080-Ac1069). This fact explains the observation that our Sweet Belle-derived a7::rdt sh2 stock is heterogeneous for these two php70080 alleles. The a7-dl allele is a stable recessive allele obtained in coupling with etchedl (et7) from Pioneer Hi-Bred (Ames, IA). Ac1068 was a gift from C. Scofield (Ames, IA), Ac1069, were gifts from the Asgrow Seed Company (Kalamaio, MI), and Ac1069, were gifts from the Asgrow Seed Company (Kalamaio, MI).

DNA Isolation, DNA Gel Blot Analyses, and DNA Probes

Maize DNA was isolated from immature plant leaves, as described by Saghai-Maroof et al. (1988). DNA gel blots were conducted via standard protocols (Sambrook et al., 1989). The php70080, a7-, and sh2-specific probes were isolated from p-php70080, pALC2 (Schwarz-Sommer et al., 1987), and pSh2-850 (or pSh2-1000) derived from a sh2 cDNA clone (Brave et al., 1990). These clones were gifts from Pioneer Hi-Bred International Inc. (Johnston, IA), Alfonso Gierl (Technische Universität, Munich, Germany), and Curt Hannah (University of Florida, Gainesville, respectively).

Sequencing the a7-mum2 and a7::rdt Alleles

An "old" lysate of the phage c10Mu, which contains a 78-kb EcoRI fragment that spans the a7-mum2 allele sequence (O'Reilly et al., 1985), was generously provided by Alfonso Gierl (Technische Universität). Unfortunately, this lysate no longer contained viable phage. To rescue the a7-mum2 allele, phage DNA was extracted from the lysate, digested with NotI and EcoRI, and subcloned into pUC9 (Veira and Messing, 1982). The 3.0- and 4.8-kb subclones were termed pYEN1 and pYEN2, respectively. The a7::rdt allele was obtained as a 10-kb EcoRI fragment in the pUC9 vector and was a generous gift from N. Shepherd (DuPont, Wilmington, DE). This clone was termed pE10. The DNA sequence of the 1.2-kb interval of the a7-mum2 allele that is defined by the two transposon insertion sites was found to be identical to the corresponding interval of the existing sequence of the A1-LC allele (GenBank accession number X05088). The sequence of the corresponding interval from the a7::rdt allele was obtained and submitted to GenBank (accession number U23161). All sequences are based on at least three sequencing reactions. Sequencing was performed at the Iowa State University Nucleic Acid Facility on an ABI 373A automated DNA sequencer (Applied Biosystems, Foster City, CA) using dyeodeoxy terminators.

Generating Intragenic Recombinants

Rare intragenic a7 recombination events were selected from crosses 1 and 2. Cross 1 was conducted by planting the two parents in near isolation from other maize pollen sources at the Ross and Iden research farms (Ames, IA) during the 1991 and 1993 summer seasons. To conduct this cross, the female parent (which is listed first) of cross 1 was detasseled (emasculated) before anthesis. Therefore, the seed produced by the female parent was generated via cross 1. Most kernels isolated from this cross would be expected to have the genotype of either a7-mum2 Sh2::rdt sh2 or a7::rdt Sh2::rdt sh2. These genotypes would condition colorless, round and colorless, shrunken kernel phenotypes, respectively. However, rare intragenic recombination events at the a7 locus that occur within the 1.2-kb interval between the two transposon insertion sites in the a7 gene can generate chimeric A1' alleles (Figure 2B) that condition colored kernels (Figure 3). The intragenic recombinants isolated from the 1991 experiment were reported by Civardi et al. (1994). The recombination events between the A1-LC Sh2 and a7::rdt sh2 chromosomes analyzed in this report were isolated by Civardi et al. (1994). Briefly, cross 2 was conducted via controlled hand pollinations performed in our genetics nursery in 1992 at the Curtiss research farm (Ames, IA). Putative recombinants were selected as colored, shrunken and colorless, round kernels.

The recombination break point associated with each recombinant was mapped relative to the first EcoRI site 5' of the A1-LC alleles, using DNA gel blot analyses in which an a7-specific probe was utilized. This EcoRI site is marked with an asterisk in Figure 5. Recombination break points within the 134-kb interval defined by the marked EcoRI site and the sh2 locus would be expected to yield a7-hybridizing restriction fragments identical to those of A1-LC or a7::rdt. In contrast, break points within the 6-kb interval defined by the 700-bp rdt insertion in a7::rdt (position +1083) and the marked EcoRI site would be expected to generate novel restriction fragments. The a7-hybridizing EcoRI restriction fragments associated with the A1' sh2 and A1' Sh2 chromosomes would be expected to be 700 bp smaller than those associated with a7::rdt and 700 bp larger than those associated with A1-LC, respectively. Using
this method, we identified those recombinants that potentially arose via intragenic recombination within the \( a1 \) locus.

