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While studying spontaneous mutations at the maize *bronze* (*bz*) locus, we made the unexpected discovery that specific low-copy number retrotransposons are mobile in the pollen of some maize lines, but not of others. We conducted large-scale genetic experiments to isolate new *bz* mutations from several *Bz* stocks and recovered spontaneous stable mutations only in the pollen parent in reciprocal crosses. Most of the new stable *bz* mutations resulted from either insertions of low-copy number long terminal repeat (LTR) retrotransposons or deletions, the same two classes of mutations that predominated in a collection of spontaneous *wx* mutations [Wessler S (1997) *The Mutants of Maize*, pp 385–386]. Similar mutations were recovered at the closely linked *sh* locus. These events occurred with a frequency of 2–4 × 10^{-5} in two lines derived from W22 and in 4Co63, but not at all in B73 or Mo17, two inbreds widely represented in Corn Belt hybrids. Surprisingly, the mutagenic LTR retrotransposons differed in the active lines, suggesting differences in the autonomous element make-up of the lines studied. Some active retrotransposons, like *Hopscotch*, *Magellan*, and *Bs2*, a *Bs1* variant, were described previously; others, like *Foto* and *Focou* in 4Co63, were not. By high-throughput sequencing of retrotransposon junctions, we established that retrotranposition of *Hopscotch*, *Magellan*, and *Bs2* occurs genome-wide in the pollen of active lines, but not in the female germline or in somatic tissues. We discuss here the implications of these results, which shed light on the source, frequency, and nature of spontaneous mutations in maize.

LTR retrotransposon | male sporogenesis | spontaneous mutation | maize | inbred variation

The ability to readily sequence entire genomes has shifted the study of spontaneous mutation from the single gene level, characteristic of the previous century, to the genomic level. Taking advantage of carefully developed genetic tools, such as mutation accumulation lines, and relatively inexpensive high-throughput sequencing technologies, researchers have been able to study genome-wide spontaneous mutations arising in a series of model organisms with small genomes, from yeast to *Arabidopsis* (1–5). Interestingly, the rate of mutations per site per cell division did not vary greatly across the range of eukaryotic model organisms examined, except for *Chlamydomonas*, where the intraspecific rate varied by almost two orders-of-magnitude (5). However, the spontaneous mutations analyzed in these studies have been largely limited to single-nucleotide changes and small indels. Only in yeast were a few large-scale (>1 kb) changes identified: 11 insertions and 4 deletions (1). Because several of these changes were flanked on both ends by transposable elements, the authors concluded that a substantial fraction of chromosomal instabilities in yeast were associated with mobile elements.

Single-gene spontaneous mutations that lead to a change in phenotype are rare and have been characterized extensively in only a few organisms. In *Drosophila*, most spontaneous mutations originate by large-scale chromosomal changes, the most common being insertion of gypsy long-terminal repeat (LTR) retrotransposons (6–12). In plants, the most comprehensive analysis to date has been that of the maize *wx* gene, whose mutations can be easily identified by a change in the appearance of the endosperm. Taking advantage of a collection of existing *wx* spontaneous mutations, the Wessler laboratory established in a series of landmark papers that most spontaneous mutations in maize arose from gross changes, such as deletions or LTR-retrotransposon insertions (13–16). However, most of those mutations were of unknown origin, as they had been isolated over the years by several researchers in a variety of genetic backgrounds, precluding estimates of spontaneous mutation frequency.

Because of the difficulties in screening large gamete populations in higher plants, few studies of spontaneous mutations in specific genes have been conducted with populations much larger than 10^5 gametes. In maize, even studies of induced mutations rarely incorporate a large untreated control because the goal of most mutagenesis experiments is to obtain and analyze mutants that arise from a given treatment (17–19). L. J. Stadler, who codiscovered the mutagenic effect of radiation with H. J. Muller, performed the largest study of spontaneous mutation at specific loci yet reported in plants (20). That experiment was significant enough to be still discussed in the mutation literature at the turn of the 21st century (21). Stadler analyzed populations

Significance

Spontaneously arisen mutations provide the raw material for natural selection and evolution in all organisms, yet their frequency of occurrence is so low that special "mutation accumulation" lines are used to study them. In the course of conventional genetic experiments, we have found that spontaneous mutations arise with unexpectedly high frequency in the pollen of some maize lines, but not of others. Most of them result from insertions of retrotransposons present in a few copies in the genome or from deletions. Most interestingly, the mutagenic retrotransposons differ from line to line. The work reported here sheds light on the source, frequency, and nature of spontaneous mutations in maize.

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See Commentary on page 10617.

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of up to 2.5 million gametes and obtained estimates of spontaneous mutation frequencies in eight maize genes. Except for two genes, mutation frequencies were in the order of one in a million. Mutations in the two exceptions, *R* and *I*, were subsequently shown to be either due to unequal crossing over (22) or to be not heritable (23).

Stadler (20) conducted his experiments in open-pollinated corn fields, in which female rows carrying dominant factors were pollinated with plants carrying multiple recessive markers. Because of its relative ease, this experimental format has been repeated often in experiments that have looked at spontaneous mutation in different loci (23). In other words, prior experiments have measured spontaneous mutation frequencies in female gametes.

The possibility that the picture might be different in male gametes was suggested to us by results from experiments originally designed to isolate new *bronze* (*bz*) mutations from different *Bz* alleles. Crosses of *Shrunken* (*Sh*) *Bz* plants as females or males to a *sh bz* stock produced putative *bz* mutations only in crosses with the *Bz* parent as male. Genetic and molecular characterization of these mutations (*Results*) established that, surprisingly, several had resulted from insertions of low-copy number LTR retrotransposons (24). This unexpected observation, coupled with the earlier finding by the Wessler laboratory that several *wx* spontaneous mutations had been caused by LTR retrotransposon insertions (13, 15, 25, 26), prompted us to reexamine the issue of spontaneous mutations in maize. We set up a series of large genetic experiments to isolate new *bz* mutations from several *Bz* stocks used as male or female parents. These experiments showed that spontaneous mutations in maize arise much more frequently in the pollen parent than in the ear parent, that most of them result from either insertions of low-copy number LTR retrotransposons or deletions, that the frequency of these events varies greatly among maize lines, and that the mutagenic retrotransposons differ from line to line. The results of those experiments, which shed light on the source, frequency, and nature of spontaneous mutations in maize, are discussed in this paper.

Results

Spontaneous *bz* Mutations. New *bz* mutations arising in either the female or the male parent were isolated from reciprocal crosses between *Sh Bz* stocks and an *sh bz-R* tester. The recessive *sh* mutation serves as a marker for self-pollen contamination in crosses of *sh bz* ear parents by *Sh Bz* pollen parents. The recessive *bz-R* allele is an internal 340-bp deletion in the *bz* gene (27) and serves to identify and distinguish newly arisen *bz* mutations.

Our first experiments were set up to isolate new mutations from *Bz-B73* and *Bz-McC*, two alleles differing extensively in sequence and haplotype structure (28). *Bz-B73* is the allele present in the B73 inbred: it was back-crossed three times to a stock carrying the *sh-bz-X2* multigenic deletion (29) to preserve the B73 *Sh-Bz* region for studies of recombination in the interval (30, 31). The *sh-bz-X2* deletion had been introduced into a W22 background by two backcrosses to *Bz-W22* followed by selfing. This reduced inbreeding allowed the weak *sh-bz-X2* deletion line to preserve enough vigor and fertility to function effectively as an ear and pollen parent, even under suboptimal field conditions. *Bz-McC* is an allele extracted from a Barbara McClintock stock and introgressed into a W22 *bz-R* line obtained from J. L. Kermicle (Department of Genetics, University of Wisconsin–Madison, Madison WI). So, although the two *Bz* lines have W22-convergent genetic backgrounds, they were derived differently. The results of the experiments with these two lines, identified as W22 *Bz-B73* and W22 *Bz-McC*, are reported in Table 1.

