Molecular Analysis of Multiple Mutator-Derived Alleles of the Bronze Locus of Maize

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ABSTRACT

Very few mutations derived from Mutator maize lines have been studied at the molecular level. The variety of Mu elements that can induce mutations, the relative frequency of mutant induction by insertion of a given class of Mu elements or by a Mu-induced genomic rearrangement, a possible intragenic insertion site specificity, and the molecular nature of reversion events are all unknown in the Mutator system. To address these questions, we have isolated several partially or fully inactivated bronze alleles from Mutator maize lines, and structurally characterized them by gel blot hybridization of genomic DNA. The mutations were induced in three parental Bronze alleles which differ by polymorphisms flanking the coding region. Each of the 14 inactivated bronze mutants characterized was found to contain an insert which cross-hybridized with the transposable element Mu1. Detailed maps of 11 of these alleles revealed a 1.4-kb insert with restriction sites characteristic of Mu1. These Mu1 insertions were found dispersed throughout both of the Bronze exons and in either orientation relative to Bronze transcription. Stable and somatically unstable (mutable) mutant alleles differed with respect to the covalent modification of restriction sites within the inserted Mu1 element. Several germinally revertedants of one mutable bronze allele, bzMum-4, were isolated. These all were associated with excision of the Mu1 element from the affected locus.

A uniquely high frequency and low specificity of de novo mutant induction is the definitional characteristic of the Mutator transposable element system (Robertson 1978, 1985; Robertson and Mascia 1981; Bennetzen et al. 1987a). The numerous mutations isolated from Mutator stocks, particularly those alleles which show phenotypic reversion events in somatic sectors (i.e., mutability), have proven useful for studies of Mutator regulation (Robertson 1981; Bennetzen 1985; Walbot, Chandler and Taylor 1985; Chandler and Walbot 1986; Walbot 1986; Bennetzen, Brown and Springer 1988). Mutator-associated mutations have been isolated in genes which determine such phenomena as disease resistance, seed dormancy, embryo development, seed starch and/or protein deposition, and the synthesis of anthocyanin, carotenoid or chlorophyll pigments (Robertson 1978, 1981, 1985; Bennetzen 1985; Walbot, Chandler and Taylor 1985; Walbot, Briggs and Chandler 1986; Bennetzen, Brown and Springer 1988; Bennetzen et al. 1988). In order to understand these important physiological processes, several laboratories have set out to molecularly clone Mutator-derived alleles by the process of transposon tagging, with some initial success (O’Reilly et al. 1985; McLaughlin and Walbot 1987; McCarty and Carson, 1988). Implicit in these tagging experiments is the assumption that the transposable element most closely associated with Mutator activity, Mu1 (Bennetzen 1984; Bennetzen et al. 1984, 1987b; Alleman and Freeling 1986), will be inserted at the mutated locus. This general expectation has been borne out by the observation that Mu1, or a 350 bp larger relative of Mu1 called Mu1.7 (Taylor and Walbot 1987), has been found inserted in each of seven independent mutations at A1, Adhl, Bronze, and Bronze2 (Strommer et al. 1982; Bennetzen et al. 1984; O’Reilly et al. 1985; Taylor, Chandler and Walbot 1986; McLaughlin and Walbot 1987). However, all maize lines contain numerous sequences homologous only to the ends of Mu1 (Chandler, Rivin and Walbot 1986). Two of these elements, Mu3 and Mu8, have been cloned as insertions in Mutator-derived Adhl (Oishi and Freeling 1988) and Waxy alleles (Varagona, Fleenor and Wessler 1988), respectively. These results demonstrate that other classes of elements can be involved in mutant induction in a Mutator background.

Insertion site specificity is the rule, rather than the exception, for all transposable element systems investigated (Calos and Miller 1980; Greenblatt 1984; Mori et al. 1988). Mutator is unusual in its ability to induce mutations at all loci investigated, and at

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roughly similar frequencies (ROBERTSON 1985). The high mutagenic rate compared to the low copy number (10–70) of Mu1 transposable elements in Mutator lines led to the prediction that Mu elements would have either a high transposition frequency, a gene specific insertion preference, or both (BENNETZEN 1984). The latter prediction has been substantiated by subsequent studies (ALLEMAN and FREELING 1986; BENNETZEN et al. 1987b; BENNETZEN, BROWN and SPRINGER 1988). The initial observation that three independent Mu1 insertions of Adh1 were all in the same intron (STROMMER et al. 1982; BENNETZEN et al. 1984), and that all seven independent Mu1 or Mu1.7 insertions in the A1, Adh1, Bronze or Bronze2 loci have been in the same orientation relative to transcription of the mutated allele (BENNETZEN et al. 1984; O’REILLY et al. 1985; TAYLOR, CHANDLER and WALBOT 1986; MCLAUGHLIN and WALBOT 1987), suggested that Mu1 may exhibit some microspecificity for insertion within a given gene.

