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RESEARCH ARTICLE

The Late Developmental Pattern of *Mu* Transposon Excision Is Conferred by a Cauliflower Mosaic Virus 35S–Driven MURA cDNA in Transgenic Maize

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The *MuDR* element responsible for *Mutator* activities in maize encodes two genes, *mudrA* and *mudrB.* Each encodes multiple transcripts hypothesized to regulate, directly or indirectly, the unique late timing and switch in transposition mechanism during maize development. *mudrA*, which encodes the MURA transposase, is unstable in bacterial plasmids, a technical problem solved by using phage M13 as a vector to prepare DNA for biolistic transformation. In transgenic maize, a single 2.7-kb *mudrA* cDNA predicted to encode an 823–amino acid protein is sufficient to catalyze late somatic excisions, despite removal of the native promoter, alternative transcription start sites, known introns, polymorphic 5′ and 3′ untranslated sequences, and the *mudrB* gene. These results suggest that post-translational regulation confers *Mu* excision timing. The transgene is active in lines containing silencing *MuDR* elements. This suggests that endogenous *MuDR* transposons do not measurably immunize the host against expression of a homologous transgene.

INTRODUCTION

Mutator lines of maize contain a high-copy-number DNA transposon family (Robertson, 1978). Nine *Mu* element subfamilies exist. All share homologous flanking ~215-bp terminal inverted repeat (TIR) sequences. Subfamilies *Mu1* to *Mu8* are nonautonomous and require a source of transposase to catalyze transposition (reviewed in Bennetzen et al., 1993). The *Mu* transposase is encoded by the 4.9-kb *MuDR* element (Chomet et al., 1991; Hershberger et al., 1991; Qin et al., 1991; Hsia and Schnable, 1996), which is present in multiple copies in highly mutagenic *Mutator* lines.

Mu elements are an efficient transposon-tagging tool, because multicopy *MuDR* lines have a forward mutation frequency 20- to 50-fold higher than either *Ac* or *Spm* (Robertson and Mascia, 1981). Moreover, *Mu* elements transpose equally to linked and unlinked sites (Lisch et al., 1995). They exhibit an extremely high insertion bias (>90%) for low-copy-number transcribed regions of the genome (Cresse et al., 1995). Finally, *Mu* germinal insertion events occur late, resulting in independent insertions in sibling progeny (Robertson, 1981, 1985).

A fascinating component of *Mutator* biology is that *MuDR* catalyzes distinct transposition behaviors of *Mu* elements in somatic and germinal cells. The full somatic program involves activation, activity, and epigenetic silencing. In a line with methylated *Mu* elements, introduction of a transcriptionally active *MuDR* results in *Mu* element TIR demethylation in leaves (Chandler and Walbot, 1986; Bennetzen, 1987). Demethylated *Mu* elements can then excise at high frequencies, but only during the terminal cell divisions of somatic tissues, as observed in anthers, aleurone, and leaves (Levy and Walbot, 1990). In the cells that give rise to gametes, *Mu* follows a different program, because germinal revertants are exceedingly rare (Schnable et al., 1989; reviewed in Walbot, 1991). Instead, *Mu* elements duplicate and insert in late pregerminal, meiotic, and gametic cells but rarely in the vegetative precursor cells that give rise to the inflorescences (Robertson, 1981; Alleman and Freeling, 1986; Lisch et al., 1995). After amplification, multiple unlinked *MuDR* elements in some progeny or leaf sectors within progeny plants undergo coordinate epigenetic transcriptional silencing, which results in the remethylation of *Mu* element TIRs and loss of *Mutator* activity (Walbot, 1991; reviewed in Fedoroff and Chandler, 1994; Martienssen and Baron, 1994).

