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Recombination Occurs Uniformly within the *bronze* Gene, a Meiotic Recombination Hotspot in the Maize Genome

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The *bronze* (*bz*) gene is a recombinational hotspot in the maize genome: its level of meiotic recombination per unit of physical length is >100-fold higher than the genome's average and is the highest of any plant gene analyzed to date. Here, we examine whether recombination is also unevenly distributed within the *bz* gene. In yeast genes, recombination (conversion) is polarized, being higher at the end of the gene where recombination is presumably initiated. We have analyzed products of meiotic recombination between heteroallelic pairs of *bz* mutations in both the presence and absence of heterologies and have sequenced the recombination junction in 130 such *Bz* intragenic recombinants. We have found that in the absence of heterologies, recombination is proportional to physical distance across the *bz* gene. The simplest interpretation for this lack of polarity is that recombination is initiated randomly within the gene. Insertion mutations affect the frequency and distribution of intragenic recombination events at *bz*, creating hotspots and coldspots. Single base pair heterologies also affect recombination, with fewer recombination events than expected by chance occurring in regions of the *bz* gene with a high density of heterologies. We also provide evidence that meiotic recombination in maize is conservative, that is, it does not introduce changes, and that meiotic conversion tracts are continuous and similar in size to those in yeast.

INTRODUCTION

Meiotic recombination is not distributed uniformly in eukaryotic genomes, with the variation in recombination between different chromosomal regions being several orders of magnitude in some species. This variation in recombination has given rise to the concept of "recombination hotspots," which are defined as regions exhibiting a much higher recombination per unit of physical distance (usually expressed as centimorgans [cM] per kilobase or its reciprocal) than the genome's average (Lichten and Goldman, 1995).

Recombination hotspots in chromosomes have been known for years. In maize, for example, there is a disproportionate frequency of crossing over in distal chromosomal regions (reviewed in Carlson, 1977). Discrepancies in other regions, particularly the centromere, have been reported in several other plants (reviewed in Puchta and Hohn, 1996). In yeast, the eukaryote in which meiotic recombination has been best characterized, regions of high and low recombination have been found to alternate along the length of an entire chromosome (Lichten and Goldman, 1995). At a much higher level of resolution, variability of recombination in fungi takes the form of gradients in gene conversion frequencies: markers located near one end of the gene convert more frequently than do markers located at the opposite end of the gene. This is the principal manifestation of the phenomenon known as conversion polarity. The high conversion end of the gene is the 5′ end for *ARG4* (Fogel et al., 1981; Nicolas et al., 1989) and *HIS4* (White et al., 1991) but the 3′ end for *HIS2* (Malone et al., 1992). Polarity gradients are generally accepted to reflect sites at the high-conversion end of the gene, where recombination (i.e., gene conversion) is initiated and from where conversion tracts are propagated in a distance-dependent manner. A polarity gradient like that seen for gene conversion has also been detected for reciprocal recombination within the yeast *LEU2* gene: segments of similar size recombined 60 times more frequently at the 5′ end than did segments at the 3′ end of the gene (Hayden and Byers, 1992).

A sharper resolution of the variability of meiotic recombination in maize has been attained in studies of intragenic recombination at various loci. These studies have revealed an extreme nonrandomness in the distribution of recombination events. Recombination in the *bronze* (*bz*) locus, for example, is at least 100 times higher than in the average segment of the maize genome (Dooner et al., 1985). Although genes comprise a small fraction of the genome, they behave in general as recombination hotspots (Dooner, 1986; Brown and Sundaressan, 1991; Civardi et al., 1994; Eggleston et al., 1995; Patterson et al., 1995). This is perhaps an unexpected finding, but it provides support for Thuriaux's (1977) hypothesis that meiotic recombination in eukaryotes is confined to

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"structural" genes (Dooner et al., 1991). The occurrence of multiple sequence polymorphisms between maize alleles has enabled investigators to identify sites within genes where recombination events resolve preferentially. Thus, Patterson et al. (1995) and Xu et al. (1995) found that most exchanges resolved in regions near the 5′ end of *B* and *A1*, respectively, whereas Eggleston et al. (1995) found that nearly all exchanges at *R* occurred in the 3′ portion of the gene. Here, we address the issue of recombination polarity at the *bz* locus, particularly with reference to the possible effects of insertion and single base pair heterologies in creating apparent recombination hotspots within the gene.

The *bz* locus is ideally suited for an analysis of meiotic recombination in plants. It conditions anthocyanin pigmentation in the aleurone layer of the seed so that *Bz* intragenic recombinants (IGRs) can be easily selected as exceptional purple seeds in bronze ears and then subjected to genetic and molecular analysis. This is an important advantage because the generally low frequency of intragenic recombination in plants makes it necessary to screen populations of tens, and even hundreds, of thousands of individuals to recover a sufficient number of recombinants for subsequent analyses. Furthermore, *bz* is flanked by two easily scored endosperm markers, *shrunken* (*sh*) and *waxy* (*wx*), making it possible to readily sort *Bz* IGRs into classes carrying either parental or recombinant arrangements of flanking markers (Dooner, 1986). We have analyzed products of meiotic recombination between heteroallelic pairs of *bz* mutations both in the presence and absence of heterologies and have sequenced the recombination junction in 130 *Bz* IGRs. In addition to examining the uniformity of recombination within *bz*, we have been able to determine the length of two conversion tracts at *bz*, that is, the length of the fragment from one parent chromosome that is transferred to the homolog during meiotic recombination, and to investigate the fidelity of the meiotic recombination process in maize.

RESULTS

Location of Mutations

Figure 1 shows the physical location of all of the mutations used in this study. The two insertion mutations are derivatives of the *Bz-McC* allele that was present in the stocks in which B. McClintock isolated her unstable *bz* mutations (McClintock, 1955). *bz-m2(DI)* harbors a 3.3-kb *Dissociation* (*Ds*) element that arose by a simple internal deletion of the 4.6-kb *Activator* (*Ac*) element present in the *bz-m2(Ac)* allele (Dooner et al., 1986). It is located at positions 755 to 762 in *Bz-McC*; that is, the 8 bp between positions 755 and 762 of *Bz-McC* are duplicated on either side of the *Ds* element. *bz-m1* harbors a 1.2-kb *Ds* element close to the 5′ end of the gene (Dooner et al., 1985). To localize this insertion precisely, DNA from the *bz-m1* mutation was amplified with primers N and F

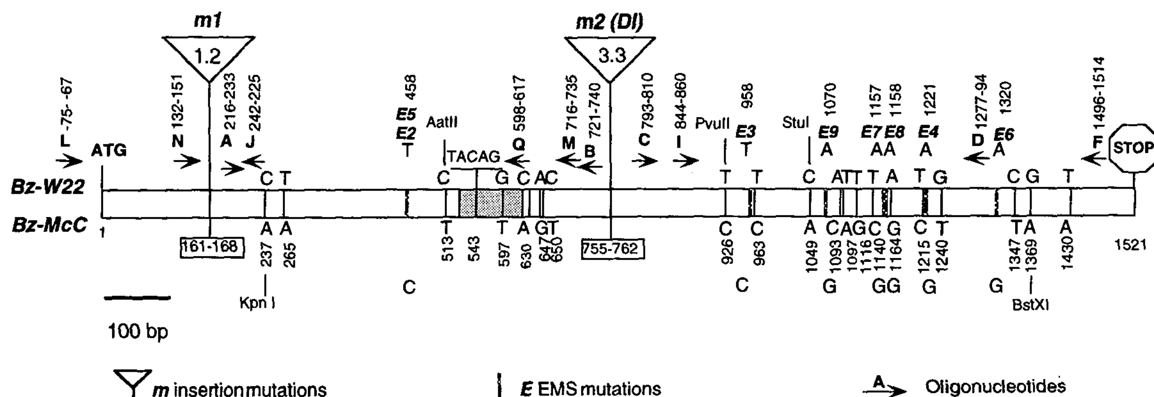


Figure 1. Composite Molecular Map of the *bz* Locus.

