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Origin of spontaneous mutations in maize has been hiding in plain sight

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Spontaneous mutations are the raw material of evolutionary change. Given their importance, it is surprising that so little is known about their origin, frequency, or molecular structure. These questions have weighed on me since my laboratory published a series of papers, with the first appearing in PNAS in 1985, on the structure of spontaneous mutations at the maize *waxy* gene (1). This and subsequent studies revealed the predominance of two classes of mutations: long-terminal repeat (LTR) retrotransposons (2) and complex deletions (3). Members of the same LTR retrotransposon families found among *waxy* mutants were also found as causative agents of spontaneous mutation at other maize loci. In all cases, the insertions contained members of LTR retrotransposon families, with fewer than 10 copies genome-wide. In contrast, the availability of an increasing amount of maize genome sequence revealed that 75% was derived from LTR retrotransposons, largely families with thousands, even tens of thousands, of copies (4). Despite comprising the vast majority of the maize genome, LTR retrotransposon activity—that is, the movement of elements in real time—had not been convincingly demonstrated in the 35 y since their discovery in spontaneous mutants. The paper in PNAS by Dooner et al. (5) reports that the mechanism to activate LTR retrotransposons in maize has been hiding in plain sight in the fields of geneticists and breeders. The authors couple their mastery of maize genetic resources with modern genomic and computational analyses to produce a large collection of spontaneous mutations, both at targeted genetic loci and throughout the genome. They demonstrate that low-copy retrotransposons are likely responsible for virtually all observed spontaneous mutations, including the unusual deletions. Furthermore, they determine that genetic backgrounds differ in the spectrum of activated retrotransposon families, with some elements moving in only one background and no elements moving in others. Most important is the finding that mutations only occur during pollen development; no mutations could be isolated through the female lineage of any tested line. This latter finding provides a convincing rationale for the failure of prior studies to detect spontaneous mutations, as virtually all followed the pioneering crossing strategy of Stadler (6) who used female rows with dominant markers open-pollinated with plants containing several recessive markers.

Dooner et al. (5) begin with a simple question: What is the frequency of spontaneous mutation at the *Bz* locus? To this end, they set up reciprocal crosses of two *Bz* stocks with *bz* testers where rare spontaneous mutations could be easily identified as bronze kernels in a purple-kernel background. For each of the four crosses, they screened at least 400,000 kernels (~1,000 ears). To identify the molecular lesions, putative mutant *bz* kernels were planted and DNA was isolated from leaf tissue for PCR amplification with *Bz* primers. This straightforward experimental design produced several surprising results. First, *bz* mutants were only detected when the dominant *Bz* alleles were in the male parent. Second, for the two structurally distinct dominant alleles tested (*Bz-B73* and *Bz-McC*), the frequency of mutation was unexpectedly high (4.3 and 3.6 per 100,000 gametes, respectively). This is over an order of magnitude greater than estimates by Stadler (6) of spontaneous mutation frequencies at six maize genes. Third, the majority of new mutants contained low-copy LTR retrotransposons or deletions that were reminiscent of the *waxy* mutations reported decades earlier. For example, several insertions of members of the *Magellan* and *Hopscotch* families of LTR retrotransposons were among the new *bz* and previously characterized *wx* mutants. Taken together, these results suggested that low-copy LTR retrotransposons were mobile during pollen (male), but not female gamete, development. However, a striking difference in the spectrum of LTR retrotransposons inserted in the two *Bz* alleles was noted. Insertions in *Bz-B73* included six *Magellan* and 12 *Bs2* LTR retrotransposons, while insertions in *Bz-McC* included two *Hopscotch* LTR retrotransposons and four soloLTRs (sLTRs), presumably derived by internal deletion, from previously undescribed LTR retrotransposons.

Strain-Specific LTR Retrotransposon Activity

To understand the origin of the allele-specific differences and to determine whether mobility of LTR

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retrotransposons during pollen development occurs in other strains, Dooner et al. (5) designed additional genetic crosses using, as the male parent, three Corn Belt inbreds: B73, Mo17, and 4Co63. The authors did not detect any *bz* mutations among the ~200,000 kernels when B73 or Mo17 served as the male parent. Notably, the B73 inbred harbors the same *Bz-B73* allele that was used in their original crosses. However, for those crosses, the *Bz-B73* allele had been backcrossed into the W22 inbred background, suggesting that it was the genetic background and not the dominant allele that determines the spectrum of mutations. Additional support for this hypothesis came from the analysis of the cross results using the third inbred, 4Co63, as male parent. Not only did the cross result in a high frequency of *bz* mutations (2.6×10^{-5}), including deletions and insertions of low-copy LTR retrotransposons and sLTRs, but none of the nine insertions in the *Bz-4Co63* allele had been found previously among maize mutants.