As shown in Figure 1A, *MuDR* consists of two convergently oriented genes, *mudrA* and *mudrB*, flanked by promoter-containing TIRs (Hershberger et al., 1991; Benito and Walbot, 1994). By homology and analysis, the function of *mudrB* remains unknown. In contrast, *mudrA* is the candidate transposase gene, because it is related to bacterial

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transposons (Eisen et al., 1994). Furthermore, analysis of lines carrying deletions in *MuDR* demonstrated that *mudrA*, but not *mudrB*, is required to catalyze somatic excisions (Lisch et al., 1999).

mudrA encodes diverse transcripts resulting from alternative transcription initiation, intron splice failure, and alternative polyadenylation sites (Figure 1B; Hershberger et al., 1995). Thus, *mudrA* produces transcripts with polymorphic 5′ and 3′ untranslated regions (UTRs) and a coding region predicted to produce at least two large polypeptides of 736 and 823 amino acids. Although *MuDR* was identified in 1991 (Chomet et al., 1991; Hershberger et al., 1991; Qin et al., 1991) and fully sequenced (Hershberger et al., 1991; James et al., 1993; Hsia and Schnable, 1996), there has been no progress in using a transgenic approach to determine which transcripts are sufficient to catalyze or regulate specific *Mu* activities. The major limitation is that all *mudrA* plasmids grown in *Escherichia coli* develop frameshift or deletion mutations (reviewed in Bennetzen, 1996). For this reason, it has also not been possible to transfer *Mutator* activity to heterologous hosts for transposon-tagging experiments.

In this article, we demonstrate that bacteriophage M13 is a suitable vector to manipulate *mudrA* and to make transgenic plants. We then use transgenic maize to test the function of the fully spliced transcript, capable of encoding the 823– but not the 736–amino acid protein. When expressed in yeast, this cDNA encodes a 120-kD polypeptide that has been shown to specifically bind a *Mu* TIR sequence in vitro (Benito and Walbot, 1997). To determine whether the polymorphic noncoding sequences of *mudrA* are required for developmental regulation, we excluded the alternative 5′ and 3′ UTRs from the transgene and replaced the native promoter with a heterologous cauliflower mosaic virus (CaMV) 35S promoter (Figure 1C). In this report, we analyze transgenic plants expressing full-length and truncated versions of this cDNA to determine whether these transgenes are sufficient to program the four molecular and developmental activities catalyzed by *MuDR*: demethylation, somatic excision, germinal insertion, and epigenetic reprogramming.

RESULTS

mudrA Clones Are Unstable as Plasmids in *E. coli* but Stable in M13

Since the identification of *MuDR*, several groups independently reported the frustrating inability to maintain any *mudrA* plasmid cDNA or genomic clone in *E. coli* (reviewed in Bennetzen, 1996). The *mudrA* genomic clone with introns is toxic, perhaps as a result of internal initiation in the second exon at two Shine-Dalgarno-like sequences at +938 and +1437 (numbering according to Hershberger et al., 1991); the resulting translational product of up to 619 amino acids includes the bacterial transposase–related region (Figure 1A). Attempts to stabilize *mudrA* in low-copy or transcriptionally repressed pET vectors (R.J. Hershberger, R. Taylor, and V. Walbot, unpublished results) or in *Agrobacterium tumefaciens* (A. Lloyd, C.D. Goodman, and V. Walbot, unpublished data) have failed; to date, all sequenced plasmids have contained internal deletion or frameshift mutations that disrupt the second exon long open reading frame. The *mudrA* cDNA corresponding to fully spliced transcript is stable in *Saccharomyces cerevisiae* in a low-copy vector (Benito and Walbot, 1997); however, the low yield of the yeast plasmid made it difficult to manipulate or use for the biolistic transformation of maize callus. By restriction enzyme analysis, we first discovered that this cDNA clone was stable in an M13 vector through two rounds of cloning. Four randomly chosen colonies produced full-length translation products in vitro and therefore did not contain frameshift mutations; one of these was sequenced and contained no mutations. We conclude that M13 is a useful vector for the cloning of this otherwise toxic gene.

Analysis of Transformed Callus Lines

We used biolistic transformation of HiII (hybrid of two inbred lines; A188 × B73) type II embryogenic callus (Armstrong and Green, 1985) to make transgenic maize (Fromm et al., 1990; Gordon-Kamm et al., 1990). This genotype lacks intact copies of *MuDR* by DNA gel blotting or by sequence analysis of polymerase chain reaction (PCR) products from degenerate *MuDR* elements (G. Rudenko and V. Walbot, manuscript in preparation). As shown in Figure 2A, hybridization analysis failed to detect *mudrA* or *mudrB* RNA transcripts. The M13-based *mudrA* expression construct was cotransformed with *Bar* gene plasmid pAHC20 (Christensen and Quail, 1996), which confers Basta (AgrEvo USA, Apple Valley, MN) herbicide resistance (De Block et al., 1987; Thompson et al., 1987). RNA from herbicide-resistant calli was analyzed for expression of *mudrA* by RNA gel blot hybridization. Of 64 herbicide-resistant lines, 18 expressed reasonable levels of the expected 2.7-kb *mudrA* transcript, and these were termed cA (for cDNA *mudrA*) lines.

