Haplotype structure strongly affects recombination in a maize genetic interval polymorphic for *Helitron* and retrotransposon insertions

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We have asked here how the remarkable variation in maize haplotype structure affects recombination. We compared recombination across a genetic interval of *9S* in 2 highly dissimilar heterozygotes that shared 1 parent. The genetic interval in the common haplotype is ≈100 kb long and contains 6 genes interspersed with gene-fragment-bearing *Helitrons* and retrotransposons that, together, comprise 70% of its length. In one heterozygote, most intergenic insertions are homozygous, although polymorphic, enabling us to determine whether any recombination junctions fall within them. In the other, most intergenic insertions are hemizygous and, thus, incapable of homologous recombination. Our analysis of the frequency and distribution of recombination in the interval revealed that: (*i*) Most junctions were circumscribed to the gene space, where they showed a highly nonuniform distribution. In both heterozygotes, more than half of the junctions fell in the *stc1* gene, making it a clear recombination hotspot in the region. However, the genetic size of *stc1* was 2-fold lower when flanked by a hemizygous 25-kb retrotransposon cluster. (*ii*) No junctions fell in the *hypro1* gene in either heterozygote, making it a genic recombination coldspot. (*iii*) No recombination occurred within the gene fragments borne on *Helitrons* nor within retrotransposons, so neither insertion class contributes to the interval's genetic length. (*iv*) Unexpectedly, several junctions fell in an intergenic region not shared by all 3 haplotypes. (*v*) In general, the ability of a sequence to recombine correlated inversely with its methylation status. Our results show that haplotypic structural variability strongly affects the frequency and distribution of recombination events in maize.

Ac | *bz* locus | genome | hotspots | methylation

There is ample evidence that most recombination in maize takes place in or around genes (1–3) and that little, if any, recombination takes place in the repetitive and methylated retrotransposon DNA that makes up the bulk of the genome (4, 5). The evidence that retrotransposons are largely recombinationally inert is 2-fold: (*i*) when homozygous, the standard situation in inbreds, they do not contribute significantly to genetic length (4), and (*ii*) when hemizygous, a common situation in hybrids, no recombination junctions fall in intervals containing them (5).

The unprecedented haplotype diversity recently discovered in maize (6–8) raises several questions regarding the effect of local structural polymorphisms on recombination. In a recent pairwise comparison of 8 *bz1* haplotypes, each one consisting of 8 genes spread over a stretch of DNA that averaged ≈90 kb, the percentage of shared sequences ranged from 25% to 84% (9). The lines differed by the existence of many polymorphic insertions in introns and intergenic regions, the main ones being LTR retrotransposons, often arranged in nests (10), miniature inverted repeat transposable elements (MITEs) (11), and *Helitron* transposons carrying fragments of several genes (12, 13). In addition, high SNP and indel heterozygosity would occur in most intergenic regions of hybrids made from those lines.

This level of structural polymorphisms could affect recombination in multiple ways and, thus, contribute to the 2- to 3-fold variation in estimates of map distances for single genetic intervals that has been reported in different maize mapping populations (14–16). For example, the highly methylated retrotransposon clusters are probably heterochromatic, so retrotransposon hemizygosity could reduce recombination in adjacent genes; the level of small insertion and SNP affects overall sequence homology and, thus, could determine whether recombination occurs in intergenic regions lacking retrotransposons; and *Helitrons* could lead to the expansion of an interval's genetic length if recombination occurred within the gene fragments they carry.

Cis-effects were demonstrated in a study that examined recombination rates across the *a1-sh2* genetic interval in 3 heterozygotes containing the same maize haplotype and different teosinte-derived haplotypes in a common maize background (17). This region had several large insertion/deletion polymorphisms relative to maize, including 2 LTR retrotransposons. The analysis identified up to 3-fold differences in recombination rates and statistical differences in the distribution of recombination junctions across subintervals among haplotypes. Recently, we examined the local effect of intergenic retrotransposons on recombination between 2 genes located at the proximal end of the characterized *bz1* haplotypes (18). Recombination in a genetic interval defined by markers in the adjacent genes *bz1* and *stc1* was compared in heterozygotes between haplotypes that differed by the presence and absence of a large retrotransposon cluster in the *bz1-stc1* intergenic region. There was a 2-fold suppressing effect of retrotransposon hemizygosity on recombination across the interval and an even stronger effect in the *bz1* and *stc1* intervals immediately flanking the cluster.

In the present work, we have analyzed recombination between genes at opposite ends of the characterized *bz1* haplotypes (9) in 2 heterozygotes that share 1 common haplotype but differ extensively in the level and type of structural polymorphisms in the intergenic regions. In one heterozygote, most structural polymorphisms are homozygous, although different enough to allow placement of any recombination junctions that might fall within them. In the other one, most structural polymorphisms are hemizygous and, thus, incapable of homologous recombination.

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Data deposition: The Ac7077 haplotype sequence reported in this paper has been deposited in the GenBank database (accession no. FJ624873).