This report describes the results of gel blot hybridization analysis of 14 independent bronze mutations isolated from Mutator maize lines. We determine the nature, position and orientation of the element inserted in Bronze in 11 of these mutant alleles. In addition, we present gel blot hybridization data which indicate that germinal reversion of one Mutator-derived bronze mutation, bzMum4, is associated with excision of the Mu1 element.

MATERIALS AND METHODS

Isolation of bronze mutations from Mutator maize lines: The bronze mutations bzMum1-bzMum8, bzMus1-bzMus3, and bzMus5-bzMus10 were isolated in a maize plot containing detasseled plants of the genotype c sh bz wx/e sh bz wx and purple Mutator plants of the genotype C Sh Bz Wx/C Sh Bz Wx. The 334,392 kernels from the detasseled plants were screened for purple or mutable bronze kernels. Mutants bzMum9-bzMum11 were identified on ears derived from self-pollinated progeny of the detasseled plants. Twelve of these mutant stocks segregated a 3:1 ratio of purple to bronze or bronze mutable kernels.

Isolation of germinal phenotypic revertants of bzMum4: Plants hemizygous for bronze were constructed by crossing to a bronze-shrunken deletion line (BENNETZEN 1985). Hemizygous bzMum4 stocks were self-pollinated or crossed to siblings or the bronze-shrunken deletion line. To minimize the possibility of contaminating pollen, hand pollinations were performed in a plot isolated both spatially and temporally from any other maize lines. Plants derived from putative revertant (i.e., purple) kernels were self-pollinated or outcrossed to the bronze-shrunken deletion line to confirm the revertant phenotype.

Hybridization experiments: Maize genomic DNA was prepared according to the method of SHURE, WESSLER and FEDOROFF (1983). Restriction enzymes were purchased from Bethesda Research Labs, New England Biolabs or Boehringer Mannheim Biochemicals and used in a two-fold excess under conditions specified by the manufacturer. Genomic DNA (6–8 µg) was digested with the appropriate restriction enzyme(s) and resolved on 0.7% agarose gels. HindIII digested lambda DNA was used as a molecular weight marker. The DNA was transferred to a nitrocellulose or nylon (Micron Separations, Inc.) filter which was hybridized and washed as described previously (BENNETZEN 1984). Radiolabeled DNA was prepared using the hexamer labeling procedure (FEINBERG and VOGELSTEIN 1983). Unincorporated nucleotides were removed by Sephadex G-75 gel filtration chromatography. Plasmids pMBZP1 and pMBZPR5, containing the cloned Bronze gene (FEDOROFF, FURTEK and NELSON 1984), were provided by DRS. D. FURTEK and O. NELSON. Probe Pi is an 842-bp PstI fragment and probe PR5 is a 1.8-kb PstI/EcoRI fragment of the cloned Bronze allele (FURTEK 1986; see Figure 3). The purified 1.0-kb TdiI111 fragment of Mu1 was used as a probe to detect genomic Mu1 elements (BARKER et al. 1984; BENNETZEN 1984).

RESULTS

Isolation and mapping of Mutator-associated bronze mutations: Seventeen bronze mutations were isolated in a screen of 334,392 maize kernels. Three additional bronze mutant alleles (bzMum9, bzMus10, bzMum11) were obtained from self-pollinated ears of different outcross progeny in a purple Mutator background. Eleven of these mutant alleles were mutable, exhibiting the small revertant sectors characteristic of Mutator-induced mutations (ROBERTSON 1981; WALBOT, BRIGGS and CHANDLER 1986). These were termed “Mum” alleles. One of these mutants, bzMum7, yielded pale purple kernels with dark purple spots. Nine of the bronze inactivations isolated were somatically stable (no revertant sectors) and were termed “Mus” alleles. Mutations bzMus1-bzMus3, bzMus5-bzMus7 and bzMus9-bzMus10 could be activated to a low frequency of mutability by crossing to an active Mutator stock, while bzMus8 was not amenable to such a trans-activation. We decided to prepare detailed restriction maps of bzMum1-bzMum11, bzMus1, bzMus2 and bzMus8. Each of these mutants and their parental Bronze alleles were mapped by genomic gel blot hybridizations using the 1.8-kb PstI/EcoRI fragment of Bronze (PR5) as a probe for the 5' end of the Bronze locus and the 842-bp PstI fragment of Bronze (P1) as a probe for the 3' end of the Bronze locus (FURTEK 1986; RALSTON, ENGLISH and DOONER 1988). The results indicated that 11 of the mutants were induced in two different parental Bronze alleles, each of which is distinct from the first Bronze alleles to be cloned and sequenced (FEDOROFF, FURTEK and NELSON 1984; FURTEK 1986; RALSTON, ENGLISH and DOONER 1988). Almost all restriction sites within the transcribed region of Bronze were conserved. Despite extensive mapping of all the Bronze alleles from the different inbreds known to have contributed to the stocks from which these mutant alleles were selected, we could not identify a progenitor allele for bzMum6, bzMus8 and bzMum10. Mapping of these alleles indicated, in each case, an insertion at the Bronze locus which cross-hybridized with the central TdiI111 frag-
We have isolated bronze mutations from a Mutator stock at a rate of about 1/20,000. This frequency, and the fact that just under half of these mutant alleles were somatically unstable, is in agreement with the standard mutagenic properties of an active Mutator stock (Robertson 1978). Since all but one of the nine stable bronze alleles identified in this screen could be induced to somatic mutability by crossing to an active Mutator stock, it appears that Mutator does not commonly cause viable deletions encompassing the Bronze locus as an initial mutagenic event.

