Epigenetic silencing and unstable inheritance of MuDR activity monitored at four bz2-mu alleles in maize (Zea mays L.)

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Maize MuDR/Mu elements are one of the most active Class II transposons and are widely used for transposon tagging for gene cloning. The autonomous MuDR encodes a transposase, while diverse non-autonomous elements share similarity to MuDR only within their ~215 bp terminal inverted repeats (TIRs). Four independent Mu-induced mutable alleles of the anthocyanin pigment pathway Bronze2 (Bz2) locus have been sequenced; bz2-mu1, bz2-mu2, and bz2-mu3 contain Mu1 element insertions while bz2-mu4 contains a MuDR insertion. Somatic excision activity can be monitored for each allele as a purple spotted phenotype on the otherwise beige epidermal layer of the kernel. To study epigenetic silencing of Mu elements, we investigated inheritance of somatic transposition of these four reporter alleles, and using samples from leaves just below the ear and the tassel, DNA methylation status and mudrA expression were quantified through three consecutive generations. Percentages of spotted kernels when crossed into Bz2 tester as pollen parent were lower than those when crossed with Bz2 tester as ear parent in all bz2-mu alleles. The propensity for silencing in kernels with different frequencies of spotting was investigated. In the inactive lines, both MuDR and Mu1 elements were de novo methylated. The transposition frequency was negatively correlated with the level of Mu methylation and positively correlated with the level of mudrA transcript. The most reliable indicator of incipient silencing was a decrease in mudrA transcript levels in the leaf below the tassel, and this transcriptional silencing could precede methylation of Mu elements.

Key words: bronze2, methylation, Mutator transposable element, silencing, Zea mays L.

INTRODUCTION

The Mutator (MuDR/Mu) family of maize is one of the most active DNA transposable elements in any genome (Robertson, 1978). Its ability to increase the spontaneous mutation rate up to 100-fold led applications for efficient gene tagging and isolation in maize (Chandler and Hardeman, 1992; Walbot, 1992). RescueMu, a genetically engineered Mu1 element containing a bacterial plasmid, further accelerates gene cloning and functional genomics in maize (Raizada and Walbot, 2001). The Mu family consists of the autonomous MuDR element and various non-autonomous elements and is grouped into at least 6 subclasses, Mu1/Mu2, Mu3, Mu4, Mu5/MuDR, Mu6/Mu7 and Mu8 (reviewed by Walbot and Rudenko, 2002). MuDR contains two protein-coding genes: mudrA encodes MURA transposase and mudrB encodes MURB helper proteins functioning in the Mu insertional activity (Lisch et al., 1999; Raizada and Walbot, 2000). All Mu elements share ~215-bp terminal inverted repeat (TIR) sequences, and the mobile Mu elements contain a highly conserved 32-bp MURA transposase binding site within both TIRs (Benito and Walbot, 1997). Only a few maize lines contain active MuDR elements, but all maize lines examined carry homologous MuDR sequences (hMuDR elements; Rudenko and Walbot, 2001). Although hMuDR homologs can be expressed, none of the hMuDR homolog-encoded proteins can substitute for the functional proteins encoded by MuDR (Rudenko and Walbot, 2001).

Two distinctive aspects of Mutator are that activity is strictly regulated in plant development and there is a high frequency of element silencing. Both somatic and
germinal transposition events occur during the last few cell divisions of tissue development (Levy and Walbot, 1990; Walbot and Rudenko, 2002). As expected the copy number of MuDR correlates with amount of mudrA and mudrB transcripts (Hershberger et al., 1991), but does not affect either the frequency or the timing of somatic excision (Lisch et al., 1999; Walbot, 1991). Second, Mutator activity is heritable, but unstable. When multicopy MuDR Mu-active plants are crossed to non-Mutator plants, some of the progeny lack Mutator activity (Robertson, 1978). Loss of activity is also observed during intercrossing among Mu-active plants and self-pollination of specific Mu-active plants (Robertson, 1983; Walbot, 1986; Brown and Sundaresan, 1992) and can occur in lines with just one Mutator element (Walbot and Rudenko, 2002). Such non-Mendelian inheritance indicates that epigenetic events cause activity loss, and early studies established that this epigenetic switch is often associated with increased methylation in Mu TIRs (Chandler and Walbot, 1986; Bennetzen, 1987; Brown and Sundaresan, 1992; Martienssen and Baron, 1994).