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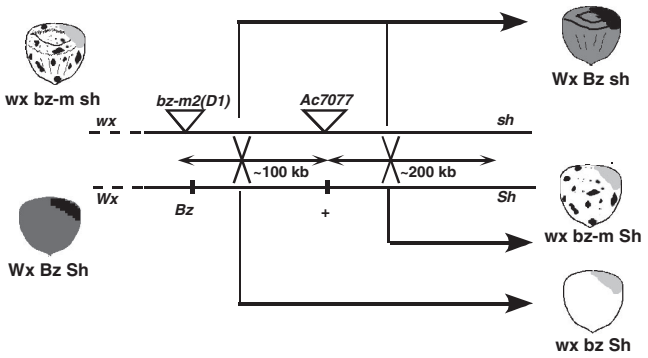

Fig. 1. Recovery of recombination products in the *bz1-Ac7077* and *Ac7077-sh1* intervals. The cartoon depicts the parental (*Left*) and recombinant (*Right*) phenotypes. The contrasting kernel phenotypes are: Bz, purple; bz-m, spotted; and bz, bronze; Wx, nonwaxy and wx, waxy, which stain dark and light, respectively, with an I-KI solution, as illustrated at the top right of each kernel (although no staining is necessary when scoring bz Sh seed); Sh, plump; and sh, shrunken. Recombination in the *bz-m2(D1)-Ac7077* interval gives rise to wx bz Sh and Wx Bz sh reciprocal recombinants. Recombination in the *Ac7077-sh1* interval gives rise to wx bz-m Sh and Wx Bz sh reciprocal recombinants. The physical distance between *Ac7077* and *bz* in B73 is from ref. 4; that between *sh* and *Ac7077* is from the maize genome sequencing project (www.maizesequence.org). These will vary from inbred to inbred and are shown for relative purposes only. Table 1 shows the seed populations screened for the Ac7077/W22 and Ac7077/B73 heterozygotes and the number of recombinants isolated in each class.

The genetic interval in the 3 haplotypes used in the study ranges in physical length from 62.7 to 111.5 kb. It consists of 5 complete genes plus parts of the 2 genes that mark the opposite ends of the interval and of 6 intergenic regions that contain the 3 main types of intergenic polymorphic insertions discussed above: *Helitrons* bearing gene fragments, nested and single retrotransposons, and MITEs. The 2 heterozygous configurations studied here have allowed us to: (*i*) determine whether recombination occurs within gene fragments borne on *Helitrons*; (*ii*) examine whether recombination junctions across the ≈100-kb multigenic interval always fall in genes and their immediate vicinity or occasionally fall in intergenic regions apparently devoid of genes; (*iii*) identify genes that behave as recombination hotspots or coldspots in the interval; (*iv*) compare recombination frequencies and the distribution of hotspots and coldspots in structurally diverse heterozygotes, and (*v*) correlate recombination with percentage sequence identity and C-methylation status.

Results

Derivation and Analysis of the *bz1-m2(D1) Ac7077* Chromosome. To analyze the effect of *Helitrons* and retrotransposon insertions on the number and distribution of exchanges across a genetic interval containing a mix of genes and polymorphic insertions, we followed a similar strategy to that described in ref. 18. Basically, we took advantage of a *Ds* mutable allele reporter whose expression depends on the presence of an *Ac* element at a second locus that is separated from the reporter by the length of the genetic interval under study. Crossing over between the transposable elements results in derivatives with a stable phenotype and a recombinant arrangement of flanking markers (Fig. 1). In this instance, the *Ds* mutable allele is *bz1-m2(D1)*, and the locus where *Ac* resides is *tac7077*. In W22 (18), *tac7077* is separated from *bz1* by ≈100 kb of DNA that contain 5 genes and a mixture of large and small insertions in the intergenic regions. This locus was defined initially by the insertion of an *Ac* transposon (*Ac7077*) in its 5′ UTR and, subsequently, by the isolation of a full-length cDNA clone by using the *Ac*-adjacent sequence as probe (19).

Our 2 main objectives at the outset of this study were to analyze the recombinational properties of the gene fragments borne on *Helitrons* and to determine the extent to which insertion polymorphisms affected recombination across the length of a relatively gene-rich ≈100-kb segment of maize genomic DNA. Thus, we anticipated that a comparison of recombination in heterozygotes between McC and the contrasting haplotypes W22, which it resembles (18), and B73, from which it differs greatly (6), should generate this information (Fig. 2). A chromosome carrying *bz1-m2(D1)* linked in-*cis* to the distal *Ac7077* element was derived for this study, and the composition of its *bz1-tac7077* region was determined by cloning it into a BAC vector (20) and sequencing it. In *bz1-m2(D1) Ac7077*, referred to hereafter as the Ac7077 haplotype, *Ds* and *Ac* are separated by 98.9 kb (Fig. 2). The proximal (left) 81 kb are identical in sequence to McC and comprise the genes *bz1*, *stc1*, *rpl35A*, *tac6058*, and *hypro1*, their respective intergenic regions, and a 66.7-kb segment between *hypro1* and *znf* that includes the 5.9-kb *HelA* and 2.6-kb *HelB Helitrons* (12) plus a 53-kb retrotransposon cluster consisting of *Opie2*, *Huck1*, and *Ji-6* nested sequences (10). However, close to the transcription start of *znf*, the sequences diverge, and the distal 17.9 kb of the Ac7077 haplotype closely resemble B73, rather than McC, in their MITE and retrotransposon make-up. The *znf-tac7077* intergenic region in Ac7077 and B73 contains a 12.4-kb *Grande1* retrotransposon and 3 previously unannotated small insertions, including a 286-bp MITE into which *Grande1* has inserted and a 360-bp *mPIF* element (21). Thus, as expected from its origin as a *Ds* derivative of the *bz1-m2(Ac)* allele (18, 22), the *bz1-m2(D1)* allele is borne on a predominantly McC *bz1* haplotype, but the recombination event that coupled it with *Ac7077* led to a reshuffling of the McC haplotype's distal end.