The locations of all of the mutations and primers used in this study are shown in a composite map of the two progenitor wild-type alleles, *Bz-W22* and *Bz-McC*. Numbers refer to the published *Bz-W22* genomic DNA sequence, with 1 corresponding to the translation start site (Ralston et al., 1988). The designations *E2* to *E9* refer to the mutations *bz-E2* to *bz-E9* induced by EMS in the *Bz-W22* allele. The nucleotide difference between a *bz-E* mutation and the contrasting wild-type site is shown under each mutation. The *Ds* insertion mutations *bz-m1* and *bz-m2(DI)* arose in the *Bz-McC* progenitor allele and are represented by triangles. The number inside each triangle refers to the size of the insertion in kilobases. The location of the 8-bp direct repeat generated by the insertion is indicated in the box beneath each insertion. The nucleotide differences between *Bz-W22* and *Bz-McC* and some of the major 6-bp restriction site polymorphisms that result from those differences are shown, respectively, above and below the lines representing the two alleles. The single intron (105 bp in *Bz-W22* and 100 bp in *Bz-McC*) is shown by stippling. Oligonucleotides used as primers in polymerase chain reaction (PCR) and sequencing are identified by boldface letters above arrows that indicate their 5′ to 3′ strand polarity.

(Figure 1), and the *Ds-bz* junctions were sequenced. We found that the *Ds* element in *bz-m1* is located at positions 161 to 168 in *Bz-McC*.

Eight different *bz* mutations induced in the *Bz-W22* allele by treatment with ethylmethane sulfonate (EMS) (Dooner, 1986) were used in this study. To place these mutations in the gene, we located them first relative to the two insertions *bz-m1* and *bz-m2(DI)* on the basis of the flanking marker constitution of *Bz* IGRs (Dooner, 1986; this study). This genetic exercise allowed us to place *bz-E2* and *bz-E5* between *bz-m1* and *bz-m2(DI)* and *bz-E3*, *bz-E4*, *bz-E6*, *bz-E7*, *bz-E8*, and *bz-E9* distal to (3′ of) *bz-m2(DI)*. To determine the exact location and nature of these EMS-induced mutations, we amplified the DNA on either the left or the right of *bz-m2(DI)* with primer combinations L and M (or A and B) and C and F (or C and D), respectively, and sequenced them (Figure 1). Table 1 summarizes these results. All of the mutations are single base pair changes, being either C→T or G→A transitions in the sense strand. Surprisingly, *bz-E2* and *bz-E5* have the same nucleotide change, a C→T transition at position 458. These mutations are known to map genetically very close to each other (Dooner, 1986). Nevertheless, to confirm that there had not been a stock mix-up since generation of the mutations, we extracted DNA from the self-progenies of the original mutants, resequenced both alleles, and verified that the same base was altered in both.

The mutants *bz-E2* and *bz-E5* synthesize a protein that cross-reacts with antibodies raised against the normal UDP-glucose 3-O-glucosyltransferase enzyme encoded by the *Bz-W22* allele (Dooner, 1986). Accordingly, they represent missense mutations that change an alanine residue to a valine. The mutants *bz-E3*, *bz-E4*, *bz-E6*, *bz-E7*, *bz-E8*, and *bz-E9* lack an immunologically cross-reactive protein. In agreement with their serological characterization, all of them represent nonsense mutations. Either the truncated polypeptides made in these mutants are broken down or the transcripts are degraded by a mechanism similar to the nonsense-mediated RNA decay that has been observed in yeast (Leeds et al., 1991), animals (Pulak and Anderson, 1993), and possibly plants (Van Hoof and Green, 1996).

Table 1. Characterization of EMS-Induced *bz* Mutations

Mutation	Anti-UFGT Cross-Reactivity[a]	Location[b]	Codon Change	Amino Acid Change
bz-E2	+	458	GCC→GTC	Ala→Val
bz-E3	−	958	CAA→TAA	Gln→ochre
bz-E4	−	1221	TGG→TGA	Trp→opal
bz-E5	+	458	GCC→GTC	Ala→Val
bz-E6	−	1320	TGG→TGA	Trp→opal
bz-E7	−	1157	TGG→TAG	Trp→amber
bz-E8	−	1158	TGG→TGA	Trp→opal
bz-E9	−	1070	TGG→TAG	Trp→amber

[a] Cross-reactivity with antibodies raised against the *Bz-W22* UDP-glucose 3-O-glucosyltransferase (UFGT; from Dooner, 1986): (+), presence of cross-reactive protein; (−), absence of cross-reactive protein.

[b] Relative to the sequence of *Bz-W22*, the progenitor allele (Ralston et al., 1988).

Intragenic Recombination between Mutations Derived from the Same Progenitor Allele

The *bz* gene has been shown to be a recombinational hotspot in the maize genome (Dooner et al., 1985; Dooner, 1986). To determine whether within the *bz* gene there are recombinational hotspots, as has been suggested for other genes (Eggleston et al., 1995; Patterson et al., 1995; Xu et al., 1995), or whether recombination across the gene is linear, we measured the frequency of recombination between point mutations of the same progenitor allele, that is, in the absence of any other potentially confounding heterologies. We have shown previously that the mutations used in this study are extremely stable and that all gametes carrying functional *Bz* alleles can be attributed to recombination between heteroallelic mutations. Thus, the frequency of *Bz* progeny provides a direct estimate of the frequency of recombination between mutations.

We analyzed intragenic recombination between several pairs of *bz-E* mutations that define different physical intervals in the *bz* gene. If recombination were not distributed uniformly across the gene, one would expect the ratio of genetic to physical distance (centimorgans to kilobase) to vary across the gene. Table 2 summarizes the results of this analysis, includes both new and published genetic data (Dooner, 1986), and reviews the genetic information in light of the precise placement of the mutations reported in this study.

We have already shown that recombination between the mutations *bz-E2* and *bz-E4* was very high (0.18 cM), at least 100 times higher than would be expected on average based on the physical size of the *bz* gene (Dooner, 1986). We can now assign a precise value to the relationship between physical and genetic distance for the interval defined by the *bz-E2* and *bz-E4* mutations. The cM/kb value for that interval is 0.23, which is ~500 times higher than the average value for the entire maize genome and approaches values observed in yeast (Lichten and Goldman, 1995). When one examines the cM/kb value for intervals defined by other pairs of mutants (*bz-E4*/*bz-E3*, *bz-E3*/*bz-E2*, *bz-E9*/*bz-E2*, and *bz-E9*/*bz-E5*), it is clear that recombination per kilobase is high and remarkably uniform in the different intervals. Figure 2 provides a graphic representation of the uniformity of recombination within *bz*. In particular, it should be noted that the genetic distances between the pairs of mutants *bz-E2*/*bz-E3* and *bz-E3*/*bz-E4* are roughly additive when compared with the genetic distance between the outermost pair *bz-E2*/*bz-E4*, and all are proportional to their physical distances. Thus, there is no indication from these data, which cover intragenic intervals stretching over half of the coding region, that there is a recombinational hotspot within the *Bz* gene.