The epigenetic silencing of Mutator includes both transcriptional inactivation of MuDR genes and methylation of the TIRs in MuDR and non-autonomous Mu elements at dispersed locations throughout the genome. Initiation of Mutator silencing seems to correlate with a developmentally progressive accumulation of transposon-encoded 21–26 nucleotide small RNAs in nuclei (Rudenko et al., 2000). One rearranged MuDR element, Mu killer (Muk) resulted from an inverted duplication of a partially deleted MuDR (Slotkin et al., 2005). Muk can heritably silence highly active MuDR (Slotkin et al., 2003), and small RNAs are processed through a hybrid hairpin transcript from Muk (Slotkin et al., 2005). The precise role(s) of the small RNAs apparently derived from MuDR or MuK in Mutator silencing is still not clear; that is, it has not been established whether these small RNAs serve to silence transcription, to guide methylation of Mu elements, and/or to guide destruction of MuDR transcripts. Inactivated elements can be reactivated in lines homozygous for a mutation in the modifier of paramutation 1 (map1) gene; map1 reverses the effects of previous methylation of the Mu elements (Lisch et al., 2002) and shows differential effect on reactivation of the silenced methylation and mudrB (Woodhouse et al., 2006). The map1 gene encodes an RNA-dependent RNA polymerase required for paramutation in maize (Alleman et al., 2006).

Although there are several reports analyzing Mu element silencing, this subject has not been addressed on a large scale with full knowledge of the gene functions in MuDR. Given the current interest in RNA-mediated silencing and its potential role in controlling endogeneous transposons, we have re-investigated the kinetics and patterns of MuDR/Mu silencing with four reporter alleles assayed over three consecutive generations in large populations. Four independent mutable alleles of bz2 (bz2-mu1 to bz2-mu4) have been sequenced (McLaughlin and Walbot, 1987; Nash et al., 1990; Hershberger et al., 1991). Bz2 acts late in the anthocyanin biosynthetic pathway; loss of function disrupts vacuolar sequestration of purple anthocyanin and results in cytoplasmic accumulation of a bronze-colored pigment (Nash et al., 1989). Somatic reversion can be readily assessed by scoring purple spotting on the otherwise bronze aleurone of kernels (Walbot, 1986). In addition to somatic excision, the inheritance of Mu element methylation status and mudrA transcripts were quantified in the same populations to determine the correlation among the three measures of Mutator activity and the quality of each assay to predict full silencing of MuDR/Mu elements in the following generation.

**MATERIALS AND METHODS**

**Plant materials** The nomenclature used for the mutant alleles (bz2-mu1, bz2-mu2, bz2-mu3, and bz2-mu4) indicates the mutant allele (bz2), type of insertion (MuDR/ Mu family), and the numerical order in which the mutant was isolated. The 1.4 kb Mu1 insertions in bz2-mu2 and bz2-mu3 occurred in the first exon of the gene, whereas the Mu1 element in bz2-mu1 and 4.9 kb MuDR element in bz2-mu4 were identified in the second exon (Nash et al., 1990). The Mutator stocks have been maintained by selfing and by outcrossing to a non-Mutator bz2 tester line in an inbred W23 background with constant selection for full activity. All the Mutator stocks used in this study had the W23 genetic background. Somatic variegation in kernels resulting from Mu1 and MuDR excision was used to score Mutator activity after crossing to and by the bz2 tester.

**Crossing and estimation of Mutator activity** Heterozygous individuals (bz2-mu1//bz2) were grown from heavily spotted kernels in summer 2002 at Stanford University and crossed to and by the bz2 tester, with the expectation of 50% spotted kernels in the progeny. With 100 kernels per ear, the 1:1 expectation was met with genetic ratios of 40:60 with 0.05 probability of error in classification; generally ears had 250–450 kernels, and hence ears with fewer than 45% spotted kernels was considered to show incipient loss. Second generation families were generated by selecting either heavily (thousands of revertant sectors per kernel) or weakly spotted kernels (< 100 sectors per kernel), and the plants were crossed to and by the bz2 tester line in the 2003 winter nursery in Molokai Hawaii; similarly, the third generation sampled strongly or weakly spotted kernels for a final generation of outcrossing in the summer 2003 nursery at Stanford University.
Inheritance of maize MuDR/Mu transposon activity