The sequences of the genes in the *bz1-m2(D1) Ac7077* interval of McC, W22, and B73 show the typical percentage of divergence of maize alleles and no particular haplotypic pattern. Thus, allelic comparisons show *bz1* and *stc1* to be more polymorphic between McC and W22 than between McC and B73 (1.5% vs. 1.2% and 1.7% vs. 1.5% divergence, respectively), *rpl35A* and *tac6058* to be less so (0.0% vs. 0.8% and 0.0% vs. 1.1% divergence, respectively), and *hypro1* to be equally polymorphic (1.3% divergence). The *znf* allele found in the Ac7077 haplotype is closer to B73 than to W22 (0.8% vs. 1.3% divergence).

Recombination Between *bz1-m2(D1)* and *Ac7077*. All 3 haplotypes used in the recombination experiment were first introduced into a W22 inbred background (18). Heterozygotes between haplotypes sharply different in overall structure were synthesized by crossing *bz1-m2(D1) Ac7077* with either *Bz1-W22* or *Bz1-B73* (Fig. 2). *bz1-m2(D1) Ac7077* and *Bz1-W22* lack a retrotransposon cluster in the *bz1-stc1* intergenic region, share the same 5.9-kb and 2.6-kb *Helitrons* and the 53-kb retrotransposon cluster in the *hypro1-znf* intergenic region, the latter accounting for half of the interval's physical length, and differ mainly in the makeup of the retrotransposons in the *znf-tac7077* intergenic region. In contrast, *bz1-m2(D1) Ac7077* and *Bz1-B73* differ by the presence in one, but not the other, of the 26-kb retrotransposon cluster in the *bz1-stc1* intergenic region, the 2 *Helitrons*, and the 53-kb retrotransposon cluster in the *hypro1-znf* intergenic region and resemble each other only in the *znf-tac7077* intergenic region, where they share the *Grande1* retrotransposon inserted in a MITE and 2 other small insertions.

The experiment set up to identify recombinants in the *bz1-m2(D1)-Ac7077* interval is diagrammed in Fig. 1. The *bz1-m2(D1)* allele produces a spotted seed phenotype in the presence of *Ac7077* and a bronze phenotype in its absence. The contrasting W22 and B73 *Bz1* haplotypes produce a purple phenotype. The interval is flanked by the outside markers *wx1* and *sh1*, so individuals in which the *bz1-m2(D1)* reporter has been separated