Table 2. *Bz* IGRs from Various *bz-E*/*bz-E* and *bz-m*/*bz-m* Heteroallelic Combinations

Genotype	Population	Flanking Marker Classes				Frequency ($\times10^{-4}$)	cM	kb	cM/kb
		P_1[a]	P_2[b]	R_1[c]	R_2[d]				
sh bz-E4 Wx / *Sh bz-E3 wx*	33,075	1	2	0	6	2.7	0.054	0.263	0.20
Sh bz-E3 wx[e] / *sh bz-E2 Wx*	63,270	2	3	1	23	4.6	0.092	0.500	0.19
Sh bz-E4 wx[e] / *sh bz-E2 Wx*	56,560	3	12	1	36	9.1	0.18	0.763	0.23
sh bz-E9 Wx / *Sh bz-E2 wx*	22,700	3	3	1	8	6.6	0.14	0.612	0.23
sh bz-E9 Wx / *Sh bz-E5 wx*	37,400	4	3	1	14	5.9	0.12	0.612	0.20
sh bz-m1 Wx[f] / *Sh bz-m2(DI) wx*	210,914	23	7	5	2	1.8	0.035	0.601	0.059
sh bz-m1 Wx[f] / *Sh bz-m2(DII) wx*	224,020	16	3	10	2	1.4	0.028	0.601	0.047

[a] P_1, parental class 1, both flanking markers from the top strand in the heterozygote.
[b] P_2, parental class 2, both flanking markers from the bottom strand in the heterozygote.
[c] R_1, crossover class 1, left marker from the top strand and right marker from the bottom strand in the heterozygote.
[d] R_2, crossover class 2, left marker from the bottom strand and right marker from the top strand in the heterozygote.
[e] Data from Dooner (1986).
[f] Data from Dooner and Kermicle (1986).

The last two entries in Table 2 document further the suppressive effect of insertions on intragenic recombination. This effect had been established earlier on the basis of mainly genetic data (Dooner, 1986). The mutations *bz-m1*, *bz-m2(DI)*, and *bz-m2(DII)* carry different *Ds* insertions in the *Bz-McC* allele. Thus, in the *bz-m1/bz-m2(DI)* and *bz-m1/bz-m2(DII)* heterozygotes, we are measuring the frequency of intragenic recombination between insertion mutations of the same progenitor allele. In these heterozygotes, the effect of the insertions is not confounded by the possible effects of other heterologies. As can be seen in Table 2, recombination per kilobase is reduced approximately fourfold by heteroallelic insertions located 600 bp apart in otherwise totally homologous alleles. In the *rosy* locus of Drosophila, large insertions located 2.7 kb apart have also been reported to reduce recombination four- to fivefold relative to the baseline recombination detected between point mutations (Clark et al., 1988).

Intragenic Recombination between Mutations Derived from Different Progenitor Alleles

Recombination Frequencies

The suppressing effect of insertions on recombination can also be discerned from examination of the IGR data obtained from *bz-m/bz-E* heterozygotes. Dooner (1986) reported that the relative size of the *bz* genetic map, measured from two series of heteroallelic combinations between *bz-E* mutations, and a *bz-m* insertion appeared larger if the insertion was at one end of the gene (*bz-m1*) rather than in the middle (*bz-m2(DI)*). We can take the estimates of genetic distance from that paper and from the new data presented in Table 3, together with the physical location data from Figure 1, to derive cM/kb ratios for the various intervals defined by the two allelic series. The results are summarized graphically in Figure 3.

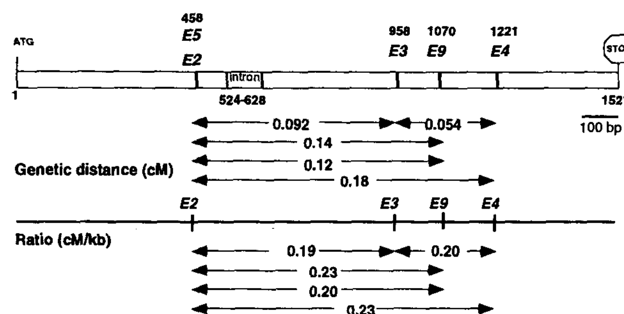


Figure 2. Recombination between Mutations Derived from the Same Progenitor Allele.

The map shows the location of *bz-E* mutations analyzed in heteroallelic pairs. The relative constancy of the cM/kb ratio in all of the intervals studied illustrates the uniformity of recombination within *bz*. Double-headed arrows indicate the extent of each interval. Numbers above the *bz-E* mutations refer to the location of the mutations in the gene.

Table 3. *Bz* IGRs from Various *bz-m1/bz-E* Heteroallelic Combinations

Genotype	Population	Flanking Marker Classes				Frequency ($\times10^{-4}$)	cM	kb	cM/kb
		P_1[a]	P_2[b]	R_1[c]	R_2[d]				
sh bz-m1 wx / *Sh bz-E7 Wx*	18,890	0	0	8	0	4	0.08	0.997	0.08
sh bz-m1 wx / *Sh bz-E8 Wx*	18,830	0	0	7	0	4	0.08	0.998	0.08
sh bz-m1 wx / *Sh bz-E9 Wx*	19,970	1	0	10	0	5.5	0.11	0.910	0.12

[a] P_1, parental class 1, both flanking markers from the top strand in the heterozygote.
[b] P_2, parental class 2, both flanking markers from the bottom strand in the heterozygote.
[c] R_1, crossover class 1, left marker from the top strand and right marker from the bottom strand in the heterozygote.
[d] R_2, crossover class 2, left marker from the bottom strand and right marker from the top strand in the heterozygote.

Two conclusions are evident from an examination of the ratios. (1) Intragenic recombination is lowest in regions immediately adjacent to the insertions. The lowest recombination per kilobase was observed in the 304-bp *E2/E5-m2(DI)* interval, which in addition contains six heterologies, including a 5-bp simple sequence repeat polymorphism in the intron. Recombination in this interval is >10-fold lower than in completely homologous intervals flanked by *bz-E* point mutations (Table 2 and Figure 2). (2) For intervals >500 bp, in which the suppressing effect of the insertions may not be as strong, recombination per kilobase seems to be relatively constant, in agreement with the results obtained with heteroallelic pairs of *bz-E* mutations (Figure 2). Nevertheless, recombination is still reduced two- to threefold relative to *bz-E* heteroallelic pairs that differ only at the two mutant sites.

The *bz-m* and *bz-E* allelic pairs differ not only by the presence versus absence of a large insertion but also by multiple single base pair heterologies. *Bz-McC* and *Bz-W22*, the progenitor alleles of the *bz-m* and *bz-E* mutations, are polymorphic at 21 positions across the 1521 bp between the start and stop codons. Of these, 20 are single base pair differences, and the remaining one is a 5-bp simple sequence repeat polymorphism within the intron. In yeast, mismatches of one to a few bases may (Borts and Haber, 1987) or may not (Symington and Petes, 1988; Malone et al., 1994) affect meiotic recombination. Because of the suppressing effect of insertions on recombination, it is not possible from the present data to determine whether single base pair heterologies affect the frequency of intragenic recombination at the *bz* locus. However, by examining the distribution of recombination junctions among the *Bz* IGRs, one can determine whether there are apparent recombination hotspots and coldspots in *bz-m/bz-E* heterozygotes.

Analysis of Recombination Junctions

To locate crossover points relative to single base pair heterologies, we amplified and sequenced the recombination junctions and adjacent polymorphisms in 86 *Bz* IGRs from the *bz-m1/bz-E* heteroallelic series and 45 *Bz* IGRs from the *bz-m2(DI)/bz-E* heteroallelic series. The data are presented in Figures 4 and 5. For each of the 131 *Bz* IGRs, we succeeded in localizing the recombination junction to a specific interval demarcated by two adjacent heterologies in the parental chromosomes.