We also performed RNA gel blot screening to identify lines with 5′ or 3′ deletions in *mudrA*, because a deleted transgene with partial function could help define essential protein domains. We isolated eight dcA (for deletion of cA) lines that hybridized to the 1.3-kb 5′ *mudrA* probe (Figure 2A) but not to the probe corresponding to the terminal 350 bp of the gene. An additional line, dcA1, expressed an abundant ~650-bp transcript, which hybridized to a probe spanning the last 350 bp of *mudrA* but not to a probe corresponding to the first 1.3 kb of the gene (Figure 2A). These results are consistent with a >2-kb 5′ deletion in the dcA1 line. Thus, after biolistic transformation, the random breakage of vector DNA during chromosome integration can generate a useful deletion series of the transgene.

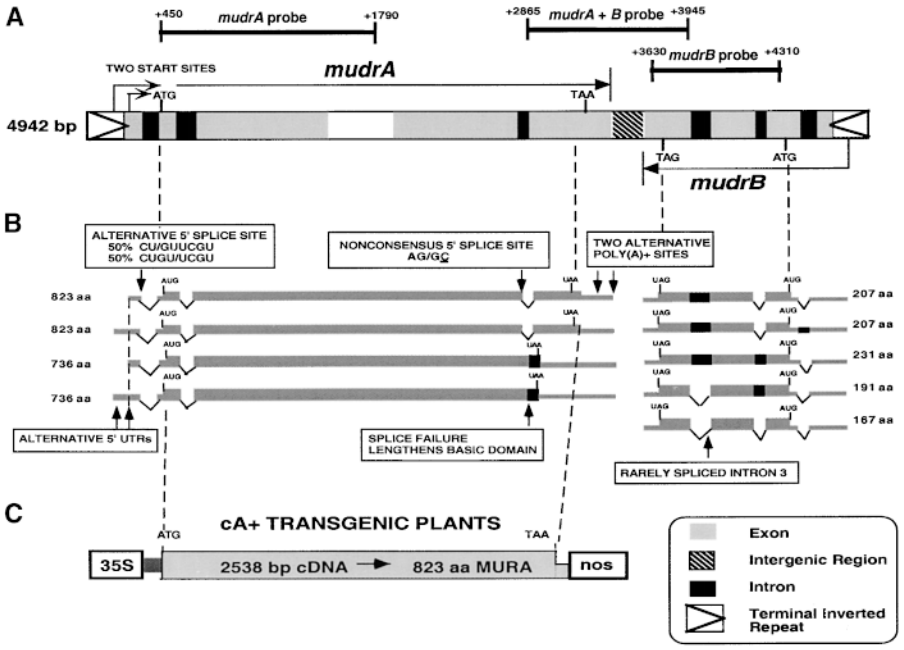

Figure 1. Structure of *MuDR*, Endogenous *mudrA* and *mudrB* Transcripts, the CaMV 35S–*mudrA* Construct in cA+ Transgenic Maize Lines, and the Probes Used for RNA Gel Blots.

(A) Structure of an endogenous *MuDR* element. The element has two open reading frames, termed *mudrA* and *mudrB*, encoded in antiparallel orientation. The intergenic region between the two genes is composed of diverse short repetitive elements. The promoters are located within the ~215-bp TIRs. The *mudrA* region with high similarity to bacterial transposases is shown in white. The DNA probes for RNA analysis in this study are located above the element. Numbering is according to Hershberger et al. (1991).

(B) The diversity of endogenous *mudrA* and *mudrB* transcripts in active *Mutator* seedlings (Hershberger et al., 1995). Intron sequences shown in solid black are in-frame with exons. Alternative *mudrA* transcription initiation sites (+169 and +252) produce transcripts with a short or long 5′ leader sequence. aa, amino acids.