DNA blot hybridization analysis of methylation status  Total DNA was isolated from one leaf just below the ear and from the flag leaf just below the tassel and digested with restriction enzymes. SstI is a methylation-sensitive enzyme that shares the same 6-bp recognition site as SacI, a methylation-resistant enzyme. The SacI-SstI-recognition site is located within the TIR of MuDR (Greene et al., 1994; Martienssen and Baron, 1994). HinII was used to assess Mu1 and a related element Mu2; sites are located within the TIRs of these elements (Lisch et al. 2002). The digested DNA was electrophoresed through an 0.8% agarose gel, and blotted onto nylon membrane (Hybond N⁺, Amersham, Piscataway, NJ). Following hybridization with 32P-labeled probes, signals were detected by autoradiography according to Rudenko and Walbot (2001). The 481-bp mudrA-specific probe was generated by PCR amplification from appropriate MuDR subclones described in Hershberger et al. (1995) and Raizada and Walbot (2000). The mudrA probe was amplified with the following primer set: 5'-TACTCTCTTCCTCGGCGAAT-3' (in position +260) and 5'-TCATCCAATACATCTGATACTAT-3' (+750). In samples from active Mutator plants, digestion with SacI generated a 4.7-kb band indicative of intact MuDR elements, plus a few other bands corresponding to hMuDR. Signal intensity differences in the 4.7-kb band between SacI- and SstI-digested samples were used to infer the methylation state of the TIRs of the population of MuDR elements. The intensity of the 4.7-kb band in the SacI- and SstI-blots was estimated at level ‘1’ (weak) to level ‘4’ (strong), and this scoring system also estimates the copy number of the intact, unmethylated MuDR elements. The Mu1 probe was the 650-bp AvaI-BstN1 internal fragment from plasmid pA/B5 (Chandler and Walbot, 1986); when elements are unmethylated, this probe yields two bands in most Mutator lines corresponding to the 1.4 kb Mu1 and 1.7 kb Mu2 elements. The signal intensity of the 1.4-kb band was estimated at level ‘0’ (none) and level ‘1’ (weak) to ‘4’ (strong) as reflecting the copy number of unmethylated Mu1 elements. The MuDR and Mu1 copy numbers were sometimes altered by their transposition during the line maintenance, and therefore we estimated the unmethylated Mu element intensity.

Real-time RT-PCR analysis of mudrA transcripts  Total RNA was isolated using RNAwiz reagent (Ambion, Austin, TX) according to the manufacturer’s protocol, and treated with RNase-free DNase I. Quantitative RT-PCR was done in an Opticon 2 (MJ Bioworks, South San Francisco, CA). For quantitative RT-PCR, we designed the following mudrA-specific primer set: 5’-TTCCTGTGTGGATTTGGCT-3’ and 5’-GTGCTGTCTCGTACCTCTGC-
Fig. 2. Frequency distribution of spotted kernels in the ears of heterozygous (bz2-mu/bz2) plants. Mu activity was classified based on the percentage of spotted kernels in testcrosses to bz2. (A)(B)(C) The first generation (summer, 2002). 255 bz2-mu × bz2 ears (50, 86, 76 and 43 ears for mu1, mu2, mu3 and mu4 allele, respectively) were analyzed. All plants were derived from fully Mu active plants, selecting heavily spotted bz2-mu/bz2 heterozygotes to initiate the study. (D)(E)(F) The second generation (winter, 2002-2003). In total 504 bz2-mu × bz2 ears (82, 105, 173 and 144 ears for mu1, mu2, mu3 and mu4 allele, respectively) and 416 bz2 × bz2-mu ears (63, 95, 138 and 120 ears for mu1, mu2, mu3 and mu4 allele, respectively) were classified. (G)(H)(I) The third generation (summer, 2003). In total 347 bz2-mu × bz2 ears (78, 46, 175 and 43 ears for mu1, mu2, mu3 and mu4 allele, respectively) and 326 bz2 × bz2-mu ears (77, 46, 160 and 43 ears for mu1, mu2, mu3 and mu4 allele, respectively) were classified. Plants with > 45% spotted kernels are fully active, while most individuals in the 35% to 45% spotted kernel category are silencing; ears with 5 to 35% spotted kernels are clearing silencing; 0% spotted kernels is fully inactive. The data are divided by the direction of the cross: ears of bz2-mu/bz2 plants crossed by bz2 tester (A, D, G) and bz2 tester crossed by bz2-mu/bz2 pollen (B, E, H). Black, shaded, open, and gray bars indicate bz2-mu1, -mu2, -mu3 and -mu4 alleles, respectively. In C, F and I, the frequency distribution of the Mu activity was based on the sum of the four examined alleles in each generation, and the shaded and open bars indicate ears crossed with bz2 pollen and with bz2-mu pollen, respectively.
Inheritance of maize MuDR/Mu transposon activity

3'. As an internal control, a 200-bp thioredoxin-like gene was used (P. Casati, personal communication). The rate of amplification was monitored using SYBR™ Green fluorescence at 83°C. All biological samples were tested in triplicate as previously reported by Goodman et al. (2004). In some assays, an actin gene was used as a second internal control for the RT-PCR analysis (Goodman et al., 2004). The PCR products were also resolved by electrophoresis to verify that only products of the expected sizes were generated.