Crosses of the *Bz* stocks to *sh bz-R* produced putative *bz* mutations, identified as rare plump bronze kernels in a purple kernel background, only in crosses with either of the two *Bz* parents as male. The frequency of their occurrence was similar in the two crosses: 4.3 and 3.6 per 100,000 gametes, respectively, in the *Bz-B73* and *Bz-McC* lines. No mutations were obtained through the female of either line, even though similarly sized populations were screened (Table 1). A statistical analysis (32) confirms that the *Bz* to *bz* spontaneous mutation frequencies differ significantly in the male and female inflorescences of the two lines.

The bronze kernel selections fell into two classes: those that germinated as bronze (*bz*) seedlings (concordant) and those that germinated as red (*Bz*) seedlings (nonconcordant). The latter result from postmeitoic or male gametophytic mutations in which a sperm carrying the new *bz* mutation fertilizes the polar nuclei in the central cell of the embryo sac and a sperm carrying the parental *Bz* allele fertilizes the egg (33). The red seedlings carrying a wild-type *Bz* allele were not studied further. DNA was extracted from the bronze seedlings of concordant mutants, PCR-amplified with different *bz* primers, and sequenced. The results of that analysis are summarized in the last column of Table 1.

The vast majority of new mutations in both lines arose from insertion of low-copy number LTR retrotransposons or from deletions. However, the proportion of LTR retrotransposon insertions and deletions differed in the two lines. Whereas 18 of 24 concordant mutations from *Bz-B73* arose from retrotransposon

Table 1. Spontaneous *bz* mutations in different maize lines

Bz genotype	Parent in cross	Effective kernel population	*bronze* mutations	Concordant mutants	Non concordant mutants	Mutation frequency ($\times 10^{-5}$)	Mutation frequency 95% limits ($\times 10^{-5}$)	Nature of *bz* concordant mutants
W22 *Bz-B73*	Male	906,621	39	24	15	4.3	3.1–5.9	6 *Magellan* and 12 *Bs2* LTR Res, 3 deletions, 1 8-bp and 1 *MULE* insertion, 1 uncharacterized
	Female	435,857	0	0	0	0.0	0.0–0.8	None
W22 *Bz-McC*	Male	1,079,667	39	32	7	3.6	2.6–5.0	2 *Hopscotch* and 4 new sLTR Res, 16 deletions, 2 8-bp insertions, 1 duplication 3 transitions, 4 uncharacterized
	Female	813,540	0	0	0	0.0	0.0–0.5	None
B73 *Bz-B73*	Male	227,491	0	0	0	0.0	0.0–1.6	None
Mo17 *Bz-Mo17*	Male	208,984	0	0	0	0.0	0.0–1.8	None
4Co63 *Bz-4Co63*	Male	583,237	15	14	1	2.6	1.4–4.2	5 RLX_*jeli*, 1 RLG and 3 RLX LTR Res, 1 deletion, 1 transition, 3 uncharacterized

insertions, only 6 of 32 mutations from *Bz-McC* did. On the other hand, 16 of the 32 mutations from *Bz-McC* arose from deletions, whereas only 3 of the 24 mutations from *Bz-B73* did. Most strikingly, the mutagenic retrotransposons differ in the two lines. Insertions of the *Gypsy* element *Magellan* (26) and of *Bs2*, a *Copia* element related to *Bs1* (34, 35), or of their respective solo LTRs (sLTRs), predominate among *bz-B73* retrotransposon mutations (6 of 18 and 12 of 18, respectively), whereas only insertions of the *Copia* element *Hopscotch* and of a novel sLTR occur among *Bz-McC* mutations (2 of 6 and 4 of 6, respectively). Other classes of mutations were observed in smaller numbers: 8-bp insertions in both *Bz* progenitor alleles, an insertion of a 264-bp *MULE* element in *Bz-B73*, and a small duplication and single nucleotide changes in *Bz-McC*. All of these mutations will be discussed in detail in the next section.

The surprising discovery that low-copy number LTR retrotransposons were unusually mobile in the pollen of the two genetic lines led us to investigate whether that was also true in other maize lines. We decided to extend our analysis to three Corn Belt inbred lines that have been used extensively in either breeding or genetic work: B73, Mo17, and 4Co63 (36). Although these inbreds produce colorless seeds because they carry mutations at the *R* and *C* genes required for anthocyanin pigmentation in the seed, they are genetically *Sh Bz* (like all other inbreds) and can be screened for male-arisen *bz* mutations by pollinating *R*;*C sh bz-R* female testers. The three inbreds were used as pollen parents on a large number of *sh bz-R* females and the resulting ears were screened for plump, bronze kernels, which were analyzed as described earlier. The results are summarized in the bottom three entries of Table 1.

No *bz* mutations were detected in either B73 or Mo17 in populations of over 200,000 kernels, which would have generated 7–10 mutants if the mutation frequency in those inbreds was as high as in the two genetic lines tested. The upper 95% confidence limits of these nil mutation frequencies are lower than the lower 95% confidence limits of the mutation frequencies in either genetic line. The fact that the B73 inbred carries the same *Bz* haplotype as the introgressed *Bz-B73* genetic line argues that, as one might expect, mobility of the *Bs2* and *Magellan* retrotransposons recovered in *bz-B73* mutations does not depend on a specific haplotype, but on the genetic background of the line.

In contrast, *bz* mutations arose in the paternal progeny of inbred 4Co63 with a frequency of 2.6×10^{-5}, which is not significantly different from those of the two active genetic lines. Most interestingly, several of the *bz* mutations from 4Co63 resulted from insertion of low-copy LTR retrotransposons that have not been previously found among maize mutants. We are naming them here according to the nomenclature for LTR retrotransposons adopted by the annotators of the B73 maize genome sequence (37). In this nomenclature, members of the *Copia* and *Gypsy* retrotransposon superfamilies are named RLC and RLG, respectively, and those that cannot be assigned to either superfamily because of unidentifiable homology to retrotransposon genes are named RLX for "unknown." Five *bz* mutations resulted from insertions of RLX-*Jeli* or its sLTR, one from insertion of a new RLG retrotransposon, and three from insertions of new retrotransposons with LTRs not related to any of the 406 families in the database (37, 38). Two other *bz* mutations in 4Co63 arose from a deletion of the entire *bz* gene and from a single nucleotide change. Thus, the types of changes found in the long-term Iowa inbred 4Co63 (36) are similar to those found in the two recently derived W22 genetic lines.

Molecular Characterization of the *bz* Mutations. All of the mutations in this study are designated by the name of the parental allele, followed by the letter "*N*" to denote their natural or spontaneous origin, and a number assigned to each mutant at germination time. Thus, *bz-B73:N1* stands for the first spontaneous bronze mutation from the *Bz-B73* genetic line seeded in the greenhouse. Of the 70 concordant *bz* mutations in Table 1, the most common class resulted from insertion of an LTR retrotransposon. Table 2 presents a detailed summary of those insertion mutations, including the *bz:N* mutant allele designation, the name of the retrotransposon family, the size of the insertion and of its LTR, the sequence of the 5-bp target site duplication and its location in the respective *Bz* allele, and the orientation of the retrotransposon relative to *bz*.