A cursory examination of the distribution of recombination junctions among the *Bz* IGRs reveals the following. (1)

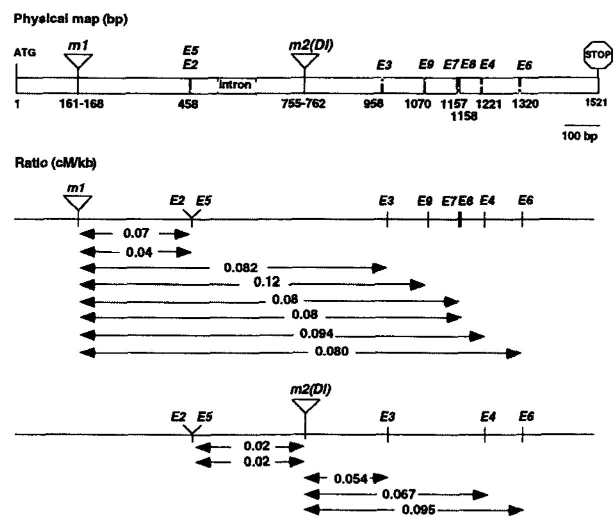
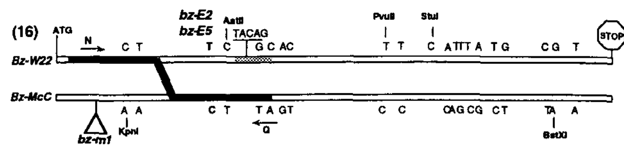


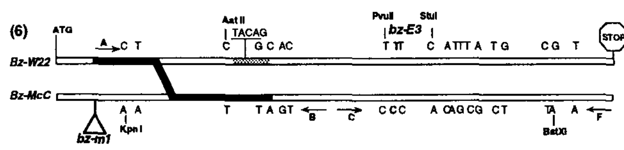
Figure 3. Recombination between Mutations Derived from Different Progenitor Alleles.

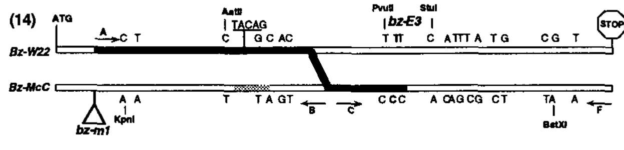
The map shows the location of *bz-E* point mutations and *bz-m* insertion mutations analyzed in heteroallelic pairs. *bz-E* mutations are derived from *Bz-W22*; *bz-m* mutations are from *Bz-McC*. The extremely low cM/kb ratios immediately adjacent to the two insertions document further the strong suppressing effect of the insertions on intragenic recombination. Symbols are as given in the legends to Figures 1 and 2.

I. *Bz* IGRs ex *bz-m1/bz-E2* and *bz-m1/bz-E5*

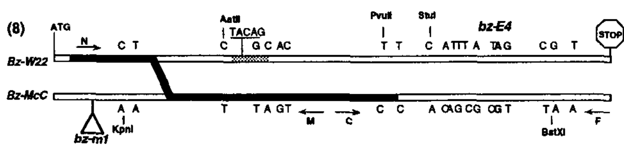


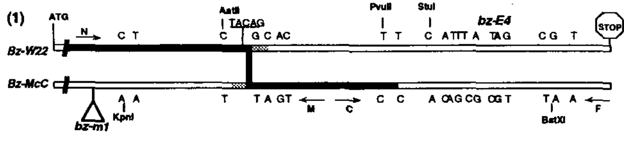
II. *Bz* IGRs ex *bz-m1/bz-E3*

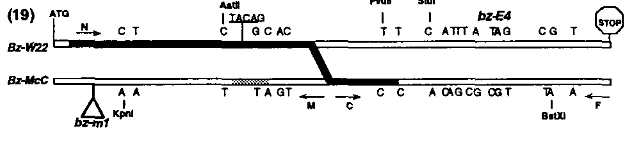


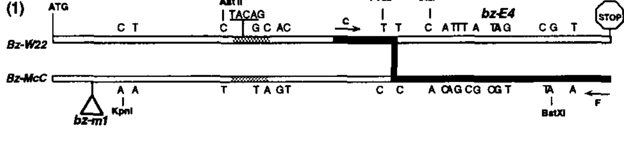


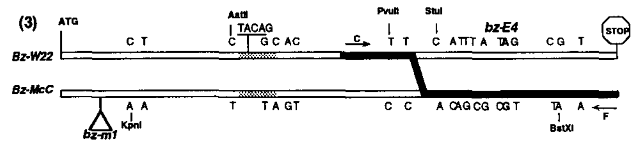
III. *Bz* IGRs ex *bz-m1/bz-E4*



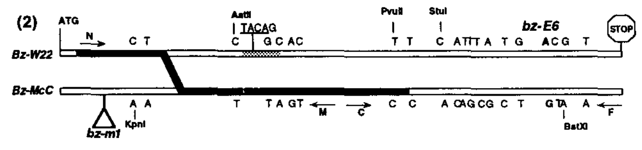


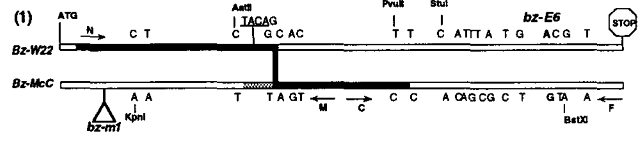


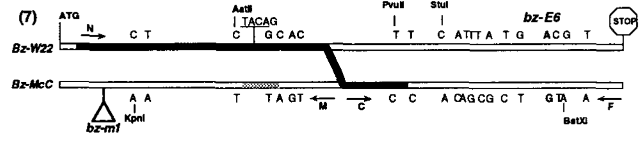


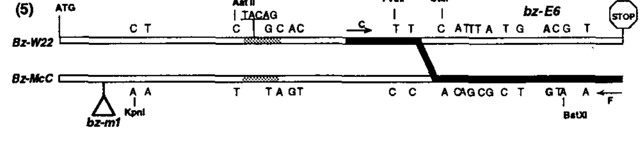


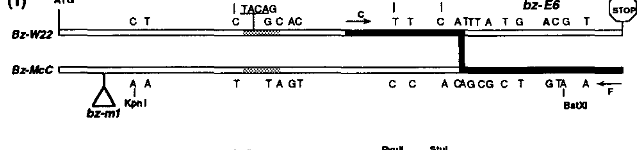
IV. *Bz* IGRs ex *bz-m1/bz-E6*

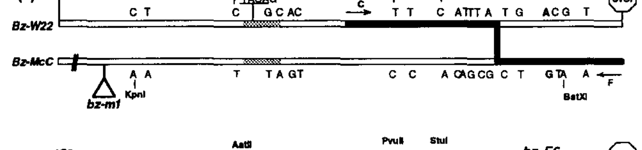












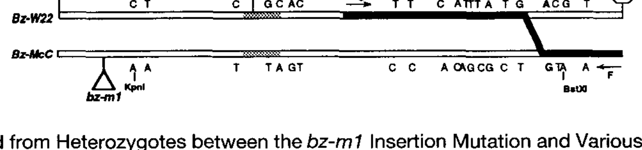


Figure 4. Location of the Recombination Junctions in *Bz* IGRs Obtained from Heterozygotes between the *bz-m1* Insertion Mutation and Various *bz-E* Point Mutations.

The two progenitor alleles are represented by open lines, and the new recombinant *Bz* alleles are represented by solid lines. The lengths of the solid lines indicate the extent of the DNA sequence readout. The stippled rectangles represent the introns. The polymorphic sites between the two parental alleles, which serve as markers in this experiment, are shown above and below the lines. Oligonucleotides used as primers in PCR and sequencing are identified by letters above arrows that indicate their 5′ to 3′ strand polarity. The single base pair change in each *bz-E* mutation is in boldface. The insertion mutation is represented as a triangle below the *Bz-McC* strand. The number of *Bz* IGRs that fall into each interval is shown in parentheses.

Exchanges can occur almost anywhere in the gene, including the intron. One recombination junction was located to a 19-bp interval and six to intervals shorter than 30 bp. Thus, a long stretch of homology of several hundred base pairs is not a prerequisite in maize for the resolution of recombination intermediates. (2) No recombination junctions occur in the 104-bp segment immediately adjacent to the *bz-m1* insertion. If exchanges were distributed purely on the basis of the physical length of the interval, 13 would have been expected among the *Bz* IGRs from all five *bz-m1/bz-E* heterozygotes. Thus, this interval would appear as a distinct coldspot in recombination experiments involving only *bz-m1* and a *bz-E* point mutation. (3) The largest number of crossovers resolve in the two longest regions of uninterrupted homology—the 248-bp segment between nucleotides 265 and 513 and the 276-bp segment between nucleotides 650 and 926. Conversely, few crossovers resolve in the intron or the 3′-most region of the gene, both of which contain multiple heterologies within a short DNA segment.