**Confirmation of \( A1' \) and \( A1^* \) Alleles**

Genetic crosses and DNA gel blotting experiments were used to confirm the validity of most of the putative \( A1' \) intragenic recombinants from cross 1 (seven colored, shrunk kernels did not germinate and therefore could not be tested). Colored, shrunk kernels from cross 1 (presumed genotype being \( A1' \) sh2/at::rdt sh2) were crossed by \( at^{-} \) Sh2/al-at Sh2. Half of the progeny kernels from this cross were expected to be colored and round. The other half were expected to be colorless and round. Plants derived from colored, round kernels were self-pollinated. Self-pollinated progenies that segregated in a 1:2:1 ratio of colored, shrunk:colored, round;colorless, round kernels were deemed to carry legitimate \( A1' \) alleles. As a further test, plants carrying the \( A1' \) alleles were subjected to DNA gel blot analysis with the RFLP marker \( \text{php}10080 \) (data not shown). Because the \( \text{php}10080 \) locus is closely linked to the \( a1 \) locus (Figure 2A), only those \( A1' \) alleles that were in coupling with one of the parental alleles of \( \text{php}10080 \) were considered to be valid recombinants.

The confirmation procedure for the progeny of cross 2 was described previously by Civardi et al. (1994). As a further confirmation, putative recombinants from cross 2 were subjected to DNA gel blot analyses using \( \text{php}10080 \)-specific and \( sh2 \)-specific probes (data not shown). Only those progeny that exhibited appropriate alleles of these loci were considered to be valid recombinants.

**Calculations of Genetic Distances**

\( A1' \) alleles arise in cross 1 via one of the two possible intragenic recombination events (see Figure 2B). The reciprocal recombination event generates \( A1' \) alleles that carry both transposon insertions and thus do not condition distinctive phenotypes, that is, they condition colorless kernels. Because only one of the two possible classes of recombination events could be identified in the case of cross 1, the genetic distance associated with the 1.2-kb interval was calculated by doubling the rate at which colored, shrunk kernels were recovered (2 \( \times \) 24/742,100 = 0.0065 cM). This calculation assumes that the two events occur at an equal frequency. Standard errors were calculated according to the equation \((p-q)/(n)^{1/2}\), where \( p \) is the map distance in centimorgans (cM), \( q \) is (100 - \( p \)), and \( n \) is the number of kernels from the testcross that were scored. To calculate the genetic distance associated with the 377-bp interval as measured by cross 1, the proportion of \( A1' \) alleles whose recombination break point mapped to the 377-bp interval was multiplied by the genetic distance of the 1.2-kb interval (14/15 \( \times \) 0.0065 cM = 0.0061 cM).

The collection of recombinants derived from cross 2 has been used to estimate the genetic distance between the \( a1 \) and \( sh2 \) loci as 0.09 \( \pm \) 0.01 cM (Civardi et al., 1994). Because not all of these recombinants were used in the present study, estimates of genetic distances for physical intervals smaller than the 140-kb \( a1-sh2 \) interval that flank the 1.2-kb interval defined by the two transposon insertion sites. The 5' ends of primers XX025 and XX026 are located at positions -122 and +1162 in the \( a1 \) gene, just upstream of the \( Mul \) insertion site in the \( a1::rdr \) allele and just downstream of the \( rdt \) insertion site in the \( a1::rdr \) allele, respectively. The sequences of the two primers are as follows: XX025, 5'-GGAAGTAGTGACGGGTGTTGGTT-3'; XX026, 5'-AGGTCGTCGACGGGTTGAGC-3'.

**Polymerase Chain Reaction Amplification of Recombinant \( A1' \) and \( A1^* \) Alleles**

Template DNA was obtained from plants derived from colored, shrunk kernels that carried \( A1' \) and \( A1^* \) alleles and that were isolated via crosses 1 and 2. The \( A1' \) and \( A1^* \) alleles from each sample were polymerase chain reaction (PCR) amplified using two primers (XX025 and XX026) that flank the 1.2-kb interval defined by the two transposon insertion sites. The 5' ends of primers XX025 and XX026 are located at positions -122 and +1162 in the \( a1 \) gene, just upstream of the \( Mul \) insertion site in the \( a1::rdr \) allele and just downstream of the \( rdt \) insertion site in the \( a1::rdr \) allele, respectively. The sequences of the two primers are as follows: XX025, 5'-GGAAGTAGTGACGGGTGTTGGTT-3'; XX026, 5'-AGGTCGTCGACGGGTTGAGC-3'.