RESULTS

Monitoring of Mutator activity in bz2-mu/bz2 individuals Somatic variegation of anthocyanin pigmentation in aleurone layer of kernels results from somatic excision of Mu elements at the bz2 locus. Heavily spotted kernels exhibit highly frequent excisions and are typical of active Mutator status (Fig. 1A and 1B). Kernels of this phenotype were used to start the experiment using the four bz2-mu alleles. In reciprocal crosses of sibling heterozygous plants to and by bz2 tester, the expected ratio of heavily spotted to bronze kernels was 1:1 in both resulting ears if the transposon family was fully active. The number of spots per kernel depends on the direction of the test cross. Because the aleurone is triploid the bz2 × bz2-mu kernels (male Mutator parent) have only one copy of the mutable reporter allele while the reciprocal test cross progeny of bz2-mu × bz2 have two copies; the two-fold difference in the number of spots per kernel did not impact calculation of the percentages of spotted kernels per ear. Some individuals showed significantly lower percentages of spotted kernels per ear (Fig. 1C and 1D). In these less variegated ears, both heavily spotted kernels and weakly spotted kernels were observed. The hypothesis is that such weakly spotted kernels retain some Mu activity but that epigenetic silencing has started, evidenced by the deficiency in the percentage of spotted kernels (< 45%, see Materials and Methods) and the presence of lightly spotted kernels. In a fully silenced plant, all kernels are bronze, devoid of Mu excision sectors.

The Mu activity status of individual plants was assigned based on the percentage of spotted kernels per progeny ear. In the first generation, 225 bz2-mu × bz2 and 257 bz2 × bz2-mu ears were analyzed. Activities in the progeny from Mu-active plants varied from 0% (inactive) to around 50% (fully active) in crosses with a Mutator ear (Fig. 2A) or pollen parent (Fig. 2B). This spectrum of Mu activities was produced within one generation as all of the plants used in the experiment were grown from fully active kernels taken from fully active (50% spotted) ears. In the bz2-mu × bz2 ears, two major classes were found: full Mu-active and nearly to completely inactive (Fig. 2A). The bz2 × bz2-mu crosses also generated these two classes, but the proportion of fully active ears was lower compared to the bz2-mu × bz2 crosses and more ears showed an intermediate percentage of spotted kernels (Fig. 2B and 2C).

To establish the second generation, individuals showing the various Mu-activity levels were chosen for the reciprocal crossing program (summarized in Fig. 2D-I; subsequent figs. track the performance of different initial
activity states). In the second generation there is an increase in the fraction of fully inactive and partially active (ears with 5–35% spotted kernels) (Fig. 2D–F). By the third generation most ears were fully inactive, and the remaining ears were found in about equal numbers in each of the intermediate activity classes and the full activity group (Fig. 2G–I). Fewer than 15% of the ear transmission cases retained full activity, and only about 8% of the pollen-transmitted cases. These data indicate that maintenance of high *Mutator* activity requires selection.

To study the effect of cross direction on *Mu* activity, we compared the percentages of spotted kernels in the complete data set for reciprocal crosses in the three generations. Percentages of spotted kernels with *bz2* tester as the ear parent were lower than in the reciprocal cross for all *bz2-mu* alleles (Fig. 3). The bias increased with each successive generation. These results indicated that the frequency and extent of loss of *Mu* activities differ between the pollen and egg cell lineages; both loss and reduction occurred more frequently when the *bz2-mu* alleles were transmitted through pollen.