The last two observations suggest that recombination junctions may not be distributed randomly across the gene. To test this, we performed a statistical analysis of the pooled data sets from *bz-m1/bz-E4* and *bz-m1/bz-E6,* two heterozygotes for mutations located at opposite ends of the gene. Figure 6 shows the number of recombination junctions that fall between adjacent heterologies across the length of DNA separating *bz-m1* and *bz-E4* versus the number expected based on a random distribution. Because the gene is fragmented by polymorphisms into too many small intervals and we were primarily interested in testing the possible effect of heterologies on recombination, we grouped adjacent small intervals into six larger intervals with different densities of heterologies, ranging from none in 276 bp to six in 172 bp (Figure 6). The χ^2 value for the comparison between the observed and expected distributions is very highly significant (χ^2 = 28.5; 5 df; $P < 0.001$), indicating that the distribution of recombination junctions is not uniform across the gene. Relatively few crossovers occur in regions with multiple heterologies, and none occur immediately adjacent to the *bz-m1* insertion—a not surprising result, given the suppressing effect of insertions on recombination. This effect most likely extends for several hundred base pairs, because the next interval (zero heterologies in 248 bp) showed no increase in recombination junctions.

It appears, therefore, that the density of single base pair heterologies may affect where meiotic exchanges occur in the *bz* gene to produce *Bz* alleles. We are currently investigating whether they have any effect on the frequency of recombination, which is an important consideration, given the extensive sequence polymorphisms found in maize.

Gene Conversion at the *bz* Locus

Gene conversion is detected in yeast as a deviation from the expected 1:1 Mendelian segregation of alleles at a locus (e.g., 3:1 or 5:3). Although conversion events cannot be formally identified in plants because only one meiotic product is usually recovered, flanking markers can be used as good indicators of the recombinational mode of origin of *Bz* IGRs. Chiasma interference in the *sh-wx* region is very high (Dooner, 1986), so parentally marked *Bz* IGRs most likely have arisen by conversion rather than by double crossing over.

We found previously that the distribution of flanking markers among *Bz* IGRs varied, depending on the nature of the mutations in the heterozygote (Dooner, 1986; Dooner and Kermicle, 1986). Heteroallelic combinations of a *Ds* insertion and a point mutation produced almost exclusively IGRs carrying recombinant flanking markers, whereas heteroallelic combinations of two *Ds* insertions produced mainly IGRs with a parental arrangement of flanking markers. Heteroallelic combinations of point mutations yielded a majority of IGRs of one recombinant class but also some parental types. This general pattern is seen once again in this study (Tables 2 and 3).

One of our objectives was to try to determine the length of a conversion tract at *bz*, that is, the length of the fragment from one homolog that is transferred to the other during meiotic recombination. Because tetrad analysis is not readily possible in maize, an indirect way of establishing conversion tract length is to determine the distance between recombination junctions in parentally marked IGRs. A requirement for this type of analysis is that the parent chromosomes be polymorphic at multiple sites, but unfortunately, our polymorphic heteroallelic combinations are precisely those (*bz-m/bz-E*) that yield the lowest number of parentally marked IGRs.

In a small-scale attempt to determine conversion tract lengths, we analyzed five parentally marked *Bz* IGRs from *bz-m1/bz-E* heterozygotes (Dooner, 1986). Three of them carry the outside markers of *bz-m1* and thus formally represent conversions of *bz-m1*. One recombination junction should fall between *bz-m1* and the respective *bz-E* mutation, and the second one should fall between *bz-m1* and the proximal marker *wx*. Indeed, all three had one recombination junction within *bz* and are included among the IGRs shown in Figure 4. To attempt to localize the second junction, we scored the nearest known marker proximal to *bz*, *tac2094*, which is located between 30 and 100 kb upstream (Ralston et al., 1989; Z. Zheng and H.K. Dooner, unpublished data). By using DNA gel blot analysis, we determined that one of the three had the *tac2094* marker of the *bz-m1* parent, suggesting a conversion tract of <100 kb; however, the other two carried the *tac2094* marker of the *bz-E* parent, suggesting that either the *tac2094* site was coconverted with *m1* and the conversion tract was >100 kb or that they represent rare double crossovers (data not shown). Currently, we are analyzing these three events further. The remaining two parentally marked *Bz* IGRs from *bz-m1/bz-E* heterozygotes carried the outside markers of the *bz-E* mutation. In one of them (*Bz-9625*), both recombination junctions, and hence the entire conversion tract, occurred inside of *bz*. Its constitution is shown in Figure 7. Based on the position of the recombination junctions relative to the parental

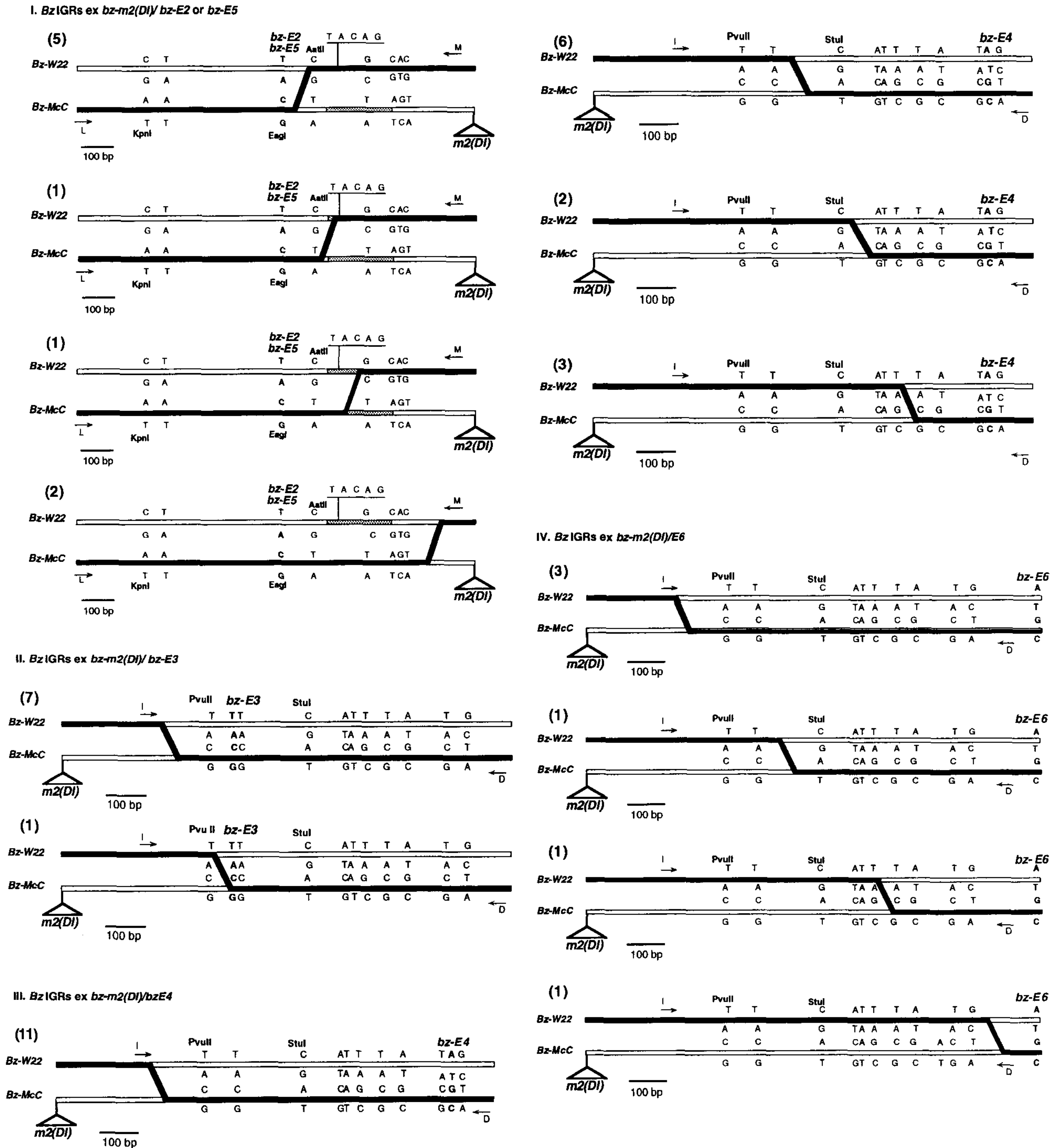


Figure 5. Location of the Recombination Junctions in *Bz* IGRs Obtained from Heterozygotes between the *bz-m2(DI)* Insertion Mutation and Various *bz-E* Point Mutations.