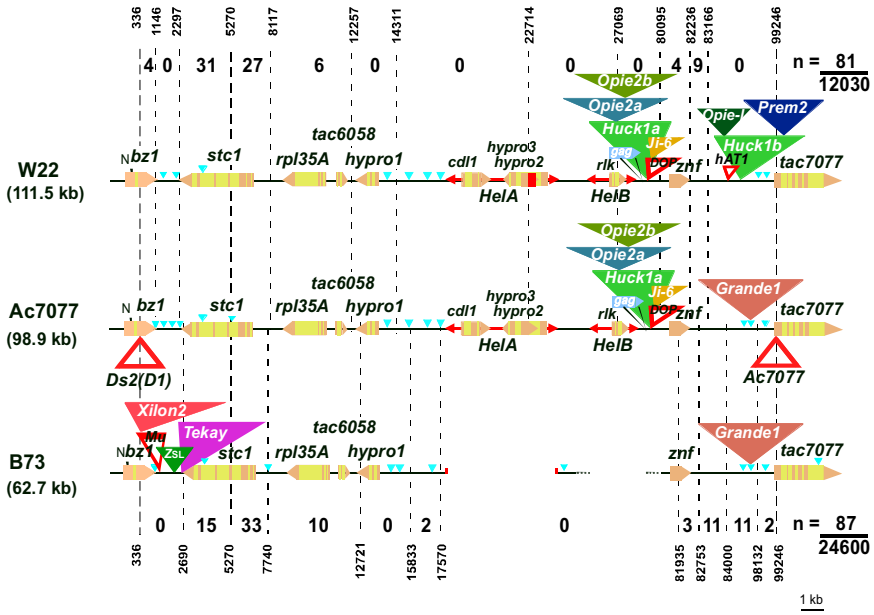

Fig. 2. Distribution of recombination junctions in the *Ds2(D1)-Ac7077* genetic interval among Sh bz recombinants from *bz-m2(D1) Ac7077/Bz-W22* + (*Upper*) and *bz-m2(D1) Ac7077/Bz-B73* + (*Lower*) heterozygotes. Each haplotype is identified by the name of the genetic line, followed by the physical size of the interval under study, in parentheses. The *9S* centromere (proximal side) is to the left; the *9S* telomere (distal side), to the right. Genes are shown as pentagons pointing in the direction of transcription; exons are in bronze, and introns in yellow. There are 7 genes in the genetic interval: *bz1*, *stc1*, *rpl35A*, *tac6058*, *hypro1*, *znf*, and *tac7077*. The same symbolism is used for gene fragments carried by *Helitrons* (*Hels*), which are represented as bidirectional arrows below the line for each haplotype. The vacant sites for *HelA* and *HelB* in B73 are provided as reference points and marked with short vertical strokes. Dashed lines represent deletions. Retrotransposons are indicated by solid triangles of different colors. DNA transposons, including *Ds2(D1)* and *Ac7077*, are indicated by open triangles of red color. Small insertions are indicated in light blue color. Only the genes have been drawn to scale. For analysis, the interval was subdivided into a series of roughly corresponding segments defined by the same polymorphic sites, where possible, or by polymorphisms located close to each other in each heterozygote. The locations of the different polymorphisms are numbered according to the *bz-m2(D1) Ac7077* sequence, starting with the NotI site in the first exon of *bz1* as position 1. The nature of the polymorphisms at each location (SNPs or indels) is identified in Tables S1 and S2. The number of recombination junctions in each subinterval is shown above (W22) and below (B73) the unique haplotype in each heterozygote. See *Results* for additional details.

from *Ac7077* by crossing over can be easily identified as stable bronze derivatives with recombined flanking markers. Heterozygotes between *wx1 bz1-m2(D1) Ac7077 sh1* and either *Wx1 Bz1-B73 + Sh1* or *Wx1 Bz1-W22 + Sh1* were hand-pollinated with a *wx1 bz1-sh1-X2* tester. The tester stock carries a deletion of the entire *bz1-sh1* region (23) and is useful for recovering selections in a hemizygous condition that facilitates their molecular analysis. Recombination between *bz1-m2(D1)* and *Ac7077* will produce wx bz Sh and Wx Bz sh seed as reciprocal products, and that between *Ac7077* and *sh1* will produce wx bz-m Sh and Wx Bz sh seed as reciprocal products. Hence, the sum of bz Sh and bz-m Sh seed should approximately equal the number of Bz sh seed, and, as shown in Table 1, it does in both heterozygotes. However, the size of the *bz1-sh1* interval, estimated from the sum of the last 2 columns in the table, is significantly larger in the Ac7077/W22 heterozygote than in the Ac7077/B73 heterozygote (2.75 vs. 2.08 cM), most of the difference being attributable to the bz Sh class. This class provides a rough estimate of recombination in the *bz1-Ac7077* interval, which was shown to be structurally more similar in the Ac7077 and W22 haplotypes (Fig. 2). Most bz Sh selections were wx, as expected from the high chiasma interference in the *wx1-bz1-sh1* region (24). Subsequent molecular analysis of the wx bz Sh selections confirmed that most arose by recombination between *bz1-m2(D1)* and *Ac7077*, although ≈10% of successfully tested selections represented coincidental *Ds* or *Ac* excision and recombination in the *bz1-sh1* interval (8/89 in Ac7077/W22 and 14/101 in Ac7077/B73).

Distribution of Recombination Junctions. The recombination junctions in an approximately equal number of wx bz Sh selections from each heterozygote were placed within the *Ds2(D1)-Ac7077* interval by PCR-genotyping a series of indel polymorphisms across the interval and sequencing the PCR products when they contained multiple polymorphisms. Supporting information (SI) Tables S1 and S2 identify the position and nature of the

Table 1. Seed selections from heterozygotes *bz1-m2(D1) Ac7077* and either W22 or B73 *Bz1+*

		Recombinant seed class phenotypes			
Heterozygous haplotype	Seed population	bz Sh	bz-m Sh	(bz+b-zm) Sh	Bz sh
Ac7077/W22	38,200	307	229	536	515
Percentage		0.80	0.60	1.40	1.35
95% C.L.		0.74–0.86	0.55–0.65	1.32–1.48	1.27–1.43
Ac7077/B73	30,090	126	177	303	323
Percentage		0.42	0.59	1.01	1.07
95% C.L.		0.37–0.47	0.53–0.65	0.93–1.09	0.99–1.16

C.L., confidence limits.