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**Fig. 4.** Inheritance of somatic excision activity from the first to second generation. (A) *Mutator* activity from plant V23-2 was 26.6% and 38.9% when the *bz-mu3* allele was transmitted from pollen and ear, respectively. In the progeny from the cross of *bz2* tester with the V23-2 plant, three different kernel phenotypes, weakly, mildly and heavily dotted kernels, could be classified. (B) Frequency distribution of the *Mu* activities from the plant V28-1. The activity of this plant was 1.9% and 12.1% when the *bz-mu4* allele was transmitted from pollen and ear, respectively. Shaded and open bars indicate ears crossed with *bz2* pollen and *bz2-mu* pollen, respectively.
Inheritance of maize MuDR/Mu transposon activity

Fine-scale analysis of inheritance of Mutator activities To study transmission of the epigenetic status of Mu activities, we grew second and third generation families selected as spotted kernels from ears showing various different levels of Mu activities; as with the first generation, plants were reciprocally crossed with the bz2 tester. For example, one individual in family number V23 (V23-2) showed 26.6% pollen and 38.9% ear; heavily spotted kernels from the ear parent V23-2 progeny yielded a range of somatic excision frequencies from 21.2 to 49.5% (as ear parents) and 5.8 to 46.8% (as pollen parents) (Fig. 4A). From the reciprocal cross bz2 × V23-2,

![Graphs showing frequency distribution of spotted kernels across generations with different levels of Mu activity](image)

**Fig. 5.** Summary of Mutator activity transmission. (A) Frequency distribution in progeny from fully active lines. This graph was constructed by summing data from all four bz2-mu genotypes examined in this study. (B) Frequency distribution in the next generation based on classification into five categories of parental Mutator activities: < 15%, 15–25%, 25–35%, 35–45% and > 45%. The four graphs represented the frequency distributions in the progenies from the first four groups, which represent the silencing classes. The progeny from the > 45% group gave a distribution similar to the fully active parent. Shaded and open bars indicate ears crossed with bz2 pollen and bz2-mu pollen, respectively.
three different kernel phenotypes were noted: weakly, mildly and heavily spotted kernels. Plants derived from weakly spotted kernels generated 0 to 34.9% (as ear parents) and 0 to 30.8% (as pollen parents) somatic excision; mildly spotted kernels yielded progeny with 0 to 43.6% (ear) and 0 to 41.1% (pollen) activities; and heavily spotted kernels yielded progeny with 26.2 to 42.2% (ear) and 4.9 to 37% (pollen) activities. Therefore, the family from the weakly spotted kernels produced ears with lower percentages of spotted kernels compared with those from the mildly and heavily spotted kernels. Similarly, plant V28-1 showed 1.9% (pollen) and 12.1% (ear) somatic excision in the first generation. All of the 1.9% Mu-active kernels were weakly spotted, and the family grown from these kernels was completely inactive (Fig. 4B). Nearly all of the heavily spotted kernels from the ear with 12.1% spotted kernels generated completely inactive plants as well but there were some active individuals.

As summarized in Fig. 5, these observations were general for all four reporter alleles: plants grown from kernels on ears deviating even slightly from the 50% spotted kernel expectation often showed a dramatic drop in Mutator activity in the next generation. Progeny from ears with > 45% spotted kernels kept the same frequency distribution as fully active lines (Fig. 5A). More inactive plants were observed in the progeny from the 25–35% Mu active parents than in families constructed from 35–45% parents (Fig. 5B). Most siblings from parental lines showing less than 25% Mu activities lost Mu activity completely after only one meiosis. This trend was even more pronounced on ears with a mixture of weakly and strongly spotted kernels: weakly spotted kernels generally yielded completely inactive progenies and even heavily spotted kernels from such mixed ears were likely to lose Mutator somatic excision activity in the next generation. These results indicated that once initiated, epigenetic silencing of Mu activity is rapid, generally requiring only one or at most two life cycles for completion.

DNA methylation states at the MuDR and Mu1 loci Inactivation of Mu elements is well known to be at least partly associated with DNA methylation of MuDR (Martienssen and Baron 1994). Hybridization of a mudrA fragment onto SacI/SstI-digested DNA detects a 4.5-kb band from intact MuDR elements and several additional hMuDR copies (Rudenko and Walbot, 2001). If the intensity of the 4.5-kb band in the SacI-digested DNA was greater than that in the SstI-digested DNA, the difference is indicative of DNA methylation in the TIRs of MuDR. For example, the intensities of the 4.5-kb MuDR fragment in the tassel leaves of plants #2 from both families VV87 and VV110 were weaker than found in the corresponding ear leaves (Fig. 6A and 6B). DNA methylation increased in the TIRs in these two individuals during plant development.