Symbols are as given in the legend to Figure 4.

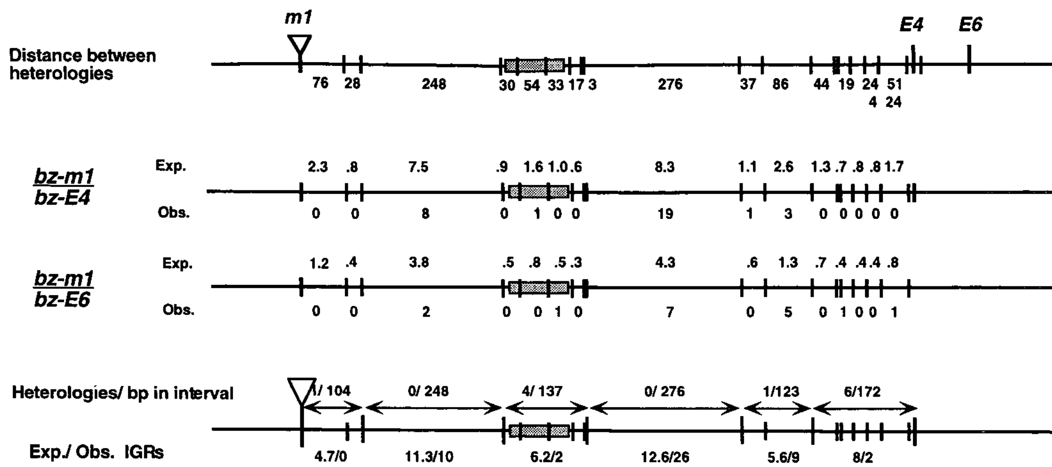


Figure 6. Comparison of the Observed versus Expected Distribution of Recombination Junctions among *Bz* IGRs from *bz-m1/bz-E4* and *bz-m1/bz-E6* Heterozygotes.

The *bz* gene has been divided into six adjacent intervals with different densities of heterologies. The intervals are delimited by heavy vertical bars. The number of heterologies in each interval and the size of the interval in base pairs are given above a double-headed arrow representing the length of the interval. The number of junctions expected in each interval has been calculated on the assumption that it should be proportional to the size of the interval. The χ^2 test of significance shows that the distribution is highly nonrandom. The triangles at the left of the map represent the *bz-m1* insertion mutation, and the stippled rectangles represent the intron. Exp., expected; Obs., observed.

heterologies, the conversion tract is taken to be between 965 and 1165 bp long. The second convertant had a conversion tract of 1.1 to 1.5 kb: it arose by a complex recombination event that generated a duplication of the *bz* locus and is the subject of a separate report (H.K. Dooner and Z. Zheng, unpublished data). The values that we have observed for the two conversion tracts in *bz* are similar to the average minimum conversion tract lengths seen in yeast (0.4 to 1.6 kb; Petes et al., 1991) and in Drosophila (0.4 to 0.9 kb; Curtis and Bender, 1991; Hilliker et al., 1994).

All of the polymorphic sites in both conversion tracts were contributed by one parent only, that is, the tracts are continuous, as has been found in yeast and Drosophila, and not patchy. Furthermore, several conversion tracts should be included among the *Bz* IGRs of Figures 4 and 5 if we assume that, as in yeast, many of them represent conversion events that resolve as crossovers. In the 130 *Bz* IGRs sequenced, all of the polymorphic sites on one side of the recombination junction were contributed by one parent, and all of those on the other side of the junction were contributed by the other parent, again suggesting continuity of conversion tracts.

DISCUSSION

Linearity of Recombination within *bz*

The *bz* gene, like other maize genes, is much more recombinogenic than would be expected from its physical size. Thus, *bz* constitutes a recombination hotspot within the maize genome (Dooner et al., 1985). In this study, our goal was to determine whether recombination was likewise nonuniform within the *bz* gene itself.

In yeast, recombination (gene conversion) hotspots have been identified at the 5′ or 3′ end of genes (reviewed in Lichten and Goldman, 1995). A recent analysis of IGR events in maize has also uncovered apparent recombination hotspots at or close to either the 5′ or 3′ ends of several genes (Eggleston et al., 1995; Patterson et al., 1995; Xu et al., 1995). The yeast recombination hotspots, that is, the high ends of conversion gradients, correspond to sites at which double-strand breaks (DSBs) initiate meiotic recombination. Therefore, their occurrence in plant genes has potential implications for the mechanism of meiotic recombination in plants.

Recombination hotspots in maize have been detected in experiments that analyzed IGR between pairs of heteroalleles

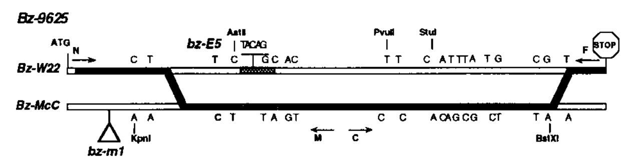


Figure 7. Constitution of the Conversion Tract in the Parentally Marked IGR *Bz-9625*.

Symbols are as given in the legend to Figure 4.

derived from different progenitors and differing from each other by at least one major insertion. Because there is a high level of sequence polymorphism in maize, these alleles differ at multiple sites along the entire gene. The density of single base pair heterologies in maize is often greater than one in 100 (e.g., Sachs et al., 1986; Furtek et al., 1988; Ralston et al., 1988). In yeast, heterologies at one-tenth that density have been reported to significantly affect meiotic recombination (Borts and Haber, 1987). Similarly, insertions have been shown to affect intragenic recombination in maize (Dooner, 1986; Xu et al., 1995), so conceivably the presence of insertions and multiple heterologies of one to a few base pairs could play a role in determining where recombination events occur, creating apparent hotspots.

To exclude potentially confounding effects from heterologies, we examined intragenic recombination between several pairs of *bz-E* point mutations induced by EMS in the same progenitor allele. We verified by sequencing that these mutations differed from the progenitor allele at only one site (Table 1). We found that in this series of *bz-E* heteroallelic pairs, recombination was proportional to the physical distance separating the mutations, regardless of the placement of the mutations within the gene (Table 2 and Figure 3). The linear relationship observed when intragenic recombination is examined in the absence of heterologies (other than at the two mutant sites) argues against a recombination polarity or a true recombination hotspot within *bz*.

Effect of Heterologies on Intragenic Recombination at *bz*

From our earlier observation that the presence of an insertion mutation in the middle of the gene results in a contraction of the *bz* genetic map, we concluded that insertions suppress intragenic recombination within *bz* (Dooner, 1986). In this study, we provide substantial evidence supporting that conclusion. (1) In heterozygotes between pairs of insertion mutations derived from the same progenitor allele and located 600 bp apart, recombination per unit of physical distance (expressed as centimorgans per kilobase) is reduced fourfold relative to point mutations (Table 2). (2) In heterozygotes between an insertion mutation and a series of point mutations located at various distances from the insertion, the cM/kb ratio is lowest in the interval defined by the insertion and the closest point mutation, suggesting that intragenic recombination is lowest immediately adjacent to the insertion (Figure 3). (3) When recombination junctions are examined in such heterozygotes, fewer junctions than expected occur in the interval immediately adjacent to the insertion, based on that interval's physical length (Figures 4 to 6).