(C) The structure of the CaMV 35S–*mudrA* cDNA in M13 transformed into maize to make cA lines. In construct phMR53, the native promoter, alternative start sites, 5′ UTR, and introns were removed. The CaMV 35S promoter and 130-bp leader sequences were substituted. The *mudrA* 3′ UTR (polymorphic region) was truncated and fused to the nopaline synthase (nos) terminator.

Expression of the 823–Amino Acid MURA-Encoding cDNA Results in Demethylation of Its Binding Site in Embryogenic Callus

A hallmark phenotype of inheriting an active *MuDR* is DNA demethylation at the HinfI site of *Mu1* TIRs (Chandler and Walbot, 1986; Bennetzen, 1987). This restriction site overlaps the MURA binding site defined in vitro (Benito and Walbot, 1997). It is hypothesized that binding of MURA in vivo blocks maintenance methylation. Because the Hill stock contains several endogenous, methylated copies of *Mu1* and *Mu2* (Chandler et al., 1986; Masterson et al., 1988), we tested the HinfI methylation status of these elements in stably transformed embryogenic calli before we proceeded to regenerate whole plants. DNA gel blot analysis was performed on callus DNA isolated 10 to 12 weeks after bombardment. As shown in Figure 2B, callus lines expressing low, medium, or high levels of *mudrA* were fully demethylated at the HinfI sites, whereas *Mu* elements in untransformed callus DNA remained more methylated. Therefore, the *mudrA* transgene contributed a known *MuDR* phenotype.

Surprisingly, four 3′ truncated dcA callus lines also underwent demethylation at the *Mu1* and *Mu2* TIRs. The 5′ deleted dcA1 line remained methylated, suggesting that the initial introduction of M13 full-length *mudrA* cDNA or lingering extrachromosomal transgene DNA was not responsible for the HinfI site demethylation in these lines. To rule out the possibility that the dcA lines also had a poorly expressed intact *mudrA* transgene, we further analyzed line dcA74 containing the shortest dcA transgene. By reverse transcription (RT)–PCR, a 3′ primer at +1090 amplified *mudrA* transcript from this line, whereas a downstream primer at +1412 failed to amplify a band; in the same experiment, full-length *mudrA*-expressing plants produced product from both primers (data not shown). Therefore, line dcA74 does not possess

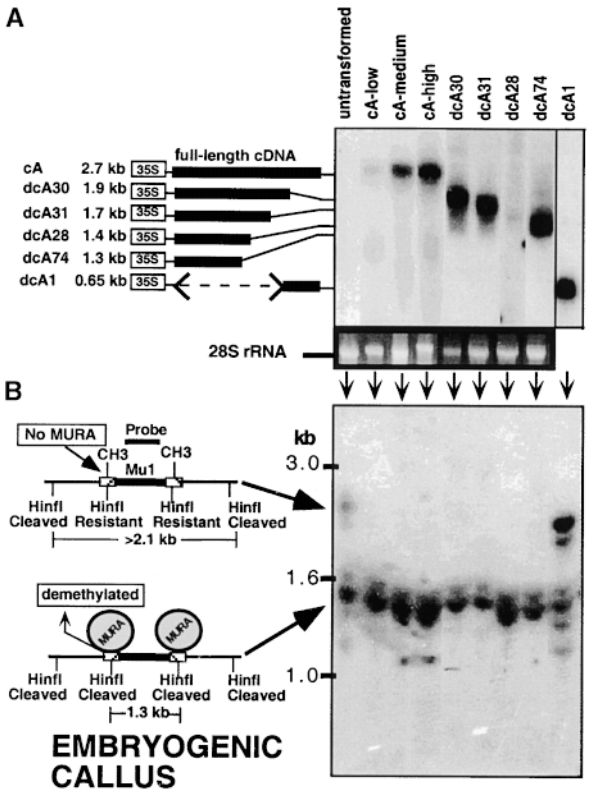

Figure 2. Test for Interaction of CaMV 35S–*mudrA*—Encoded Full-Length and Truncated Proteins with the MURA DNA Binding Sites of *Mu1* and *Mu2* TIRs in Embryogenic Callus.

(A) RNA gel blot hybridization analysis of *mudrA* transgene transcripts in maize leaves from T_0 plants. Lines tested correspond to plants regenerated from each callus line shown in **(B)**. Line dcA1 was probed with the *mudrA+B* (BX1.0) probe (Figure 1A), which hybridizes with the 3′ end of *mudrA*. All other lanes were probed with the 1.3-kb 5′ *mudrA* probe (Figure 1A). The estimated sizes of the truncated transgenes, based on RNA gel blot analysis, are shown at left. At bottom is the ethidium bromide–stained formaldehyde agarose gel indicating the 28S rRNA.