![Fig. 6. Blot hybridization analysis of DNA methylation of MuDR elements.](image)
scored with the SacI-SstI assay. First, copy number was assessed by assigning level ‘1’ for a low copy number (corresponding to 1–3 copies) in the SacI assay up to level ‘4’ (high level, corresponding to ~20 copies of MuDR) and then compared with the Mu activities. There is a loose correlation between Mu activity and MuDR copy number (Fig. 7). The level ‘4’ plants showed high Mu activities, whereas the level ‘1’ plants low activities. This result indicated that Mu activity can be controlled by the autonomous MuDR copy number, with plants having multiple copies being more likely to program strong somatic excision behavior. Overall Mu activity was more tightly and positively correlated with the number of unmethylated MuDR elements assessed by comparing the intensities of the SstI-digested samples to the same sample digested with SacI (Fig. 7A and 7C). These results indicated that the number of unmethylated MuDR copies is critical for maintenance of activity.

To explore further the methylation status of MuDR, HinfI-digested samples were analyzed; there are several sites in the internal region (at positions +35, +480, +712, +730, +812, +972, +1517, +1585, +2404, +2459, +2660, +3997, +4593 and +4904) of the transposase-encoding element (Martienssen and Baron, 1994). In the DNA blots...
of Hinfl-digested DNAs hybridized with the mudrA probe, fragments larger than expected (< 446-bp) and hence indicative of methylated mudrA fragments, were detected in silencing families. For example, all samples from the Mu-inactive VV88 family except the ear leaf of plant #1 showed larger fragments (Fig. 6C). Furthermore, the number of shifted bands was lower in Mu active family VV87 than in VV88. As before, signal intensity of the intact MuDR fragment was estimated using a ‘1’ to ‘4’ scale; a positive correlation was observed between Mu activity and unmethylated MuDR copy number after Hinfl digestion (data not shown). The level ‘3’ and ‘4’ plants showed various Mu activities, whereas level ‘1’ plants had only low activities. The correlation was confirmed in both ear and tassel leaves of the first and second generations. The intensity of the Hinfl shifted bands within MuDR was also estimated from ‘1’ (no or low methylation) to ‘4’ (high methylation) levels. There was a negative correlation between Mu activity and methylation of the internal Hinfl sites (data not shown). The level ‘1’ plants showed various Mu activities, whereas the high methylation ‘3’ and ‘4’ plants had only low activities; this

![Graphs](image-url)

**Fig. 8.** Correlation of the Mu activities of the bz2-mu1, -mu2 and -mu3 alleles with unmethylated Mu1 copy number assessed by Hinfl digestion. (A) Relationship between intensity levels of unmethylated Mu1 and the percentages of spotted kernels when bz2-mu pollen was crossed onto bz2 tester. Total DNA samples from leaves subtending the ear were digested with Hinfl. (B) Same analysis as (A) using DNA from the flag leaves. (C) The leaf DNA samples in (A) plotted against somatic activity transmission through the reciprocal cross of bz2-mu ears crossed by bz2. (D) Flag leaf DNA methylation analysis plotted against somatic activity transmission through the reciprocal cross of bz2-mu ears crossed by bz2.
correlation was confirmed in both ear and tassel leaves of the first and second generations. Substantially methylated mudrA was much more frequently observed in the flag leaf than in the leaves below the ear. This indicated that the methylation of Hinfl sites within the mudrA elements increased rapidly during plant development of a silencing individual and confirms and extends what was observed with SstI sites in the TIRs.

Similarly, we analyzed the relationship between unmethylated Mu1 and Mu2 elements and somatic excision using Hinfl-digested DNA samples. The Hinfl sites are located in the TIRs of these elements (Chandler and Walbot, 1986). Somatic excision monitored with bz2-mu1, -mu2, and -mu3, all of which contain Mu1 element insertions, is correlated with the unmethylated Mu1 copy number, whereas the correlation was not observed in the bz2-mu4 allele, which harbors a MuDR element (Fig. 8). These data suggest that loss of excision capability of the MuDR element in bz2-mu4 can occur before other Mu elements are detectably methylated. As expected in

![Diagram](image.png)