The distribution of recombination junctions across the gene can only be analyzed in heterozygotes for mutations differing at multiple silent sites. Because of the high degree of polymorphism found in maize, mutations arising in different progenitor alleles will generally differ at multiple sites. In this study, the two *Bz* progenitor alleles differed at 21 positions across the 1521-bp coding region. This high density of heterologies prompted us to question whether the distribution of exchanges could be affected by the distribution of heterologies across the gene. In discussing yeast recombination hotspots, Borts and Haber (1989) have also raised the possibility that the clustering of crossovers could be the result of the placement of the heterozygosities used in the study.

Heterologies of one to a few base pairs occurring at a density as low as one per kilobase have been reported to reduce meiotic recombination in yeast (Borts and Haber, 1987). Similarly, in bacteria (Watt et al., 1985) and mammalian cells (Waldman and Liskay, 1988), single nucleotide heterologies in short DNA fragments (37 to 230 bp) are sufficient to dramatically reduce the frequency of recombination. In maize, a meiotic recombination event selected simply because it gave rise to a new polymorphic restriction fragment turned out to have occurred within a completely homologous 534-bp region that was flanked on either side by ~900 bp of less perfect homology (Timmermans et al., 1996). Given these precedents, we investigated whether the density of single base pair heterologies affected where meiotic exchanges occurred in the *bz* gene. We found that the distribution of recombination junctions was not uniform across the gene and that relatively few crossovers occurred in regions with a higher density of heterologies. An examination of the *a1* data (Xu et al., 1995, Figure 6) reveals that the same may be true at *a1*. Unfortunately, in all of the maize intragenic recombination studies published to date, at least one of the alleles used has been an insertion mutation, so the influence of heterologies is potentially confounded with that of the insertions. We are currently analyzing the effect of heterologies of one to a few base pairs on intragenic recombination at *bz* in the absence of insertion heterozygosity.

Fidelity of Meiotic Intragenic Recombination in Maize

One of the objectives of our analysis of recombination junction sequences was to examine the fidelity of the meiotic recombination process in maize. It has been reported that repair synthesis during mitotic recombination in yeast is mutagenic (Strathern et al., 1995). To investigate whether this might also be the case for meiotic recombination in maize, we carefully read >65 kb of DNA sequence on either side of the new recombination junctions (Figures 4 and 5) but found no nucleotide changes. Because IGRs carrying *Bz* alleles were selected, a requirement for gene function was imposed in this experiment. Nevertheless, the coding sequences of different *Bz* isoalleles are known to vary by as much as 1 to 2% (Furtek et al., 1988; Ralston et al., 1988), and the removal of transposons from exons can give rise to functional *Bz* alleles with alterations corresponding to additions or deletions of 1 to 11 amino acids (Kim et al., 1987; Schiefelbein et al., 1988; H.K. Dooner and I.M. Martínez-Férez, unpublished data), so the *Bz* gene is highly tolerant to change. Yet,

there is no indication from the present data that meiotic recombination can introduce nucleotide changes in maize.

Xu et al. (1995) and Timmermans et al. (1996) have analyzed 20 other maize recombination junctions and also failed to uncover sequence alterations. Thus, DNA appears to be repaired faithfully during maize meiotic recombination. We are now examining this issue further by analyzing the nucleotide sequences of mutant *bz* exceptions produced by intragenic recombination between unique *Bz* alleles, that is, in the absence of a requirement for gene function.

Comparison of Intragenic Recombination at the *bz* Locus and in Yeast

Gradients in the frequency of meiotic recombination in yeast presumably reflect sites, usually at the 5′ end of genes, where recombination is initiated via a DSB and from where conversion tracts spread in a distance-dependent way (although a contrasting view is that conversion gradients may not primarily reflect the extension of heteroduplex intermediates but rather the direction of mismatch repair; Nicolas and Petes, 1994). The occurrence of conversion gradients led yeast geneticists to postulate a fixed initiation site for recombination at the high end of the gradient, which was later shown to correspond to the position of DSBs. Figure 8 diagrams a generally accepted version of the DSB model for initiation of recombination in yeast (Sun et al., 1991). The recombination intermediate in this model contains a double Holliday junction (d), which can be resolved in either a parental (e) or crossover (f) mode, depending on which strands are cut. As can be seen in Figure 8 (h), after repair of the heteroduplex DNA in the crossover products, the recombination junction can correspond to either the site of initiation of recombination (top) or the site of resolution of the Holliday junction (bottom).

We did not find a gradient in recombination frequency at *bz*; instead, the rates of recombination in *bz* are similar at different positions within the gene. Peculiarly, this lack of polarity also appears to be a feature of mitotic gene conversion in yeast (reviewed in Petes et al., 1991). The simplest interpretation of this lack of polarity is that there are no preferred sites for the initiation of recombination. We propose that sites of initiation of recombination in maize generally correspond to sites where the chromatin is less densely packed as a consequence of DNA hypomethylation, thus providing greater accessibility to the recombination machinery. There is considerable evidence that the hypomethylated fraction of the maize genome is associated with genes (Nick et al., 1986; Antequera and Bird, 1988; Bennetzen et al., 1994). Thus, genes in general would constitute recombination hotspots in maize but would contain no fixed initiation sites for recombination. In this view, the suppressing effect of insertions (and possibly of clustered single base pair heterologies) on recombination at *bz* could be explained by invoking

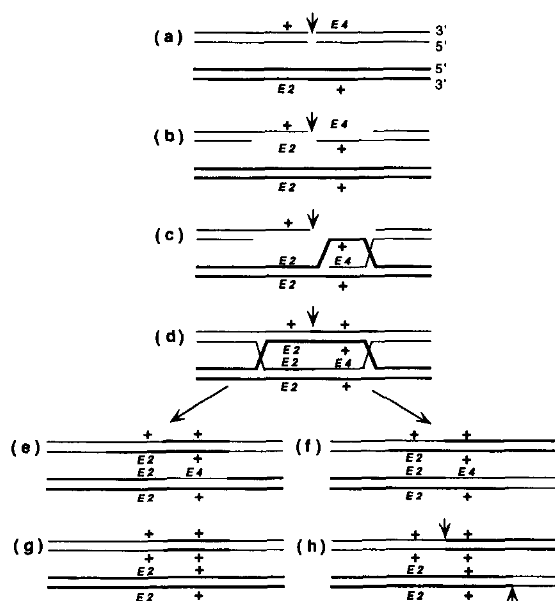


Figure 8. The DBS Model for Initiation of Recombination (Adapted from Sun et al., 1991).

The diagram shows the two interacting duplexes (chromatids) of a *bz-E2*/*bz-E4* heterozygote. The *bz-E2* duplex is represented in bold. In (a), a DSB, indicated with an arrow, is formed in the *bz-E4* duplex. In (b), long 3′ single-strand overhangs are generated by a 5′ to 3′ exonuclease. One single strand invades a homologous duplex in (c), forming a heteroduplex and displacing a D loop. In (d), the D loop is extended by DNA synthesis and annealed to the second 3′ end overhang. DNA synthesis from the second 3′ end leads to the formation of a recombination intermediate with a double Holliday junction. This recombination intermediate can be resolved in either a parental (e) or crossover (f) mode, depending on which strands are cut. In (g), the consequence of DNA repair in noncrossover products is represented. After repair of the heteroduplex DNA in the crossover products (the vast majority of IGRs in this study), the recombination junction can correspond to either the site of initiation of recombination ([h], top) or the site of resolution of the Holliday junction ([h], bottom). The arrows in (h) indicate the positions of the recombination junctions.

that the presence of heterologies, particularly large insertions, interferes with the random initiation of recombination.