polymorphisms and summarize the marker data for each recombinant in the Ac7077/W22 and Ac7077/B73 series, respectively. The results from placing 168 junctions are shown graphically in Fig. 2. The *Ds2(D1)-Ac7077* interval has been subdivided into a series of approximately corresponding intervals in the 2 heterozygotes. They are: the *bz1* gene, the *bz1-stc1* intergenic region, and the *stc1* gene—3 segments compared in detail previously (18); the *rpl35A-tac6058* segment; the *hypro1* gene; the 2 *Helitrons* and the 53-kb retrotransposon cluster in the *hypro1-znf* intergenic region of the Ac7077/W22 heterozygote; the *znf* gene, and the *znf-tac7077* intergenic region. It is important to note that the 81 recombinants analyzed in Ac7077/W22 come from a population approximately half the size of the one that generated the 87 recombinants analyzed in Ac7077/B73.

The data summarized in Fig. 2 allow us to refine the estimate of the *Ds2(D1)-Ac7077* genetic distance to 1.35 and 0.70 cM, respectively, in Ac7077/W22 and Ac7077/B73. The *stc1* gene, including ≈1 kb upstream, is a clear recombination hotspot (25) in both heterozygotes. It accounts for 70% (58 of 81) and 55% (48 of 87) of recombinants, respectively, in the Ac7077/W22 and Ac7077/B73 heterozygotes and measures close to 1 cM, ≈1/3 of the *bz1-sh1* distance, in the former. However, as reported previously (18), the distribution of recombination junctions within *stc1* differs in the 2 heterozygotes, being relatively much lower (15 of 48 vs. 31/58) in the segment of the *stc1* gene that borders the hemizygous 25-kb retrotransposon cluster in Ac7077/B73. In fact, in this heterozygote, no recombination junctions at all were recovered in the 29 kb that lie between *Ds2(D1)* and the 3′ end of the *stc1* gene at the left (proximal) end of the *Ds2(D1)-Ac7077* interval. A number of recombinants proportionate to the 2 gamete populations screened occurred in the *rpl35A-tac6058* segment of each heterozygote, but none occurred in *hypro1* in either heterozygote, making the latter a genic recombination coldspot in the interval (26).

No recombination junctions fell within either *Helitron*, although their sequences are almost 100% identical in Ac7077 and W22. Only a 680-bp deletion in the *hypro2* gene fragment of W22's *HelA* and a single SNP in the *rlk* gene fragment of *HelB* distinguish both haplotypes. Therefore, the gene fragments carried by *Helitrons* do not contribute to genetic length. Not surprisingly, no recombination junctions fell within the 53-kb retrotransposon cluster found in the *hypro1-znf* intergenic region of Ac7077 and W22, despite a >99% sequence identity. Therefore, no recombination occurs within this large retrotransposon block that makes up approximately half of the physical size of the *bz1-tac7077* genetic interval in Ac7077 and W22. In the Ac7077/W22 heterozygote, 4 junctions fell within *znf* and 9 fell in a 0.9-kb segment immediately downstream, which contains 280 bp of the *znf* 3′ UTR and is 98.2% identical between the 2 haplotypes. However, no junctions were observed in the rest of the *znf-tac7077* intergenic region, which differs in size (16 kb in Ac7077 vs. 29.7 kb in W22) and retrotransposon cluster content and shares only short stretches of interrupted homology in these 2 haplotypes.

Unexpectedly, a large number of junctions fell in the *znf-tac7077* intergenic region of Ac7077/B73 heterozygotes: 24 of 87. This region is 99.5% identical in the 2 haplotypes, so very few polymorphisms are available to map the 24 junctions. However, 2 SNPs, 1 indel, and the *Ac7077* insertion enabled us to divide the intergenic region into 3 segments. Of the 24 junctions, 11 fell in a 1.2-kb interval right after *znf*, 11 fell in a 14-kb interval that contains *Grande1* and 2 small insertions, and 2 fell in a 1.1-kb interval just upstream of *Ac7077*, which contains an *mPIF* MITE. Other than parts of the *znf* 3′ UTR and *tac7077* 5′ UTR, no genes are known to be present in these segments, so some recombination may occur in maize intergenic regions. Supporting this observation, 2 junctions were also found in a 1.7-kb segment of the *hypro1-znf* intergenic region of Ac7077/B73 heterozygotes that contains a *Tourist Zm-3* MITE in both haplotypes.

Methylation Analysis. In general, recombinogenic maize genes and single-copy sequences are not methylated at CG or CNG residues (4, 27, 28), whereas the recombinationally inert repetitive retrotransposons are (4, 29–31). To examine whether the ability of a sequence in the *Ds2(D1)-Ac7077* interval to recombine was correlated with its methylation status, we carried out an analysis of C-methylation using methylation-sensitive restriction endonucleases, which detect C-methylation in either a CG or CNG sequence context, and bisulfite sequencing, which detects any methylated C. We focused on: *stc1* and *hypro1*, a genic recombination hotspot and coldspot, respectively; the nonrecombining gene-fragment-carrying *Helitrons*; the *bz1-stc1* intergenic region, which shows no recombination in either heterozygote, and the *znf-tac7077* intergenic region, which showed a surprising amount of recombination in the Ac7077/B73 heterozygote.