The retrotransposons appear to be inserted randomly in the two *bz* exons, roughly in proportion to exon size (10 in exon 1 and 20 in exon 2, compared with 11 and 19, respectively, expected by chance) and in no preferential transcriptional orientation relative to *bz* (15 same, 13 opposite, and 2 unresolvable). The majority terminate in 5′ and 3′ LTRs in direct orientation flanked by 5-bp target site duplications (TSDs), the standard configuration of an LTR retrotransposon. Complete sequences of both LTRs were obtained for most of these insertions and, as expected from a fresh LTR retroinsertion event, they are 100% identical (39). Differences in 1 or 2 bp seen occasionally between the 5′ and 3′ LTRs most likely stem from PCR amplification or sequencing errors. Exceptions to the standard LTR retrotransposon configuration include insertions with two inverted 3′ ends (*bz-B73:N2* and *bz-B73:N13*), a simple sLTR either flanked by TSDs (*bz-McC:N29*, *bz-4Co63N1*, *bz-4Co63:N2*) or not flanked by TSDs because of adjacent deletions (*bz-B73:N6*), a compound insertion with 5′ and 3′ LTRs from two different elements (*bz-4Co63:N5*), and fractured elements missing one end and its flanking sequence (*bz-4Co63:N4* and *bz-4Co63:N14*).

***Magellan* Insertions.** Six mutations in *Bz-B73* arose from insertions of an RLG *Magellan* retrotransposon. In four of them (*bz-B73:N1*, *bz-B73:N3*, *bz-B73:N7*, and *bz-B73:N16*), the insertion is the same 5.6-kb element ending in identical 341-bp LTRs that is found in the Maize TE database (26, 38). In two of them, the insertion is much shorter. *bz-B73:N6* has a 342-bp sLTR insertion beginning with the terminal AA of the polypurine tract flanking the 3′ LTR and ending with a deletion of the 3′ terminal A of the *Magellan* LTR. Also missing are 10 bp of the *bz* first exon corresponding to bases 435–446 in *Bz*-B73. Consequently, it is not possible to identify the five bases of the TSD. No microhomologies between *bz* and *Magellan* sequences at the deletion junction could be identified that would explain the origin of this unusual insertion by a microhomology-mediated rearrangement (40, 41) at or shortly after transposition. *bz-B73:N2* has an unusual ∼0.7-kb insertion with two inverted 3′ ends. The insertion is probably palindromic as our cloned amplification products failed to produce a complete sequence and its size was estimated by Southern blot (*SI Appendix*, Fig. S1). Our clone of the 5′ *bz*-retroelement junction contained an insertion of *Magellan*'s 3′ terminal 238 bp flanked by the proximal TSD sequence on the 5′ side and a 23-bp deletion of *bz* exon 1 sequence, including the distal TSD, on the 3′ side. Our clone of the 3′ retroelement-*bz* junction contained an insertion of *Magellan*'s 3′ terminal 255 bp flanked by the distal TSD sequence on the 3′ side and an 18-bp of *bz* exon 1 sequence, including the proximal TSD, on the 5′ side.

***Bs2* Insertions.** Twelve mutations in *Bz-B73* arose from insertions of an RLC retrotransposon closely related to *Bs1* that we have termed *Bs2* (34, 35). *Bs2* is 3,269-bp long and has 330-bp LTRs that share only 61% sequence identity with those of *Bs1* over their entire length, but 90% identity over the first 100 bp. More importantly, the non-LTR sequences of *Bs2* and *Bs1* are 96% identical over their entire length and encode a partial polyprotein with a reverse-transcriptase domain and a segment of a vacuolar ATPase (42). Therefore, they belong to the same maize LTR retrotransposon family, even though their LTRs do not meet the 80% sequence identity family criterion (37). Seven mutations harbor *Bs2* elements with 330-bp LTRs: *bz-B73:N8*, *bz-B73:N11*, *bz-B73:N18*, *bz-B73:N23*, *bz-B73:N31*, *bz-B73:N32*, and *bz-B73:N39*. Four others harbor a closely related version of *Bs2* with shorter 275-bp LTRs that we have termed *Bs2-s* for *Bs2-short*: *bz-B73:N21*, *bz-B73:N25*, *bz-B73:N33*, and *bz-B73:N38*. Although the 275-bp *Bs2-s* LTR appears to have arisen by a simple deletion close to the 5′ end of the *Bs2* LTR, there is no obvious microhomology at the deletion junction to suggest an origin of the former by a DNA replication or repair mechanism.

Table 2. Independent retrotransposon insertions among the spontaneous *bz* mutations arisen in this study

bz mutant allele	Retrotransposon insertion	Insertion size (kb)	LTR (bp)	TSD	Insertion site in *Bz* allele	Orientation (relative to *bz*)
bz-B73:N mutants						
bz-B73:N1	*Magellan*	5.6	341	GCGTC	154–158 exon 1	Opposite
bz-B73:N2	*Magellan* with two inverted 3′ ends	0.7	341	CCTTC	53–57 exon 1	Cannot tell
bz-B73:N3	*Magellan*	5.6	341	ACCTG	218–222 exon 1	Opposite
bz-B73:N6	*Magellan* LTR + deletion *bz* exon 1	0.3	341	Deleted	Exon 1	Same
bz-B73:N7	*Magellan*	5.6	341	CACGG	202–206 exon 1	Opposite
bz-B73:N8	*Bs2*	3.3	330	GTGCC	1002–06 exon 2	Same
bz-B73:N11	*Bs2*	3.3	330	CGGGC	1140–44 exon 2	Opposite
bz-B73:N13	*Bs2* with two inverted 3′ ends	0.5	232	CGCGG	1298–1302 exon 2	Cannot tell
bz-B73:N16	*Magellan*	5.6	341	GCGTC	460–464 exon 1	Opposite
bz-B73:N18	*Bs2*	3.3	330	CCACC	660–664 exon 2	Opposite
bz-B73:N21	*Bs2-s*	3.2	275	CCTGC	374–378 exon 1	Opposite
bz-B73:N23	*Bs2*	3.3	330	CCTTC	1186–90 exon 2	Same
bz-B73:N25	*Bs2-s*	3.2	275	ACGAG	644–648 exon 2	Same
bz-B73:N31	*Bs2*	3.3	330	TCGCT	1072–76 exon 2	Same
bz-B73:N32	*Bs2*	3.3	330	GGGTG	1250–1254 exon 2	Opposite
bz-B73:N33	*Bs2-s*	3.2	275	CCGTG	1172–76 exon 2	Opposite
bz-B73:N38	*Bs2-s*	3.2	275	CGACG	900–904 exon 2	Same
bz-B73:N39	*Bs2*	3.3	330	ACCTC	881–885 exon 2	Opposite
bz-McC:N mutants						
bz-McC:N1	*Hopscotch*	4.8	231	GCCTC	933–937 exon 2	Same
bz-McC:N18	*Hopscotch*	4.8	231	ACCTG	218–222 exon 1	Same
*bz-McC:N29**	RLX-*Macpre* sLTR	0.5	479	GACGT	818–822 exon 2	Same
bz-4Co63:N mutants						
bz-4Co63:N1	RLX-*Fonty* sLTR	0.3	278	CTGAC	1016–20 exon 2	Opposite
bz-4Co63:N2	RLX-*Jeli* sLTR	0.2	225	CCGTG	1175–79 exon 2	Same
bz-4Co63:N3	RLG-*Foto*	5.3	297	GTGTG	1318–22 exon 2	Same
bz-4Co63:N4	Fractured RLX-*Focou* + deletion *bz* exon 1	2.4	299	CGCCT	51–55 exon 1	Opposite
bz-4Co63:N5	Compound RE (5′, RLX-*Focou*; 3′, RLX-*Jeli*)	4.2	299/224	CGCGG	1371–75 exon 2	Same
bz-4Co63:N7	RLX-*Jeli*	3.2	226	CTCTG	777–781 exon 2	Same
bz-4Co63:N8	RLX-*Jeli*	3.2	226	CTGGA	1045–49 exon 2	Same
bz-4Co63:N14	Fractured RLX-*Jeli* + deletion *bz* exon 1	2.4	226	TGCTC	83–87 exon 1	Same
bz-4Co63:N15	RLX-*Jeli*	3.2	226	CCACC	1185–89 exon 2	Opposite

*Three other mutations were identical to *bz-McC:N29*.