All PCR primers were synthesized on a 394 DNA/RNA synthesizer from Applied Biosystems at the Iowa State University Nucleic Acid Facility. PCR amplifications were conducted for 40 cycles as follows: 94°C for 2.5 min, 53°C for 1 min, and 72°C for 1.5 min.

**Mapping \( A1' \) Recombination Break Points Relative to the Diagnostic PstI Site**

DNA sequence analyses predict the presence and absence of a diagnostic PstI recognition site in the \( a1::rdr \) and the \( a1::rdr \) (and \( A1-LC' \)) alleles, respectively (Figure 6). The presence of this diagnostic PstI site in the \( a1::rdr \) allele used in this experiment was confirmed by PCR amplifying this allele from the sweet corn hybrid Sweet Belle (our source of the \( a1::rdr \) allele), using primers XX025 and XX026, and then subecting the resulting PCR product to PstI digestion.

The \( A1' \) and \( A1^* \) alleles were PCR amplified from DNA isolated from plants with the genotype \( A1'at::rdr \) or \( A1^*at::rdr \) (two of the 17 \( A1' \) alleles were not successfully amplified). The PCR primers XX025 and XX026 amplify the entire 1.2-kb interval between the \( Mul \) and \( rdt \) transposon insertion sites (Figure 6). Total PCR were subjected to electrophoresis through Tris acetate EDTA agarose gels (Figure 7A). DNA gel blot analyses using an \( a1 \)-specific hybridization probe confirmed that the resulting 1.3-kb PCR product was derived from the \( a1 \) locus (data not shown). The PCR products derived from the \( A1' \) and \( A1^* \) alleles were excised from duplicate gels and purified using the Gene-Clean kit (Bio-101, Inc., Midwest Scientific, St. Louis, MO). The 1.3-kb PCR products derived from each of the \( A1' \) and \( A1^* \) alleles were gel purified and subjected to PstI digestion and electrophoresis (Figure 7B).

Because the 1.2-kb interval of the \( a1::rdr \) allele contains a centrally located PstI site that is absent from the corresponding interval of the \( a1::rdr \) and \( A1-LC' \) alleles (marked with an asterisk in Figure 6), it was possible to map each recombination break point relative to this restriction enzyme site. Three of the predicted PstI digestion products (~381, 220, and 52 bp) are not informative. However, if a given recombination break point occurred between the \( Mul \) insertion and this diagnostic PstI site, the resulting digest should include an ~631-bp fragment. Alternatively, if the recombination break point was located between the \( rdt \) insertion and this PstI site, then the PstI restriction enzyme should cleave the 631-bp fragment into two fragments of ~389 and 242 bp.
Sequencing the A1' and A1" Alleles

Purified PCR products (derived from primers XX026 and XX025; see Figures 6 and 7) were used directly for sequencing; sequencing results are based on at least two reactions. The primers used for sequencing (and their positions within the a7 gene) are as follows: XX653, 5'-CGAGGAGGCCGAGGAAG-3' (163 to 144); XX231, 5'-GCCAAACTC-TGATCGTCCCTGTG-3' (260 to 282); and XX390, 5'-TCGCTTGGAT-TCTCATCT-3' (604 to 584).

Sequence Analyses

Sequence analyses were performed using Version 7 of the Genetics Computer Group (Madison, WI) software.

ACKNOWLEDGMENTS

We thank members of the Schnable laboratory and Joel Hansen of the Nikolau laboratory for assistance with planting, detasseling, and harvesting the isolated crossing plots. We also thank Yijih Xia, John VanDipen, Kevin Van Dee, and Weijun Chen for providing additional technical assistance; Homer Caton of AgriPro Seeds (Ames, IA) for providing access to maize ear-drying facilities; and John lmsande for technical assistance; Homer Caton of AgriPro Seeds. X.J.X., L.Z., and A-P.H. are students in the lowa State University Molecular, Cellular and Developmental Biology (X.J.X. and L.Z.), and Interdepartmental Genetics (A-P.H.) graduate programs. This is Journal Paper No. J-16288 of the lowa Agriculture and Home Economics Experiment Station (Ames, IA). Project No. 3125.

Received July 27, 1995; accepted October 16, 1995.

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*Plant Cell* 1995;7:2151-2161

DOI 10.1105/tpc.7.12.2151

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