Methylation across the interval was assayed by restricting genomic and BAC DNAs with 4 C-methylation-sensitive restriction enzymes, PstI, PvuII, SmaI, and SacII, hybridizing Southern blots with individual probes from *stc1*, *hypro1*, the *rlk* gene fragment of *HelB*, single-copy sequences just downstream of *znf* and upstream of *tac7077* in the *znf-tac7077* intergenic region, and analyzing the observed band patterns against that expected from the DNA sequence of the respective BAC clones (Figs. S1–S3). The methylation status of the 3′ (left) end of *HelA* was assayed by bisulfite sequencing (32). The results of the B73 and Ac7077 analysis, summarized in Fig. 3, reveal a strong correlation between the methylation status of a sequence and its ability to recombine. Thus, none of the assayed sites in the recombination hotspot *stc1* gene or its upstream region were methylated. All CG and CNG sites assayed in the nonrecombining *HelA* and *HelB Helitrons* and *Tekay* retrotransposon were methylated. Sites in the recombination coldspot *hypro1* were methylated in Ac7077, the common haplotype in the 2 heterozygotes, but not in B73. Last, sites in the recombination-proficient *znf-tac7077* intergenic region of Ac7077/B73 heterozygotes showed 2 distinct methylation patterns in both haplotypes: Most of those immediately adjacent to *znf* and *tac7077* were not methylated, whereas all of those within the *Grande1* retrotransposon were.

Discussion

Rates of recombination for specific chromosome segments are known to vary greatly in maize (14–16). In this and a recent study (18), we have addressed the issue of how the remarkably variable genome structure of maize affects recombination. We previously demonstrated that the presence of a large intergenic retrotransposon block in hemizygous condition reduced recombination in the adjacent genes 2- to 4-fold (18). Here, we have examined the distribution of recombination junctions in an ≈100-kb genetic interval that contains several genes mixed with intergenic *Helitron* and retrotransposon insertions, either in homozygous or hemizygous condition, and have compared recombination across the interval in 2 highly dissimilar heterozygotes.

We synthesized 2 heterozygotes that had 1 haplotype in common, but differed in the other. The common haplotype was Ac7077, and the unique one was either W22 or B73 (Fig. 2). In terms of the large insertion polymorphisms in the intergenic regions, the interval of Ac7077 can be divided in 2: the proximal 80 kb, which includes 2 gene-fragment-bearing *Helitrons* and a 3-level nested retrotransposon cluster, are shared with W22 but not with B73; conversely, the distal 20 kb, which includes a single retrotransposon, are shared with B73 but not with W22. This arrangement allows us to determine in what homozygous genic or intergenic regions recombination occurs and to examine the overall effect of insertion hemizygosity on recombination across the interval.

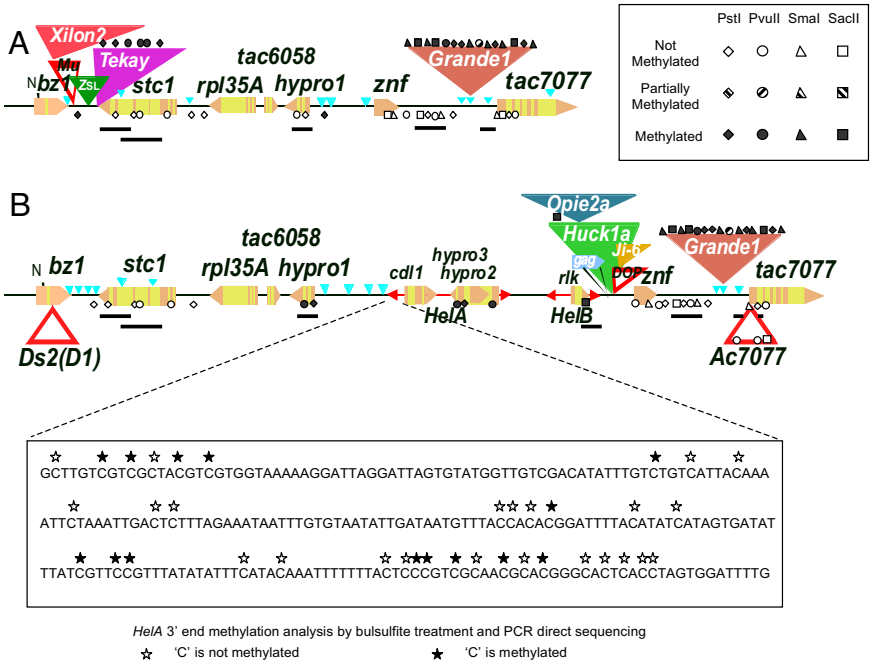

Fig. 3. Summary of C-methylation analysis in B73 (*A*) and Ac7077 (*B*) haplotypes. The results from methylation-sensitive restriction endonucleases are represented with a series of geometric symbols, whose meaning is described in the box at the top right of the figure. The probes used to hybridize the blots are shown as bars beneath each haplotype. The C-methylation status at the 3′ end of *HelA* in Ac7077 was determined by bisulfite sequencing. Unmethylated sites are represented as open stars and methylated sites, as filled stars. The C-methylation status of W22 sites between *Ds2(D1)* and *HelB* was essentially identical to Ac7077.