One mutation (*bz-B73:N13*) harbors a 0.5-kb insertion with two inverted *Bs2* 3′ ends. As with its counterpart *bz-B73:N2*, we were unable to generate a complete sequence of this insertion and its size was estimated from a Southern blot (*SI Appendix*, Fig. S2). In both the proximal (5′) and distal (3′) *bz*-retroelement junctions, the *bz* 5-bp TSD flanked a *Bs2* 3′ terminal sequence, suggesting a palindromic structure of the insertion.

***Hopscotch* Insertions.** Two mutations in *Bz-McC* arose from insertions of an RLC *Hopscotch* retrotransposon. The insertions in these two mutants (*bz-McC:N1* and *bz-McC:N18*) are identical to each other and to the *Hopscotch* retrotransposon insertion in *wx-K* (25). They all encode an apparently complete 1,438-aa gag/pol polyprotein and are, most likely, autonomous retrotransposable elements.

Insertions of New Retrotransposons. Several spontaneous *bz* mutations were caused by insertion of retrotransposons not previously found among maize mutants. Most of them arose in the inbred 4Co63, whose genome is not yet characterized and which has been used principally in studies of variegated pericarp (43, 44). Six of the nine *bz-4Co63* mutations resulted from insertions of retrotranposons belonging to the RLX-*Jeli* family (37). Of these, three (*bz-4Co63:N7*, *bz-4Co63:N8*, and *bz-4Co63:N15*) were identical 3.2-kb insertions flanked by 226-bp LTRs and encoding proteins with homology to a pyruvate decarboxylase and a PAP-specific phosphatase, rather than a gag/pol protein. Therefore, these are not insertions of an autonomous retroelement, but of an element that has captured host gene sequences. One mutation (*bz-4Co63:N2*) was caused by insertion of a 225-bp RLX-*Jeli* sLTR and is flanked by a 5-bp TSD. Most likely, this sLTR arose by intrachromosomal recombination between the two flanking LTRs of an intact element at the time of or shortly after chromosomal integration. The other two mutations were caused by insertions of more complex RLX-*Jeli* elements. The insertion in *bz-4Co63:N14* is a 2.2-kb fractured RLX-*Jeli* with an adjacent *bz* deletion: missing are the 5′ terminal 1,009 bp of the RLX-*Jeli* element found in the previous mutants, including the entire 5′ LTR, and 35 bp of adjacent *bz* sequences, including the 5′ TSD. A similar structure consisting of a fractured retrotransposon adjacent to a deletion of the host gene was described for the *Magellan*-induced *wx-M* mutation in maize (26). The insertion in *bz-4Co63:N5* is the most unusual: a 4.2-kb compound element consisting of 2852-bp from a new RLX element that we have named RLX-*Focou* at the 5′ end and 1,383-bp of an RLX-*Jeli* retrotransposon at the 3′ end. This compound insertion is flanked by the same *bz* 5-bp TSD, but ends in different LTRs: a 299-bp 5′ LTR from RLX-*Focou* and a 224-bp 3′ LTR from RLX-*Jeli*. RLX-*Focou* encodes fragments of a gag/pol and a PHD protein and is, thus, also nonautonomous.

None of the insertions in the other three *bz-4Co63:N* mutations are in the database. The 278-bp insertion in *bz-4Co63:N1* corresponds to the sLTR from a retrotransposon that we have named RLX-*Fonty*. The sLTR is flanked by a 5-bp TSD and probably arose by a similar mechanism to that of the sLTR in *bz-4Co63:N2*. The 5.3-kb insertion in *bz-4Co63:N3* is an apparently intact, *Gypsy* retrotransposon that we have termed RLG-*Foto*. Its 297-bp LTRs show no significant similarity to any sequences in the Maize TE database and its body encodes a 1,468-aa, potentially active, polyprotein, so this may be an autonomous element. Finally, the

insertion in *bz-4Co63:N4* corresponds to a 2.4-kb fractured RLX-*Focou* retroelement accompanied by an adjacent *bz* deletion. Missing from the element are the terminal 500 bp from the 5′ end, including its 5′ LTR, and missing from *bz* are 77 bp of adjacent sequence, including the 3′ or distal TSD (the insertion is in opposite orientation to the gene). *bz* and RLX-*Focou* share 4 bp of homology at the deletion junction, so the deletion could have arisen as a microhomology-mediated rearrangement at or just after integration.

The last new insertion arose in the *Bz-McC* genetic line and is unique because of its distinct premeiotic origin. Mutant *bz-McC:N29* harbors the insertion of a unique unusually long, 479-bp sLTR from a retrotransposon that we have designated RLX-*Macpre*. Three other *bz-McC:N* mutations had exactly the same 479-bp insertion at the same location in exon 2. The simplest explanation for this series of four identical mutations is that they arose in microsporogenesis from a premeiotic *Macpre* retrotransposition event that produced a cluster of pollen grains carrying the same mutation. Other than this set, all of the other retrotransposon mutations in Table 2 were unique and most likely arose at meiosis.

Other Mutations. The remaining concordant *bz* mutations briefly summarized in Table 1 consisted mostly of deletions of various sizes. A few were caused by single-base pair changes, insertions of a *MULE* element or of 8 bp, and small duplications. Table 3 presents the molecular details of these mutations.

Deletions ranged in size from 19 bp within *bz* to several kilobases, often including the entire *bz* gene and sequences from the adjacent *stk1* and *stc1* genes (45). Because some of the mutations failed to yield a PCR product with any primer pair tested, we do not know the exact size of the larger deletions, but they must stretch for at least 3-kb based on the location of the adjacent gene primers. In many of the other deletions, microhomologies of 4–7 bp could be detected at the deletion junction, suggesting that they could have arisen by a mechanism involving microhomology-mediated repair of a DNA double-strand break (40, 41). The *bz-B73:N12* mutation arose by the insertion in exon 1 of a short, and clearly nonautonomous, 264-bp *MULE*, which produced the 9-bp TSD typical of this transposon superfamily. This short element must have been mobilized by a larger autonomous *MULE* in the parental line. Three mutations carried 8-bp insertions consisting of either a TCA or TAA trinucleotide plus five bases identical to the five bases preceding the trinucleotide. Possibly, these originated by the failed insertion of *Bs2* and *Hopscotch* retrotranposons, respectively, that left behind the 5-bp TSD separated by three bases from the 5′ and 3′ ends of the LTRs. Only four mutations arose from single-base pair changes, which resulted in either an amino acid change in the highly conserved glucosyltransferase domain of the *Bz*-encoded protein or in the formation of a premature stop codon.

In a few instances, mutants from the same parent were found to carry the same mutation event, suggesting a premeiotic origin. Thus, *bz-B73:N28* and *bz-B73:N29* carried the same 19-bp deletion in exon 1; *bz-McC:N17*, *bz-McC:N23*, and *bz-McC:N25* had the same G-A single-base pair change in exon 2, and *bz-McC:N24* and *bz-McC:N26* had the same 8-bp insertion in exon 1.