Fig. 9. Inheritance of the Mutator activity and DNA methylation in MuDR from the first to second generation. An upper graph shows the frequency distribution of somatic excision activity from the V20-2; activity was 33.3% and 46% when the bz-mu3 allele was transmitted from pollen and ear, respectively. Total DNAs were isolated from ear leaf (L), tassel leaf (U), and tasscl (T) of plant V20-2, and then digested with SstI (S) and Hinfl (H). DNA blot hybridization using the mudrA probe was performed to examine the methylation state of the MuDR element population. According to the signal intensity, methylation was estimated from '1' (no evidence of methylation) to '4' (complete). The three lower graphs show the frequency distribution of Mutator activities in the progeny of V20-2. In the progeny from the cross of bz2 tester with V20-2, weakly, mildly and heavily dotted kernels were recovered.
silencing individuals larger than expected size fragments were visualized with the Mu1 probe, likely resulting from methylation of one or both TIRs. Plants with mainly the 1.4-kb expected band showed various Mu activities, whereas plants with increasing levels of larger bands showed comparatively lower activities (data not shown). High levels of Mu1-hybridizing signal in larger than expected fragments was much more frequently observed in the flag leaf than in the lower leaves. This observation confirms that once initiated methylation of Hinfl sites in Mu1 rapidly proceeds during plant development as was observed for MuDR.

To examine the stability of methylation states, we compared changes in MuDR from the first to second generations. One example is represented in Fig. 9. In this case, the Mu activity of plant #2 of family V20 was 33.3% and 46% when the bz-mu3 allele was transmitted from pollen and ear, respectively, and this plant had low MuDR methylation levels in both a leaf below the ear and in the flag leaf. Progeny from bz2-mu3 transmission through the ear were uniformly and intensely spotted, however, the progeny from V20-2 pollen were a mixture of weakly, mildly and heavily dotted kernels. Plants grown from the weakly spotted kernels were switching to

![Fig. 10. RT-PCR analysis of mudrA transcript levels in maize plants showing various Mutator activity states.](image-url)
the inactive state more completely than those from the heavily spotted kernels. The three plants from the heavily spotted kernels retained low methylation levels in the population of MuDR elements, similar to the V20-2 parent. In contrast, the three plants from the weakly spotted kernels showed higher methylation levels. Thus, MuDR DNA methylation levels can be transmitted to the next generation, but epigenetically changes can also be imposed. Moreover, the inherited methylation levels -- rather than the status of the parent in the previous generation -- corresponded to the transposon activity status.

**Accumulation of mudrA transcript in individuals with different levels of Mutator activity** A selection of bz2/bz2-mu heterozygous plants in generation 2 was evaluated by quantitative RT-PCR for levels of mudrA transcript (Fig. 10A). Leaves from individual active plants generally showed high mudrA expression, but little transcript was detected in inactive plants. Individual plants of the silencing group accumulated an intermediate level of the mudrA transcript. Progeny seedlings in the third generation exhibited the same pattern vis-à-vis the classification of Mutator status (Fig. 10B). Note that detectable mudrA transcripts were present in progeny seedlings of Mu inactive plants; this could result from either residual MuDR transcription or the constitutively expressed hMuDR elements.

Relative levels of mudrA transcript were calculated by quantitative RT-PCR analysis compared to the RNA sample isolated from the flag leaf of plant V66-1; a thioredoxin-like gene mRNA was used as an internal control. There is a clear positive correlation between the relative amounts of the mudrA expression levels in the flag leaf and somatic excision activity in the subsequent generation (Fig. 11). All examined bz2-mu alleles showed this positive correlation. mudrA expression levels were significantly reduced in plants used as pollen donors that transmitted Mu active status to fewer than 30% of their progeny.

**DISCUSSION**

MuDR/Mu transposons induce high mutation rates in the maize genome, and therefore are useful in tagging and cloning genes and for functional genomics (Walbot, 1992; Raizada and Walbot, 2001). On the other hand, unstable inheritance of Mutator activity is a well-known phenomenon (Robertson, 1978, 1983; Walbot, 1986; Brown and Sundaresan, 1992) that makes it difficult to maintain high activity for gene tagging applications. Because new Mu insertions occur very late in development in pre-germinal cells, during meiosis, and in gametes, picking which plants will support a high level of mutagenesis relies on evaluating the somatic tissues of that individual or analyzing its lineage.