Given that recombination in maize takes place mostly in genes, we were particularly interested to test whether *Helitrons* contributed to genetic length. *Helitrons* are recently discovered transposons that presumably move by a rolling-circle mode of transposition (33). In maize, many *Helitrons* have trapped host gene fragments of variable length and mobilized them around the genome (34, 35). Like retrotransposons, *Helitrons* are highly polymorphic and contribute to the remarkable haplotype variability of modern maize (9, 12, 13, 36). In terms of abundance, they are estimated to comprise ≈1.4% of the sequenced B73 genome (C. Du, personal communication). To determine whether the gene fragments in *Helitrons* recombined, we paired Ac7077 and W22, haplotypes that share the same 2 *Helitrons.* Together, these *Helitrons* make up ≈10 kb of the *hypro1-znf* intergenic region and contain fragments from 4 genes (12). The *Helitrons* in the 2 haplotypes are structurally homozygous, although polymorphic, enabling us to determine whether any recombination junctions fall within them, and, as seen in Fig. 2, none did. Methylation analysis revealed that all CG and CNG sites assayed in the nonrecombining *Helitrons* were methylated (Fig. 3), in agreement with what has been observed with retrotransposons (4, 29–31). We conclude, then, that recombination within *Helitrons* is not likely to contribute to the variability in recombination rates that has been observed in maize. Because *HelA* and *HelB* are found in the same intergenic region as a 53-kb retrotransposon cluster, our experimental setup does not allow us to determine whether *Helitron* hemizygosity in Ac7077/B73 heterozygotes affects recombination in nearby genes.

The frequency of recombination across the entire *bz1-Ac7077* interval was significantly lower in the Ac7077/B73 heterozygote than in the Ac7077/W22 heterozygote (0.70 vs. 1.35 cM). The main reason for the difference is that recombination within the *stc1* gene, a clear recombination hotspot in the interval, is much lower in the former. The *stc1* gene and its upstream region measure 0.96 cM in the Ac7077/W22 heterozygote, but only 0.39 cM in the Ac7077/B73 heterozygote, most likely because hemizygosity for the large retrotransposon cluster adjacent to *stc1* in the B73 haplotype reduces recombination in the adjacent intervals (18). The most distal retrotransposon in the cluster, *Tekay*, is actually inserted within the 3′ UTR of the *stc1-B73* allele, leading to transcription termination within the 5′ LTR (C. Lin, personal communication). We have shown here that this retrotransposon is heavily methylated, but the *stc1* sequences next to it are not. The whole 25-kb cluster is probably heterochromatic and may interfere with proper homologous alignment of the adjacent *stc1* and *bz1* regions (18).

Some level of recombination was detected in all of the genes in the interval, except for *hypro1*, which behaves as a genic recombination coldspot in both heterozygotes. This observation supports the conclusion of Yao et al. (5) from their analysis of recombination in the *a1-sh2* region that not all maize genes are recombination hotspots. Peculiarly, the *hypro1* gene was found to be methylated in Ac7077 and W22 but not B73. A possible reason for this difference is that in the first 2, the gene lies adjacent to a large intergenic region that contains methylated *Helitrons* and a 53-kb retrotransposon cluster, but in B73, it lies adjacent to a short intergenic region that contains no large insertions. Possibly, recombination across the *stc1-znf* interval would be higher in a heterozygote between B73 and a structurally closer haplotype, such as Mo17 or A188 (9).

The initial conclusion that little, if any, recombination takes place within homozygous retrotransposons was based on the observation that the ratio of genetic to physical distance between 2 markers derived from the same haplotype and separated by a 94-kb retrotransposon nest was close to the genome's average (4). Here, we have examined recombination between markers in different haplotypes that share a 53-kb retrotransposon cluster of >99% sequence identity. The use of SNP and indel polymorphisms on either side of the cluster enabled us to determine that no recombination junctions fell within it, confirming that most retrotransposons are probably recombinationally inert. They are heavily methylated and probably organized into a condensed

chromatin (37) that would not be readily accessible to the cell's recombination machinery.