Spontaneous *sh* Mutations. Having found that mutations at the *bz* locus by either retrotransposition or deletion occurred frequently in the pollen of our two genetic lines, we next asked whether similar mutations could also be recovered at the closely linked *sh* locus. To identify and subsequently PCR-isolate new *sh* mutations, we took advantage of the *sh-bz-X2* deletion stock, where the entire *sh-bz* region has been deleted (29), so any new mutations can be unambiguously characterized in the hemizygous condition. We pollinated *sh-bz-X2* females with the W22 *Sh-B73*

Table 3. Spontaneous *bz* mutations not caused by a retrotransposon insertion

bz mutant allele	Nature of mutation	Relevant details	*Bz* site location (from ATG)
bz-B73:N12	264-bp *MULE*	9-bp TSD	279–287 exon 1
bz-B73:N14	156-bp deletion	7-bp microhomology at junction	627–782 exon 2
bz-B73:N27	8-bp insertion	TCA insertion between 5-bp TSD	660–661 exon 2
*bz-B73:N28**	19-bp deletion	6-bp microhomology at junction	339–357 exon 1
*bz-B73:N29**	19-bp deletion	6-bp microhomology at junction	339–357 exon 1
bz-McC:N3	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-McC:N4	1,028-bp deletion	5 bp at junction = *Hopscotch* end	1434–2461 exon 2 + 3′ end
bz-McC:N5	905-bp deletion	7/8-bp homology at junction	−321 to 585 exon 1
bz-McC:N6	320-bp duplication	Complex: three new junctions	1151–1248, 929–1150 exon 2
bz-McC:N7	777-bp deletion	4-bp microhomology at junction	632–1408 exon 2
bz-McC:N9	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-McC:N10	860-bp deletion	2-bp microhomology at junction	317 exon 1 to 1176 exon 2
bz-McC:N11	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-McC:N12	467-bp deletion	No microhomology at junction	99 exon 1 to 565 intron
bz-McC:N13	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-McC:N14	1,679-bp deletion	No microhomology at junction	−1641 (*stk1*) to 38 *bz* exon 1
bz-McC:N17†	1-bp change	G392 to D392	1275 in exon 2
bz-McC:N23†	1-bp change	G392 to D392	1275 in exon 2
bz-McC:N24‡	8-bp insertion	TAA insertion between 5-bp TSD	149–150 exon 1
bz-McC:N25†	1-bp change	G392 to D392	1275 in exon 2
bz-McC:N26‡	8-bp insertion	TAA insertion between 5-bp TSD	149–150 exon 1
bz-McC:N27	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-McC:N28	104-bp deletion	No microhomology at junction	335–439 exon 1
bz-McC:N30	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-McC:N31	582-bp deletion	19-bp unknown filler in junction	688–1269 exon 2
bz-McC:N32	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-McC:N35	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene
bz-4Co63:N6	1-bp change	C942 to A942 creates stop	942 in exon 2
bz-4Co63:N9	>3-kb deletion	No PCR product with *bz* primers	Entire *bz* gene

*These two mutant alleles had identical mutations.
†These three mutant alleles had identical mutations.
‡These two mutant alleles had identical mutations.

Bz-B73 and W22 *Sh-McC Bz-McC* lines and isolated shrunken purple kernels against a background of plump purple kernels. Any self-contaminant in the *sh-bz-X2* stock would produce a shrunken bronze kernel and thus be ignored. The shrunken phenotype is much more variable than the bronze phenotype, so we applied a lax criterion in the selection of putative mutants. Consequently, many candidates turned out to be false-positives and we cannot distinguish in this experiment true nonconcordant mutations from false-positive selections. The results of the genetic analysis of putative *sh* mutants are presented in Table 4.

Heritable *sh* mutations were recovered in both crosses, although less frequently than *bz* mutations, which is not surprising given the greater variability and line-dependent penetrance of the shrunken phenotype. The mutations were analyzed with a set of eight primer pair combinations covering the entire length of the ∼7-kb *sh* gene (46). Similar types of mutations at *sh* and *bz* were observed in each line: a deletion plus two *MULE* insertions in *Sh-B73* and two deletions plus five *Hopscotch* insertions in *Sh-McC*. The *MULE* element recovered in the two *sh* mutations was the same as that recovered in the *bz* mutation of the W22 *Sh-B73 Bz-B73* line. Similarly, the same retrotransposon, *Hopscotch*, was recovered in *sh* and *bz* mutations of the W22 *Sh-McC Bz-McC* line. We conclude, therefore, that high pollen mutability is a line-dependent, rather than locus-specific, phenomenon.

The molecular characterization of *sh* mutants is reported in *SI Appendix*, Table S1. Two of the *sh* mutants in the W22 *Sh-B73 Bz-B73* genetic line, *sh-B73:N2* and *sh-B73:N4*, arose from insertions of the same *MULE* element in exons 12 and 13 of the *sh* gene, respectively. This element is identical to the one causing the *bz-B73:N12* mutation in the same line. Therefore, this line must carry an autonomous *MULE* element encoding the transposase responsible for mobilization of the short elements. Curiously, of the five *sh-McC* mutations carrying *Hopscotch* insertions, three have insertion in the 225-bp exon 12. These data suggest that the comparatively short *sh* exon 12 may be particularly susceptible to transposon insertions.

Generality of Retrotransposition in Pollen. To determine the extent of pollen retrotransposition of *Hopscotch*, *Bs2*, and *Magellan* on a genome-wide scale, we used a combination of high-throughput sequencing and computational techniques. Because we had established genetically that retrotransposition occurs exclusively in the male of W22 *Bz-McC* and W22 *Bz-B73* and not at all in the B73 inbred, we created four different F1 populations by reciprocal crosses between B73 and each of the two genetic lines. Then, we looked for new retrotransposition events among 1,000 F1 progeny from each of the four reciprocal crosses: (*i*) B73 × W22 *Bz-McC*, (*ii*) W22 *Bz-McC* × B73, (*iii*) B73 × W22 *Bz-B73*, and (*iv*) W22 *Bz-B73* × B73. Crosses (*ii*) and (*iv*) serve as negative controls because we do not expect new transpositions from the inbred B73 used as male. Cross (*i*) should identify new transpositions of *Hopscotch*, the retroelement shown to move in W22 *Bz-McC*: new junctions should be present only in cross (*i*) and absent from the other three. Similarly, cross (*iii*) should identify new transpositions of *Bs2* and *Magellan*, the two retroelements shown to move in W22 *Bz-B73*: new junctions should be present only in cross (*iii*) and absent from the other three.

We deep-sequenced retrotransposon junctions in pools of 1,000 individuals from each of these F1 populations with the objective of identifying endogenous and new junctions on the basis of their relative abundance, the new ones expected to be about 1/1,000th as abundant on average as the endogenous ones. The details of this experiment are described in *SI Appendix, SI Materials and Methods*. In brief, an equal amount of young leaf tissue from a total of 1,000 individuals from each F1 population was harvested and pooled. A modified Splinkerette-PCR method (47) was used to isolate the insertion junctions of the three different retroelements. Sequencing libraries were constructed and sequenced on an Illumina Miseq system. The sequence output was analyzed using InsertionMapper modules (48).

Read number data for each of the three retrotransposon junctions in each of the four F1 populations are given in *SI Appendix*, Tables S2–S4, which also show the location of the junction sequences in the B73 genome (49). The experiment successfully resolved endogenous and new retroelement junctions on the basis of abundance. The presence of most endogenous elements in the respective parental line was confirmed by PCR, as indicated under the column "PCR results" in each table. In some cases, they were also confirmed in the recently sequenced version of W22 carrying the *Bz-W22* haplotype (50). New junctions were present only in the expected F1 population: that is, cross (*i*) for *Hopscotch* (*SI Appendix*, Table S2) and cross (*iii*) for both *Bs2* (*SI Appendix*, Table S3) and *Magellan* (*SI Appendix*, Table S4). The new junctions varied in abundance from 0.01 to 0.0001 of the average endogenous elements, so we adopted an empirical cut-off to accept a retrotransposon junction as representing a new germinal transposition or not. We ran PCRs on low-abundance candidates and chose as a cut-off limit the lowest abundance junction that could be successfully amplified in the respective DNA pool (*SI Appendix*, Tables S2–S4). This empirical approach resulted in the acceptance of practically all of the low-read candidates that met the initial selection criterion of being present only in the expected cross. The results of the next-generation sequencing (NGS) analysis are summarized in Tables 5 and 6.