Another difference between recombination in maize and in yeast is the requirement for transcription factors. At the yeast *HIS4* locus, where the 5′ end corresponds to the high end of the conversion gradient, the transcription factors *RAP1*, *BAS1*, and *BAS2* are required for wild-type levels of meiotic recombination (White et al., 1993). Similarly, at the *PHO5* gene, repressors and inducers of gene activity alter nucleosome positioning as well as the distribution of DSBs in the upstream region (Wu and Lichten, 1994). In contrast, recombination per kilobase in *bz* is approximately the same whether measured in the presence or absence (Dooner, 1986; Table 2) of *R*, a regulatory gene of anthocyanin biosynthesis

that encodes a basic helix-loop-helix transcription factor (Ludwig et al., 1989). This observation is perhaps not surprising because it is unlikely that the anthocyanin biosynthetic pathway is expressed in the megaspore mother cells undergoing meiosis, and again, it suggests that the basis for the recognition of substrate DNA by the cell's recombinational machinery is different in yeast and maize.

Yet a third difference is that in yeast, meiotic IGRs are borne with approximately equal frequency on parental or crossover chromosomes; however, in maize, the majority of IGRs are borne on crossover chromosomes (Nelson, 1962; Dooner, 1986; Dooner and Kermicle, 1986; Patterson et al., 1995; Xu et al., 1995). Therefore, convertants—identified as parentally marked IGRs in random spore analysis—are infrequent in maize. A great excess of crossover IGRs is seen in various types of heteroallelic combinations, including heterozygotes between *bz-E* point mutations (Dooner, 1986; Tables 2 and 3). Only in some *Ds* insertion heterozygotes are parentally marked IGRs recovered with a significant frequency (Dooner and Kermicle, 1986). It appears that in maize there is a strong preference for a crossover resolution of recombination intermediates. Resolution of the double Holliday junction postulated as the intermediate in current models of recombination (Radding, 1982; Sun et al., 1991) would not be random but instead would tend to involve opposite strands in the two junctions. Possibly, maize has evolved such a recombination system to maximize crossing over and thus ensure the proper distribution of chromosomes at meiosis.

The minimum average length of a meiotic conversion tract in yeast has been estimated to be between 0.4 and 1.6 kb (Petes et al., 1991). As mentioned above, convertants in maize can be identified among parentally marked IGRs. We have measured the conversion tract length in two of these recombinants by determining the distance between the two recombination junctions. We found the tracts to be between 1 and 1.5 kb, that is, similar to the sizes seen in yeast. To enlarge this sample, we have begun to generate convertants from heteroallelic combinations more amenable to the recovery and analysis of conversion tracts than the ones used in this study. As in yeast too, the conversion tracts are continuous, that is, all of the polymorphic sites between the two recombination junctions were contributed by one parent, suggesting that only one strand serves as a template in mismatch repair.

METHODS

Plant Materials

Description of bronze *Alleles*

The *bronze (bz)* alleles used in this study were in the common genetic background of the inbred W22. The aleurone phenotypes conditioned by the various alleles in the presence of all of the complementary factors for anthocyanin pigmentation are given in parentheses.

Bz-W22 (purple) is the normal *Bz* isoallele carried in the W22 inbred. *bz-E2* to *bz-E9* (bronze) are ethylmethane sulfonate (EMS)–induced mutants from *Bz-W22* (Dooner, 1986). *Bz-McC* (purple) is the normal progenitor allele of the *bz-m2(Ac)* mutation. *bz-m2(Ac)* (purple spots on a bronze background) is an allele that arose from the insertion of the 4.6-kb *Activator* (*Ac*) element at positions 755 to 762 in the second exon of *Bz-McC* (McClintock, 1955; Ralston et al., 1988). *bz-m2(DI)* (bronze in the absence of *Ac*; spotted in its presence) is the first derivative from *bz-m2(Ac)*, harboring a 3.3-kb internally deleted *Dissociation* (*Ds*) element at the same position as *Ac* in *bz-m2(Ac)* (McClintock, 1962; Dooner et al., 1986). *bz-m1* (bronze in the absence of *Ac*; spotted in its presence) is an allele that arose from insertion of a 1.2-kb *Ds* element at positions 161 to 168 of *Bz-McC* (McClintock, 1951; Dooner et al., 1985; Martínez-Férez and Dooner, 1997). *bz-R* (bronze) is the *bz* reference allele associated with a 340-bp deletion that extends from within the single intron to the second exon of *bz* and includes the *Ac* insertion site in *bz-m2(Ac)* (Rhoades, 1952; Ralston et al., 1988).

The mutations *sh* (shrunken endosperm) and *wx* (waxy endosperm) were used as markers flanking *bz*. They map, respectively, ~3 centimorgans (cM) distal and 25 cM proximal to *bz* in *9S*. The *sh-wx* region exhibits high chiasma interference (Dooner, 1986), so double crossovers in the region are rare.

Selection and Analysis of Bz *Intragenic Recombinants*

bz heteroallelic combinations were pollinated with a *sh bz-R wx* tester either by controlled crosses or in an isolated detasseling plot. Putative *Bz* intragenic recombinants (IGRs) were selected as single purple seed in ears otherwise containing only bronze seeds. The selections were classified for outside markers and backcrossed to the male parent to verify their heritability and the recovery of the pollen markers.

DNA Extraction and Sequencing

Leaf DNA was isolated by the urea extraction procedure of Greene et al. (1994). Genomic DNA was amplified by polymerase chain reaction (PCR; Saiki, 1990) in a GeneAmp system (model 2400; Perkin-Elmer), using a variety of primers based on the sequence of *Bz-W22* (Ralston et al., 1988). Figure 1 shows the location of all of the primers used in either amplification or sequencing; Figures 4 and 5 show the combinations of primers used in the amplifications of specific recombination junctions; and Table 4 gives the primer sequences. After incubating the genomic DNA at 95°C for 5 min, most *bz* sequences were amplified with 35 cycles of 30-sec denaturation at 95°C, 20 sec of annealing at 55°C, and a 1-min extension at 72°C. The PCR reactions were terminated with a 7-min incubation at 72°C and stored at 4°C. With primers N and F, the conditions for amplification were changed to 30 sec of annealing at 60°C and a 2-min extension at 72°C.

The amplified DNA corresponding to *bz* mutations was purified on a 0.7% agarose gel, cloned into either pGEM-T or pBS (both from Stratagene), and sequenced manually by the dideoxy chain termination method, using universal primers (Sanger et al., 1977; Messing et al., 1981). The DNA of each EMS-induced *bz* mutant was amplified in two separate reactions and sequenced twice in both directions to confirm the single base pair change associated with each mutation. The DNA from *Bz* IGRs was purified with a Wizard PCR purification

Table 4. Primers Used in This Study

Primer	Location in *Bz-W22*[a]	Sequence (5′ to 3′)
A	216 to 233	GAACCTGCGCTTCGTCGA
B	740 to 721	GAGGTTGATGACGTAGTTGA
C	793 to 810	CTCAACACGTTCCCAGGC
D	1294 to 1277	TCATCCGCTGGTCGCCGA
F	1514 to 1496	CGACAGACTATCTCCACGA
I	844 to 860	GAGATCCTGCCCAACTG
J	242 to 225	TCCGGTACCTCGACGAAG
L	−75 to 67	ATCGCATTCGCATCGCATC
M	735 to 716	GATGACGTAGTTGAAGTCGC
N	122 to 141	CACGCTCTCGTTCCTCTCCA

[a] Based on the sequence of *Bz-W22*.

system and sequenced directly by the dideoxy method, using *bz* primers and the Sequenase kit (U.S. Biochemical Corp.).

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