Perhaps the most unexpected outcome of this study was the relatively large number of junctions that fell in the *znf-tac7077* intergenic region of Ac7077/B73 heterozygotes (Fig. 2). Although this region is 99.5% identical in the 2 haplotypes, we were able to divide it into 3 subintervals through the use of polymorphic markers. Of the 24 junctions, 11 fell in a 1.2-kb interval right after *znf*, which contains the long *znf* 3′ UTR, and 11 others fell in a 14-kb interval that contains *Grande1* and 2 small insertions but no known genes. There are no polymorphisms in the interval to formally rule out recombination within the homozygous 12.4-kb *Grande1* retrotransposon, but its heavily methylated status relative to the rest of the *znf-tac7077* intergenic region (Fig. 3) would suggest that, like other retrotransposons, *Grande1* does not recombine. From the discussion above and the observation that the sequence of the *znf-tac7077* intergenic region in W22 is very different, it appears that some recombination in maize can occur in chromosomal segments apparently lacking genes, an observation that supports earlier conclusions based on more limited sequence data (5, 28). Not surprisingly, no recombination occurred in the highly dissimilar corresponding segment in the *znf-tac7077* intergenic region of Ac7077/W22 heterozygotes, so differential recombination in intergenic regions also contributes to the overall variation in recombination rates observed in maize. Coupled with the differences observed in the *bz1-stc1* segment, this comparison highlights the fact that haplotypic structural variability will strongly affect the frequency and distribution of recombination events in maize.

Materials and Methods

Genetic Lines. All of the stocks used in this study carry *bz1* haplotypes introgressed into the common genetic background of the inbred W22 and, except for the *bz1-m2(D1) Ac7077* stock, have been described previously (18). The *bronze1* alleles and the aleurone phenotypes of the various stocks are as follows. *bz1-m2(D1)* (bronze in the absence of *Ac*; spotted, in its presence) harbors a 3.3-kb *Dissociation* (*Ds*) element at position 755–762 in the second exon of the *Bz1-McC* allele (22, 38). *bz1-m2(D1) Ac7077* (spotted): a version of *bz1-m2(D1)* carrying a transposed *Ac* element in the 5′ UTR of the distal *tac7077* gene (19), at position 216489–216496 of GenBank accession no. AF391808. This stock was derived by recombining the *bz1-m2(D1)* allele, an McC haplotype derivative, with the nearby *Ac7077* insertion present in a *Bz1* haplotype of unknown origin. A 145-kb NotI BAC clone containing the entire *bz1-m2(D1) Ac7077* region was isolated and sequenced as described below. *bz1-sh1-X2* (bronze, shrunken): an X-ray-induced deletion of a large chromosomal fragment that includes the *bz1* and *sh1* loci (39) and, therefore, the entire *bz1-tac7077* region under study. *Bz1-W22* (purple): the normal allele of the color-converted version of the inbred W22 (40). *Bz1-B73* (purple): the normal allele of the inbred B73 introgressed into W22 (18).

Selection and Characterization of *bz1-Ac7077* Recombinants. The mutations *wx1* (waxy endosperm) and *sh1* (shrunken endosperm), located ≈25 cM proximal and 2 cM distal to *bz1*, respectively, were used as flanking markers. The *wx1-sh1* region exhibits high chiasma interference (24), so double crossovers in the region are rare. The *wx1 bz1-m2(D1) Ac7077 sh1/Wx1 Bz1-B73 + Sh1* and *wx1 bz1-m2(D1) Ac7077 sh1/Wx1 Bz1-W22 + Sh1* heterozygotes were hand-pollinated with a *wx1 bz1-sh1-X2* stock. Sh bz recombinants were selected as single seed with a plump, solid bronze phenotype in ears segregating spotted and purple seed. The selections were grown in the greenhouse, back-crossed to *wx1 bz1-sh1-X2* to confirm heritability, and characterized molecularly as described below.

DNA Extraction, PCR, and Sequencing. Leaf DNA from all selections was made by a CTAB extraction procedure and used for subsequent PCR amplification. PCR was performed by using Qiagen *Taq* polymerase and the primers listed in Table S3. The PCR products were run on either 1% agarose gels or 8% polyacrylamide gel based on their size and the polymorphisms to be discriminated. For sequencing, PCR products were purified by isopropanol precipitation and 70% ethanol washing. The PCR amplification primers were also used to directly sequence purified PCR products by using ABI BigDye Terminator V3.1 reagent (Applied Biosystems). DNA sequencing was carried out in an ABI 3730xl DNA analyzer.

BAC Isolation and Sequencing. NotI BAC clones of the *bz* genomic region of the *bz1-m2(D1) Ac7077* stock were isolated as previously described (20). The BAC clones were sequenced by the shotgun sequencing strategy, assembled, analyzed, and annotated as described (9). The GenBank accession no. for the Ac7077 haplotype BAC sequence is FJ624873.

Southern Blot Analysis. Restriction-digested genomic DNA (10 μg) was resolved in 0.8% agarose gels and transferred to Hybond XL nylon membranes (GE Healthcare). The membranes were hybridized with random-primer-labeled ^{32}P probes from various genes. Conditions for hybridization, high stringency washing, and exposure to X-ray film were standard.

Methylation Analysis by Bisulfite Sequencing. Bisulfite treatment of DNA was performed with the EZ DNA Methylation kit (Zymo Research). Purified DNA was used for PCR amplification by primers designed through the MethPrimer program (University of California, San Francisco). The PCR product was then cleaned and directly sequenced with the same primer in an ABI 3730xl DNA analyzer.

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