The number of endogenous insertions in the active lines varies from five to seven, most of them being inserted in nonrepetitive DNA (Table 5). By comparing the presence or absence of each endogenous element in the parental lines with the known retrotransposon activity of each line, we were able to identify potentially autonomous elements. Thus, a unique *Hopscotch* insertion in chromosome 2 of W22 *Bz-McC* (*SI Appendix*, Table S2) and a unique *Magellan* insertion in chromosome 5 of W22 *Bz-B73* (*SI Appendix*, Table S4) may be autonomous elements. Interestingly, *Bs2*, the most active retroelement in our study, has four potentially autonomous insertions: one each in chromosomes 1, 2, 5, and 8 of W22 *Bz-B73* (*SI Appendix*, Table S3).

The NGS data for *Magellan* are supported by Southern blot data (*SI Appendix*, Fig. S3). The restriction enzyme HindIII cuts within *Magellan*, separating the two LTRs, so a HindIII digest of the various lines should produce twice as many LTR-hybridizing bands as the number of endogenous insertions. NGS uncovered

Table 4. Spontaneous *sh* mutations from *Sh* lines used as male parents

Sh genotype	Effective kernel population	*Shrunken* selections	*Shrunken* mutations	Mutation frequency ($\times 10^{-5}$)	Nature of *sh* mutants
W22 *Sh-B73*	439,943	24	5	1.1 (0.4–2.6)*	One deletion Two *MULE* Two undefined
W22 *Sh-McC*	408,468	14	8	2.0 (0.7–3.4)*	Five *Hopscotch* Two deletions One undefined

*95% Confidence limits.

Table 5. Endogenous retrotransposon sites in lines analyzed by NGS for transposition in pollen

Retro-transposon	Line*	No. endogenous sites	No. sites with B73 hits†	No. sites in genes‡	No. sites in IGRs§	No. sites in repetitive DNA¶
Hopscotch	W22 *Bz-McC*	5	4	0	2	2
Bs2	W22 *Bz-B73*	5	5	2	3	0
Magellan	W22 *Bz-B73*	7	6	4	1	1

*Line where retrotransposon is active.
†>85% Identity.
‡±500 bp.
§Intergenic regions.
¶≥Five copies.

7 endogenous *Magellan* insertions in W22 *Bz-B73* and 6 in W22 *Bz-McC*, and the Southern blot detected 13 bands in the former and 11 in the latter. Interestingly, the hybridization patterns of the two W22-converted genetic lines are very similar, supporting their largely common genetic background. An ∼11-kb band present in most W22 lines, including the recently sequenced *Bz-W22* version of W22 (50) but absent from W22 *Bz-McC*, may correspond to the active autonomous *Magellan* element, putatively assigned to chromosome 5 by the NGS analysis. Not surprisingly, *Magellan* insertions have been found in at least two other mutations arisen in W22 lines: *pl1-987* (GenBank AF015269.1) and *bz-s39.71* (51).

The number of new insertions in the active lines was more variable (Table 6). Among the 1,000 progeny from W22 *Bz-McC* males, we found 18 new insertions of *Hopscotch*, that is, 1.8% of the progeny have a new *Hopscotch* transposition event. Among the 1,000 progeny from W22 *Bz-B73* males, we found 300 new insertions of *Bs2* and 91 new insertions of *Magellan*, for overall transposition percentages of 30% and 9.1%, respectively. Our observed frequencies of *bz* mutations (Table 1) are in line with these numbers. The single gene and genome-wide analyses establish that *Bs2* is the most active retroelement of the three, possibly because of the larger number of putative autonomous elements in W22 *Bz-B73* (*SI Appendix*, Table S3). Significantly, the vast majority of new insertions are in nonrepetitive sequences. Of the 409 new insertions identified in this study, 370 are in unique sequences with ≥85% identity in the B73 genome and of them, 264 or 71% fall within 500 bp of gene models.

Somatic Retrotransposition. A possible explanation for the low-coverage retrotransposon junctions detected in our NGS experiments, particularly with *Bs2* and *Magellan*, is that they arise from somatic retrotransposition events in the individual seedlings. To test this possibility, we looked for novel junctions at five different developmental stages of two individual plants from cross (*iii*) B73 × W22 *Bz-B73*. The developmental stages included the 3rd, 7th, and 11th leaf, the immature tassel (∼11 cm), and the immature ear (5–6 cm). In total, 30 samples (2 individuals × 5 stages × 3 retroelements) were amplified and sequenced (*SI Appendix*, Tables S5–S7).

The same endogenous retroelement junctions detected in the 1,000-seedling pool were seen at each of the five developmental stages of the two plants for each transposon. The only exception was a *Hopscotch* insertion (endogenous 6) seen in the 1,000-seedling pool, but not in this experiment, which was probably segregating in the population. As expected from the earlier genetic and genome-wide studies, no new *Hopscotch* junctions were observed in either of the two B73 × W22 *Bz-B73* plants (*SI Appendix*, Table S5). New *Bs2* and *Magellan* junctions were observed in the immature tassel samples from both plants (*SI Appendix*, Tables S6 and S7). These probably represent independent transposition events in different pollen grains, as the immature tassels had already gone through meiosis when collected. In agreement with the earlier NGS study, more new junctions were seen with *Bs2* than with *Magellan* (23 vs. 5), evenly distributed between the two plants. Peculiarly, one new *Bs2* junction was detected in the immature ear of plant no. 2, suggesting that *Bs2* retrotransposition may occur infrequently in the female germline (*SI Appendix*, Table S6). The only evidence for a somatic retrotransposition event was a new *Magellan* junction in the 11th leaf of plant no. 2 (*SI Appendix*, Table S7). We conclude that somatic retrotransposition in maize occurs rarely, if at all.

Discussion

Spontaneous Mutations. We have shown here in large population experiments that stable mutations at the maize *bz* locus can arise spontaneously with the surprisingly high frequency of $\sim 4 \times 10^{-5}$ in the pollen, but not ear, progeny of some lines. Previous large-scale studies of spontaneous mutation in maize had been largely restricted to the female parent because of crossing convenience, so a high spontaneous mutation frequency in the pollen was unexpected. This high-mutation frequency was found to be a property of some lines, but not others. Specifically, two genetic lines, derived recently by introgressing the *Bz-McC* and *Bz-B73* haplotypes into a W22 genetic background, and the long-standing inbred 4Co63 (36) showed this property, but B73 and Mo17 Corn Belt inbreds representing the two heterotic groups commonly used in hybrid corn production (52) did not. Thus, spontaneous mutation frequency in the pollen is a polymorphic trait in maize. The high-pollen mutation frequency of the two genetic lines was not restricted to *bz*, as it could also be detected at the closely linked *sh* locus.