This study was designed to more fully document the inheritance patterns of Mutator activities in large populations, employing four Mutator lines carrying different bz2-mu alleles to search for common attributes of active, silencing, and fully inactive materials. In agreement with previous studies within one or two generations using individual alleles, we confirmed that active plants tend to produce active progeny while silencing individuals are likely to produce inactive progeny (Fig. 5; Robertson, 1983; Walbot, 1986; Brown and Sundaresan, 1992; Walbot and Rudenko, 2002). Most progeny from parental Mu lines showing less than 25% Mu activities were completely inactive after only one meiotic event; these data indicate that in tagging experiments it is important to select lines that exhibit the expected (in our case 1:1)
segregation of mutable individuals. Moreover, plants grown from weakly spotted kernels were more rapidly switching to the inactive state than those from the heavily spotted kernels (Fig. 4); these data indicate that the assay for mutability should include classification of frequency to enrich for individuals most likely to retain Mutator activity. Epigenetic silencing of Mu activity is swift once silencing initiates and can reach near completion within an individual, as documented here by the increased methylation of MuDR and Mu1/Mu2 elements in comparing leaves near the ear and the later developing tassel and the loss of somatic excision in the next generation in such plants.

Silencing occurs progressively during the plant life cycle (Martienssen and Baron, 1994) and preferentially in the tassel, the terminal organ (Walbot, 1986). In this large-scale study, the reduction rates of the Mu activities were different between pollen and egg cell lineages, and silencing of the Mu activity occurred more frequently when the bz2-mu alleles were transmitted through pollen (Fig. 3). Previous study of suppression of hcf106-mu1 and Lex28 mutations by Mu silencing, that is reactivation of expression of the mutant allele during plant growth, was interpreted as developmental loss of Mu activity being caused by longer cell division cycles late in shoot growth (Martienssen and Baron, 1994). It is also possible that Mutator status is influenced by plant maturation, as the ears and subtending leaves differentiate as the plant switches from juvenile to adult phase while the tassel and its subtending flag leaf differentiate at the end of adult development.

In an effort to find a molecular marker that predicts inceptive loss of Mutator activity, we examined both DNA methylation and mudrA transcript levels. Both molecular phenotypes result from epigenetic regulation of the transposon family. DNA methylation states of the Mu1 elements were well correlated with Mu activities in bz2-mudb2 heterozygous plants containing bz2-mu1, bz2-mu2 or bz2-mu3 alleles, but there was no significant relation with those carrying bz2-mu4 allele (Fig. 8). The bz2-mu4 allele contains a MuDR insertion, and the other alleles have the Mu1-insertion (Nash et al., 1990). We assume that de novo methylation of the non-autonomous elements reflects the loss of MURA transposase protein, which in turn results from the silencing of mudrA transcription. We assert that the accumulation of methylated Mu1 elements is an excellent indicator of loss of activity, but may occur too slowly to be reliable in identifying plants that are silencing. The proportion of unmethylated MuDR copies showed a better correlation with somatic excision activity in all four bz2-mu/bz2 stocks, although some inactive plants retained some unmethylated MuDR copies in their genomes (Fig. 7). These results indicate that the epigenetic modification of MuDR is more strictly associated with the epigenetic silencing of Mu activity than that of Mu1 elements and that when even a subset of MuDR elements is methylated silencing has started. Plants with low percentages of spotted kernels rapidly reach a fully inactive state in the following generations (Figs. 5 and 9). In such plants, de novo methylation eventually occurred within the TIRs of MuDR (Fig. 9); our observation of this de novo methylation during silencing confirms a previous report using two other reporter genes (Martienssen and Baron, 1994).

The best indicator of retention of Mutator activity was the presence of substantial mudrA transcript in the flag leaf. In previous studies, no MuDR transcripts were observed in non-Mutator or inactive Mutator stocks (Hershberger et al., 1991, 1995), and MuDR transcript levels were proportional to the MuDR copy number in active Mutator lines (Rudenko and Walbot, 2001). In this study, the relative amount of mudrA transcripts was estimated by real time RT-PCR in plants with various Mu activities. There is an excellent, positive relation between retention of Mutator activity and mudrA expression levels especially in tassel leaves (Fig. 11). Epigenetic silencing reflects inactivation of mudrA expression, a step affecting a progressively larger fraction of the MuDR population, as well as a progressive increase in DNA methylation of the TIRs and internal sites in MuDR elements. It is possible that future studies exploiting mutations in components of RNA-mediated transcriptional silencing and de novo methylation can distinguish definitively which process is a direct cause of epigenetic silencing of the Mu activity. Currently, we note that some exceptional cases were observed in the correlation between loss of the Mu activity and unmethylated MuDR copy number (Fig. 7), whereas the mudrA transcript levels showed a much better correlation with the activity status (Fig. 11). These results suggest that the inactivation of mudrA expression precedes de novo DNA methylation of MuDR TIRs.

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