Our molecular analyses indicate that the factor responsible for most mutations in Mutator plants is Mut or a Mut-like element. This is important for gene tagging experiments since the transposon is required for the expression of the bronze phenotype.

**DISCUSSION**

We have isolated bronze mutations from a Mutator stock at a rate of about 1/20,000. This frequency, and the fact that just under half of these mutant alleles were somatically unstable, is in agreement with the standard mutagenic properties of an active Mutator stock (Robertson 1978). Since all but one of the nine stable bronze alleles identified in this screen could be induced to somatic mutability by crossing to an active Mutator stock, it appears that Mutator does not commonly cause viable deletions encompassing the Bronze locus as an initial mutagenic event.

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as a hybridization probe for the isolation of a tagged gene. *Mu1* has been employed successfully as a tag in the molecular cloning of the maize genes *AI* (O'Reilly et al. 1985), *Bz2* (McLaughlin and Walbot 1987) and *Vp1* (McCarty and Carson 1988). In our analyses, no *Mu1.7* element insertions were observed at *Bronze*, although a *Mu1.7* element has been found inserted in one *bronze* allele from another *Mu-

**Figure 2.**—Restriction maps of *Bronze* alleles and *Mu1* insertions. Abbreviations are: Bg, BglII; Bs, BstEII; H, HindIII; K, KpnI; Nc, Ncol; Nt, NotI; E, EcoRI; S, SsiI; X, XbaI. The relative positions of the probes used (P1 and PR5) and of the *Bronze* mRNA (determined for the *Bronze* gene isolated by Fedoroff and coworkers [1984]) are indicated. The position and extent of the *Bronze* intron is represented by an open box.
stocks from which we derived these *brons*e alleles (data not shown). Other classes of *Mu* elements, with termini like those of *Mu1* and *Mu1.7* but with nonhomologous internal sequences, are found at combined copy numbers of over forty per diploid genome in all maize lines examined (Chandler et al. 1988; Chandler, Rivin and Walbot 1986; J. L. Benetzen, unpublished observation), but were not associated with any of the *bzMu* alleles that we analyzed. This demonstrates that these elements, abundant in both *Mutator* and standard maize stocks, transpose rarely into *Bronze* in response to the *Mutator* transposase.

Since transposition by a replicative mechanism, like that observed for *Mu1* (Alleman and Freeling 1986; Benetzen et al. 1987b), leads to an increase in genomic element copy number, this may explain the consistent amplification of *Mu1* and the rare amplification of other *Mu* elements in *Mutator* stocks. Since these classes of *Mu* elements differ primarily in their internal sequences, the preferential transposition of *Mu1* could be due to an enhanced recognition of *Mu1* termini mediated by the internal regions of the element. Alternatively, *Mu* elements other than *Mu1* or *Mu1.7* may not show the gene-specific insertion preference associated with *Mu1/Mu1.7* (Benetzen, Brown and Springer 1988) or may be trapped in a form (e.g., modified) or chromosomal location that is unavailable to transposase.