An analysis of the *bz* and *sh* mutations revealed that they resulted mostly from either insertions of low-copy number LTR retrotransposons or deletions (Tables 1 and 2). These are the same two main classes of mutations found in the collection of spontaneous *wx* mutations by the Wessler laboratory (13, 15, 25, 26), suggesting that those mutations also arose in microsporogenesis and that perhaps most spontaneous stable mutations in maize do. Oliver Nelson had

Table 6. Retrotransposon insertion sites identified by NGS in pollen progeny of active lines

Retrotransposon	Line*	No. new sites	No. sites with B73 hits†	No. sites in genes‡	No. sites in IGRs§	No. sites in repetitive DNA¶
Hopscotch	W22 *Bz-McC*	18	16	12	4	0
Bs2	W22 *Bz-B73*	300	270	187	73	10
Magellan	W22 *Bz-B73*	91	84	65	17	2

*Line where retrotransposon is active.
†>85% Identity.
‡±500 bp.
§Intergenic regions.
¶≥Five copies.

collected the majority of these mutations from geneticists and breeders, who would send them to him on a regular basis, but without a record of whether they had arisen in the male or the female germline. However, surprisingly few other retrotransposon-caused mutations have been described in maize, perhaps because most mutations have been isolated from the female germline. An interesting case is *adh1-S5446*, which harbors *Bs1*, the first LTR retrotransposon described in maize (34). This mutation occurred as a single kernel in the paternal progeny of a barley stripe mosaic virus-treated plant whose pollen was exposed to allyl alcohol selection. Other examples of spontaneous mutations with retrotransposon insertions are: brown midrib3-*1* (*bm3-1*) with a *B5* element of undetermined size (53); *d3-4*, with a 327-bp *Sleepy* solo-LTR (54); *pl1-987*, with a 1.2-kb *Magellan* element (GenBank AF015269); and *ig-O*, with a *Hopscotch* insertion of undetermined size (55). One common feature of all these retroelements is that they are present in a low number of copies (<10) in the maize genome. In the genetic lines used in this study, W22 *Bz-McC* and W22 *Bz-B73*, these same elements were shown to be mobile: *Hopscotch* in W22 *Bz-McC* and *Magellan* and a close relative of *Bs1* that we have dubbed *Bs2* in W22 *Bz-B73*.

A few mutations were unrelated to retrotransposon insertions or deletions. The premeiotic single base pair missense *bz* mutation detected in W22 *Bz-McC* came from a screen of over a million gametes and the single base pair nonsense *bz* mutation detected in the inbred 4Co63 came from a screen of over half a million gametes. In contrast, the gamete populations screened for B73 and Mo17 were slightly larger than 200,000, so the failure to detect similar single base pair mutations in these inbreds may be purely a frequency issue. Similarly, nonautonomous *MULE* insertions in *bz* and *sh* were detected only in W22 *Bz-B73*, most likely because only this line carries the corresponding autonomous *MULE* transposon.

LTR Retrotransposon Mobility. The most plausible interpretation for the retrotransposon mobility in these lines is that only the active line carries the autonomous element encoding the retrotransposition functions required for movement of elements in the same family (56). Thus, W22 *Bz-McC* should carry at least one autonomous *Hopscotch* element, but the B73 inbred and W22 *Bz-B73* should not. Similarly, W22 *Bz-B73* should carry at least one autonomous element of both *Magellan* and *Bs2*, but B73 and W22 *Bz-McC* should not. Our genome-wide survey of endogenous insertion sites of these elements (*SI Appendix*, Tables S2–S4) supports this. Unique retrotransposon–host junctions were detected for *Hopscotch* in chromosome 2 of W22 *Bz-McC* (*SI Appendix*, Table S2) and for *Magellan* in chromosome 5 of W22 *Bz-B73* (*SI Appendix*, Table S4). For *Bs2*, the most active retroelement in our study, four unique retrotransposon–host junctions in different chromosomes were detected in W22 *Bz-B73* (*SI Appendix*, Table S3). These unique retrotransposons junctions, or at least one of them in the latter case, most likely correspond to the autonomous elements. It is interesting that the B73 and Mo17 inbreds widely used by breeders lack retrotransposon activity. One is tempted to speculate that, in addition to their desirable agronomic properties, these inbreds might have been selected for their genetic stability and against the low-level spontaneous variation reported in some long-time inbred lines (57, 58).

The analysis of new retrotransposon junctions in the male progeny of W22 *Bz-McC* and W22 *Bz-B73* (*SI Appendix*, Tables S2–S4) allowed us to conclude that high retrotransposition in pollen was a genome wide phenomenon, and not just restricted to the loci that had been assayed genetically. Among the pollen progeny from W22 *Bz-McC*, 1.8% carried a new *Hopscotch* transposition event and among those from W22 *Bz-B73*, 30% carried a new *Bs2* and 9.1% a new *Magellan* transposition event, respectively. Most new retrotranspositions are in nonrepetitive sequences: 90% are in unique sequences and of them, 71% are within 500 bp of gene models. Clearly, like the HIV and murine leukemia virus human LTR retroviruses (59, 60)—and the stress-activated *Tos17*, *LORE1*, and *Onsen* retrotransposons in rice, *Lotus*, and *Arabidopsis*, respectively (61–63)—these low-copy maize retrotransposons integrate preferentially into genic regions.

Our genetic study with the inbred 4Co63 identified LTR retrotransposons not previously described in the B73 genome (37). This is not surprising and, most likely, countless new mobile retrotransposons will be identified in other maize genomes, as they are analyzed, just as they have been identified in *Arabidopsis* ecotypes other than the Col-0 reference (64). We have named the new mobile elements by combining parts of the inbred's singular name (Four County Sixty-three). *Foto* is a 5.3-kb, apparently intact and possibly autonomous *Gypsy* retrotransposon encoding a 1,468-amino acid polyprotein, *Focou* is a 2.9-kb RLX element encoding fragments of a gagpol and a PHD protein, so it resembles *Bs1* and *Bs2* in having captured a host gene fragment (42), and *Fonty* corresponds to a novel 228-bp sLTR.

We can only speculate as to why low-copy number maize LTR retrotransposons are mobile in the male, but not the female, germline. The simplest explanation is that the promoters in the LTRs of these retrotransposons are pollen-specific, as is the *LORE1* promoter from *Lotus japonicus* (65). Alternatively, these LTR retrotransposons may be less methylated in the male germline than in the female germline. In *Arabidopsis*, CHH methylation is lost from retrotransposons in microspores and sperm cells and restored in the embryo after fertilization (66).

Structure of Recently Mobilized Retrotransposons. The majority of the LTR elements that transposed into the *bz* locus (21 of 30) end in two LTRs in direct orientation, the standard LTR retrotransposon configuration, and are flanked by 5-bp TSDs. The two LTRs are identical in sequence, as expected of newly transposed elements (39). However, a sizable minority of new insertions had a different structure. The most common exception was a solo LTR flanked by a 5-bp TSD (three of nine: *bz-McC:N29*, *bz-4Co63:N1*, *bz-4Co63:N2*). These most likely originated by an intrachromosomal recombination event between the two retrotransposon LTRs at the time of integration or shortly thereafter. Their occurrence as new, and not historical, products indicates that sLTRs can originate at the time of transposition, rather than in subsequent generations following the integration of a complete retrotransposon (67–69).

Three insertions were accompanied by deletion of adjacent *bz* sequences. *bz-B73:N6* has an insertion of what appears to be a *Magellan* sLTR, but the insertion is not simple: its first two bases, AA, are remnants of the *Magellan* polypurine tract immediately preceding the 3′ LTR and terminal A of the LTR is missing, together with 10 bp of the *bz* first exon. Two mutants have insertions of fractured elements missing one end and its flanking sequence. *bz-4Co63:N4* has an insertion of a 2.4-kb truncated RLX-*Focou* element missing its 5′ end, including the entire 5′ LTR, adjacent to a 77-bp deletion of the *bz* first exon, including the TSD. *Focou* and *bz* share 4 bp of homology at the deletion junction, so the deletion presumably arose by microhomology-mediated recombination at or just after integration. *bz-4Co63:N14* has an insertion of a 2.2-kb truncated RLX_*Jeli* missing 1 kb from its 5′ end relative to other RLX_*Jeli* insertions (Table 2) adjacent to a 35-bp deletion of the *bz* first exon, including the TSD. *Jeli* and *bz* share 5 bp of homology at the deletion junction, so this deletion also probably arose by a microhomology-mediated recombination event around the time of integration.