Common Origin of Insertions and Deletions

Comparative analysis of the lesions from the entire collection of spontaneous *bz* mutations provided clues to the underlying mechanism. Taken together, 30 of the 70 spontaneous *bz* mutations harbored intact LTR retrotransposons with identical 5′ and 3′ LTRs, which is a hallmark of new insertions, likely occurring during pollen development. But what of the origin of the remaining mutations, a majority of which were deletions? The authors make a compelling argument that a common mechanism, namely retrotransposition, underlies deletions and insertions because they occur either together in one genetic background (e.g., W22, 4Co63) or not at all in others (B73, Mo17). Strengthening their argument is the unusual structure of many of the smaller deletions, with most containing extra DNA at the deletion junctions. They hypothesize that these structures are the result of a previously described cellular mechanism that uses microhomologies to repair double-strand breaks (7) generated, in this case, by retrotransposon endonucleases in failed attempts to integrate element copies into the chromosome. Furthermore, they propose that the origins of other unusual insertions, including several sLTRs, elements with two 3′ LTRs, or even an insertion with two halves from different LTR retrotransposons, reflect other examples of "sloppy" retrotransposition.

Active Strains Have Autonomous Family Members

Detailed analysis of the 30 intact LTR retrotransposons led to a testable model that could account for the strain-specific activity of these mutagenic element families. Of the 30 elements, many were clearly nonautonomous, as they did not encode all of the protein functions necessary for retrotransposition. The authors speculate that a line that generates, for example, a nonautonomous *Magellan* insertion into the *Bz* gene, must have an autonomous *Magellan* element elsewhere in the genome. From that, it follows that inactive lines only contain nonautonomous, immobile family members. This model is appealing because it provides an explanation for why only low-copy-number LTR retrotransposons are activated. Unlike high-copy elements, host-silencing machinery has not yet recognized low-copy elements and, for small families, it is reasonable that all members be nonautonomous. Further, the model also accounts for the inactivity of B73 and Mo17, two inbreds that are favorites of maize breeders and geneticists. For these strains, it is hypothesized that selection for phenotypically uniform progeny has gradually led to the inactivation of autonomous family members, perhaps through silencing or removal by segregation.

To test their model, the authors devised an audacious genome-wide approach to locate both endogenous and new insertions of *Hopscotch*, *Magellan*, and *Bs2*. This was accomplished by first performing reciprocal crosses of each of the two lines that had generated new *Bz* insertions of either *Hopscotch* (W22 *Bz-McC*) or *Magellan* and *Bs2* (W22 *Bz-B73*) with the B73 inbred. For each of the four crosses, 1,000 F1 progeny were pooled, and regions containing the desired retrotransposon insertion junctions were captured by a targeted PCR strategy and sequenced with sufficient depth to identify junctions from both the shared endogenous family members and rare new insertions in individual F1 progeny. As expected, new insertions were only recovered in progeny from the two crosses where strains W22 *Bz-McC* and W22 *Bz-B73* served as the male parent. For the W22 *Bz-McC* cross, recovery of 18 new *Hopscotch* insertions meant that 1.8% of the progeny contained a new insertion. No new insertions of *Magellan* or *Bs2* elements were detected. Recovery of new insertions from the W22 *Bz-B73* cross was far more dramatic, with 91 *Magellan* and 300 *Bs2* insertions representing new insertions in 9.1% and 30% of the progeny, respectively. No *Hopscotch* insertions were recovered. In a separate experiment, the authors ruled out somatic retrotransposition as the source of the new insertions.

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In contrast, insertion junctions from endogenous family members were orders of magnitude more numerous, with approximately five to seven loci identified per line. Many of these loci were validated by their presence in previously sequenced maize genomes. The authors used a combination of DNA sequence and DNA (Southern) blot analyses to identify the putative autonomous *Hopscotch* element uniquely found in the W22 *Bz-McC* line and the putative autonomous members of the *Magellan* and *Bs2* families in the W22 *Bz-B73* line. Consistent with the finding that 30% of F1 progeny contained new *Bs2* insertions was the tentative identification of not one, but four, potentially autonomous *Bs2* elements in this line. However, as the authors note, the identification of autonomous elements is, at this stage, only correlative. For their model to be ready for inclusion in genetics textbooks (which speaks to its potential importance), it will require the unambiguous identification of the autonomous LTR retrotransposons hypothesized to drive the generation of high-frequency spontaneous mutations exclusively in male gametes. Fortunately, experimental protocols necessary to determine if these elements exist are clearly spelled out by the elegant genetic, genomic, and computational strategies employed by Dooner et al. (5).

Irrespective of whether all the details of their model prove to be correct, the authors have discovered a mechanism for the generation of high-frequency spontaneous mutations in maize, and perhaps in other crops. Further, the methodology used to identify active LTR retrotransposons in select maize lines should be easily replicated in any other line. In this regard, the discovery of nine previously unidentified insertions in the 4Co63 background speaks to the treasure trove of potentially active LTR retrotransposon families awaiting discovery.

On a personal note, the mechanistic connection proposed by the authors between deletions and LTR retrotransposons was particularly gratifying. The PNAS paper from my laboratory reporting that spontaneous *waxy* mutations contain structurally

unusual deletions was communicated by Barbara McClintock (2). In several conversations, we tried but ultimately could not come up with a satisfactory explanation for these disparate outcomes. I suspect that, like myself, McClintock would have been satisfied by the ingenuity and hard work that went into solving this cold case as reported by Dooner et al. (5).

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