*Mu1* insertions were found in at least six distinct sites in a 2-kb region of the *Bronze* locus. This demonstrates a lack of strong *Mu1* insertion specificity and may partly explain why *Mutator* induces mutations at similar frequencies in many genes (Robertson 1985). This property may give *Mutator* an advantage over other systems for gene tagging since most transposable elements show significant insertion site preferences (Calos and Miller 1980; Greenblatt 1984; Mori et al. 1988). The pairs of mutant alleles *bzMum1/* *bzMum4*, *bzMum9/* *bzMum11*, and *bzMum3/* *bzMum8* were indistinguishable at our level of detection (less than 50 bp). This may be either an indication of some degree of intragenic *Mu1* insertion specificity or it may be interpreted as evidence that these pairs of alleles were not independently derived. The alleles *bzMum9* and *bzMum11* arose in different fields and in unrelated *Mutator* stocks and, hence, must be due to independent events. However, *bzMum1/* *bzMum4* and *bzMum3/* *bzMum8* were identified in the same windpollinated isolation plot. Since *Mutator* commonly induces mutations in the ear prior to meiosis (Robertson 1980, 1981), it seems likely that a tassel sector containing several mutant pollen derived from the same premeiotic event might also occur. Detailed sequence analysis of genomic clones of *bzMum1*, *bzMum4*, *bzMum9*, *bzMum11*, *bzMum3* and *bzMum8* will be required to absolutely determine the similarity of insertion sites in these six alleles.

All *Mu1* insertions in the *brons*e alleles analyzed were very near or within the two *Bronze* exons. *Bronze* expression at 1% of wild-type levels yields a kernel that is fully purple in color (Schiefelbein 1987). Hence, many *Mu1* insertions that partially inactivated *Bronze* would have gone undetected in our mutant screen. Insertions of *Mu1* in the first intron of *Adh1*, for instance, led to about 40% of the normal level of *Adh1* expression (Strommer et al. 1982; Benetzen et al. 1984). Mutation *bzMum7* is our only partial inactivation allele of *Bronze* and also contains our most 5′ *Mu1* insertion. This insertion may be in the promoter or transcript leader. Most of the other insertions, which are clearly or probably in exons, yield a null, somatically mutable phenotype. This indicates that, in these alleles, *Mu1* does not serve as an intron (Kim et al. 1987; Wessler, Baran and Varagona 1987) to allow efficient reconstruction of a functional *Bronze* mRNA. These data also demonstrate that the presumed somatic excision of *Mu1* from several different coding regions of *Bronze* can yield a somewhat functional protein. The two stable *bzMus* alleles analyzed in detail were both found to have internal modification of sites within *Mu1*. The lack of somatic reversion in these stable alleles, then, is probably an outcome of an inability to excise *Mu1* rather than a low likelihood that such an excision would yield a functional *Bronze* allele.

The first six *Mutator* -derived mutations analyzed were found to contain *Mu1* or *Mu1.7* insertions in the same orientation relative to transcription of the affected locus (Benetzen et al. 1984; O'Reilly et al. 1985; Taylor, Chandler and Walbot 1986; McLaughlin and Walbot 1987). Of the eleven *Mutator*-derived *brons*e mutants we have studied in detail, six contain *Mu1* insertions in the same orientation as previously described in the six cases analyzed at *Al*, *Adh1*, *Bronze*, and *Bronze2*. The other five *brons*e alleles in our study, however, contained a *Mu1* insertion in the opposite orientation. Hence, although *Mu1* may have an orientation bias for insertion into some maize genes, this bias is not absolute.

We have isolated several germinal revertants of *bzMum4* at a frequency of about 10⁻⁴. One of these revertants was found as a three-kernel ear sector and, hence, is unlikely to be due to a pollen contamination. This three-kernel-sector revertant was correlated with excision of the *Mu1* element from *bronze*. This indicates that a *Mu1* element can excise from DNA at a reasonable frequency and that these events can occur premeiotically, as do many *Mutator*- induced insertion events (Robertson 1980). Since *Mu1* appears to transpose primarily via a replicative, nonexcisive mechanism, it is not clear whether an excised *Mu1*
element would be lost or would reinsert into chromosomal DNA. An excised Mu1 might be one source of the circular Mu1-related elements detected by Sundaresan and Freeling (1987) in the developing tassel of active Mutator stocks.

Mu1 insertion results in a 9-bp duplication of host DNA (Benetzen et al. 1984). Exact excision of the transposable element would maintain the reading frame if insertion occurred in an exon and so may not have a deleterious effect on protein function. In addition, 1% of the normal level of Bronze enzymatic activity yields a wild-type phenotype (Schieffelin 1987). Thus, an in-frame excision of Mu1 could lead routinely to phenotypic reversion at Bronze. Current work is underway to sequence the appropriate regions of b2Mum4 revertants in order to determine whether an insertion "legacy" has been left behind by the departed element (Sachs et al. 1983). In this regard, the b2Mum4 "revertant" identified as a three kernel sector does produce a kernel with a pale purple phenotype.

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LITERATURE CITED


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