Two of the most unusual mutations had insertions with inverted 3′ ends flanking 5-bp TSDs. *bz-B73:N2* had a 0.7-kb insertion consisting of the inverted 3′ ends of two *Magellan* LTRs and *bz-B73:N13* had a 0.5-kb insertion consisting of the inverted 3′ ends of two *Bs2* LTRs. For these two insertions, we were able to obtain the sequences of the junctions, but not of the entire element, and estimated their sizes from Southern blots (*SI Appendix*, Figs. S1 and S2). Not having the entire sequence of the insertions, it is difficult to envision how these complex structures arose during the retrotransposition process.

Perhaps the mutation with the most complex insertion is *bz-4Co63:N5*. It carries a compound insertion with 5′ and 3′ LTRs from two different elements. The 4.2-kb element consists of 2.8-kb from a *Focou* element at the 5′ end and 1.4-kb of a *Jeli* element at

the 3′ end. The compound insertion is flanked by the same *bz* 5-bp TSD, but ends in different LTRs: a 299-bp 5′ LTR from *Focou* and a 224-bp 3′ LTR from *Jeli*. This hybrid element probably arose by recombination between a *Focou* and a *Jeli* retrotransposon during transposition, by a process similar to retroviral transposition where two distinct RNA templates copackaged in a single virus-like particle can lead to new combinations of sequences (70). A high level of pairwise recombination between members of an LTR retrotransposon family was also reported recently in *Arabidopsis* (71).

The simple 8-bp insertions detected in three mutants—*bz-B73:N27*, *bz-McC:N24*, and *bz-McC:N26*—can also be considered to be the products of abortive retrotransposition events. They consist of either a TCA or TAA trinucleotide flanked on either side by a 5-bp direct repeat. Possibly, they originated from the failed insertion of *Bs2* and *Hopscotch* retrotranposons, respectively, that left behind three bases from their 5′ and 3′ ends separated by a 5-bp TSD.

Timing of Retrotransposition. About one-third of all new insertions have noncanonical LTR retrotransposon structures and many of them can be explained by recombination events during transposition, suggesting that, as in fission yeast (72), retrotransposition occurs at the time of DNA replication, probably at meiosis I of the maize male germline. The observation that all but one of the 30 mutations listed in Table 2 were unique supports this inference. The one exception was mutant *bz-McC:N29*, which harbors a novel 479-bp *Macpre* sLTR insertion in exon 2. Three other *bz-McC:N* mutations had the same insertion at the same location, suggesting that premeiotic retrotransposition events that produce sectors with the same mutation can also occur during microsporogenesis. Like its maize counterparts, the *Lotus LORE1* retrotransposon also transposes preferentially in the male and does so late in development because all new insertions are unique (65). The situation in *Arabidopsis* is slightly different. Retrotranspositions of *Evadé* in epigenetic recombinant inbred lines are also unique, although they appear to arise in the female reproductive lineage (73), and the heat-induced retrotranspositions of *Onsen* in siRNA-deficient plants also occur in sporogenous tissues, but are more common premeiotically (74).

Germline vs. Somatic Retrotransposition. Our genetic and genomic data indicate that the mobile LTR retrotransposons *Hopscotch*, *Bs2*, and *Magellan* move in the male germline but not the female germline. To examine if these retrotransposons move in somatic tissues, we looked for new retroelement–host junctions for the three elements at five different developmental stages, including seedling, juvenile, and mature leaf, immature tassel, and immature ear. The only evidence for a somatic retrotransposition event was a new *Magellan* junction in the mature leaf of one of the two plants used in the experiment (*SI Appendix*, Table S7). We conclude that somatic retrotransposition in maize is a very rare event and that the LTR retrotransposons studied here move mainly in the male germline.

Deletions and Retrotranspositions: A Common Mechanism? Retrotranspositions and deletions combined were, by far, the predominant *bz* mutations that arose in the pollen of W22 *Bz-B73*, W22 *Bz-McC*, and 4Co63, although deletions outnumbered retrotranspositions in W22 *Bz-McC*. However, no mutations of either kind were recovered in either B73 or Mo17 in populations large enough to have recovered several mutants if the mutation frequency in those inbreds was as high as in the lines tested. The absence of both mutant classes in these inbreds prompts us to speculate that deletions and retrotranspositions share a common origin.

LTR retrotransposition is clearly a sloppy process, as one-third of the insertions recovered at the *bz* locus lacked the standard structure of an LTR retrotransposon and showed evidence of recombination within the retroelement or between the retroelement and adjacent *bz* sequences. Others, like the 8-bp insertions, lacked a clear retroelement vestige, but could be explained by abortive transpositions that left behind three nucleotides from the 5′ and 3′ ends of the LTRs.

The deletions ranged in size from 19 bp within *bz* to at least 3 kb outside *bz*, based on the position of the primers used in PCR amplification. In many of the smaller deletions, microhomologies of 4–7 bp could be detected at the deletion junction, suggesting an origin by microhomology-mediated repair of a DNA double-strand break (40, 41). Similar microhomologies at deletion junctions were found among spontaneous *wx* deletions (14) and newly originated *dMuDR*, *Ds*, and *dTED* elements (75–77) in maize. We propose that these deletions arise from the cell's DNA repair machinery in the process of repairing the break created by the retrotransposon endonuclease as it attempts to integrate the replicated retrotransposon into the chromosome. Larger deletions could result from two endonuclease-induced double-strand breaks several kilobases away that get repaired by fusion of the broken chromosome ends and deletion of the intervening fragment. The 320-bp complex rearrangement with three new junctions seen in *bz-McC:N6* could have arisen, similarly, by multiple breaks. Double breaks induced by a retrotransposon endonuclease many kilobases apart were first proposed to explain the large deletions and inversions associated with integration of the L1 retroelement in humans (78, 79). The lower ratio of retrotranspositions to deletions seen in W22 *Bz-McC* could be explained by invoking a lower integration efficiency for *Hopscotch*, the mobile retrotransposon in that line. A possible basis for this lower efficiency could be that *Hopscotch* ends in a noncanonical TA/CA dinucleotide instead of the "obligatory" TG/CA inverted dinucleotide seen at the 5′ and 3′ ends of most LTR retrotransposons (80) and might form a less-stable preintegration complex.

Conclusions

From a study of spontaneous mutation in maize, we have serendipitously caught maize LTR retrotransposons moving "in real time" (81), rather than on an evolutionary time scale. These mobile retrotransposons are low copy number, reside in nonrepetitive DNA, and move at a relatively high frequency in the pollen of certain maize lines. Unlike previous reported cases of mobile plant retrotransposons, they are not stimulated to move by any stress or known epigenetic factors. Rather, in analogy to the well-characterized DNA transposons, they appear to move in lines that most probably carry an autonomous element expressing the proper retrotransposition functions. A prediction of this work is that mobilization of potentially mobile low-copy nonautonomous retrotransposons residing in nonrepetitive—and most likely hypomethylated—DNA could be transferred to an inactive maize line from an active one simply by crossing. This is clearly a controlled way of mobilizing retrotransposons, an attractive and largely untapped source of genetic variation (81). It remains to be seen whether similar retrotransposon mobility polymorphisms will be found in other crops.

Materials and Methods

Because of their importance to this study, the genetic lines used are described in *Results*. Detailed description of other plant materials and molecular methods can be found in *SI Appendix*. Included in the latter are the molecular characterization of mutations and the high-throughput sequencing experiments to identify and map retroelement insertion sites. Sequences reported in this paper have been deposited in GenBank with accession nos. MK532510–MK532536 (82).

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