(B) At left is a model to explain the effect of MURA binding on the methylation status of HinfI sites in the TIRs of *Mu1* and *Mu2*. MURA binds to an ~30-bp region overlapping the HinfI site in each TIR (Benito and Walbot, 1997). At right is a DNA gel blot of callus DNA digested with methylation-sensitive HinfI and hybridized with probe pA/B5, which recognizes both *Mu1* and *Mu2*. DNA was prepared from calli 10 to 12 weeks after stable transformation with the CaMV 35S–*mudrA* vector.

an intact, expressed *mudrA* transgene. The simplest explanation of the demethylation by lines with *mudrA* 3′ deletions is that the DNA binding domain of MURA is present at its N terminus; this hypothesis must be confirmed in vitro by gel shift experiments.

The demethylation data imply that MURA can bind to its target motif in the absence of other *MuDR*-encoded proteins, as expected from gel shift binding assays in vitro using the 823–amino acid protein (Benito and Walbot, 1997). Rapid TIR demethylation in embryogenic callus further suggests that the MURA transposase is both properly translated and competent to bind its target in undifferentiated cells. This is interesting, because *Mu* element excisions are restricted to terminally differentiated somatic cells undergoing their last few cell divisions (Levy and Walbot, 1990), even though *MuDR* transcripts and MURB protein are abundant in meristematic tissues (Donlin et al., 1995; Hershberger et al., 1995; Joanin et al., 1997). Among hypotheses to explain the absence of excisions early in development are the following: that MURA transposase is not translated, or that it does not interact with its TIR binding site in undifferentiated dividing cells. Our callus demethylation data indirectly rules out both of these theories.

Characterization of Regenerated Transgenic Lines

Because full-length and 3′ truncated *mudrA* transgenes resulted in *Mu* element TIR demethylation, a subset of cA and dcA lines (seven and seven, respectively) was regenerated and carried forward into the T_3 and T_4 generations; two cA lines and two dcA lines with low plant fertility or immediate transgene silencing were discarded. As shown in Table 1, only lines in which the presence of *mudrA* transcripts perfectly cosegregated with herbicide resistance were chosen. Herbicide resistance is indicated by a (+). We anticipated large-scale field experiments, and a tightly linked, scorable marker facilitated tracking the transgene locus. In the lines selected, the herbicide resistance plasmid appears to have inserted at the same single locus as the *mudrA* transgene. For example, in line cA36, a total of 36 outcross progeny at generation T_1 were analyzed for herbicide resistance and expression of *mudrA* by RT-PCR. Of these, 16 were herbicide resistant, and the same 16 plants but no others expressed *mudrA.* In our experience, at least 90% of cobombarded plasmids integrate at tightly linked sites, although one member of the pair may not be expressed (data not shown).

Three transgenic lines with full-length *mudrA* but distinctive transcript abundance and susceptibility to silencing were used for *Mu* transposition assays. An RNA gel blot of these lines at the T_0 generation is shown in Figure 3. Line cA36 is highly prone to silencing but can produce significant levels of *mudrA* transcript. Sister lines cA75A and cA75B were independently regenerated and maintained, but they express *mudrA* transcript at different levels. In addition, line cA75B undergoes epigenetic silencing infrequently compared with line cA75A. In our experience, two plants regenerated from the same maize callus line can express the transgene of interest at very different levels; hence, each T_0 plant and its progeny must be analyzed and tracked individually.

Table 1. Characterization of Genetic Lines Used in This Study

Line	Linkage Test	Generation T_1 to T_4 Behavior		DNA Gel Blot Analysis	
	Cosegregation[a] of CaMV 35S–*mudrA*[b] with Herbicide Reisistance[c]	Transgene/Herbicide Stability	Segregation of Herbicide Resistance	No. of Copies of *Bar* Gene[d]	No. of Copies of CaMV 35S–*mudrA* Transgene[e]
cA36	16+/16+ (36)	Poor	<1:1	>11	4
cA75A	17+/17+ (31)	Good	1:1	>4	4
cA75B	17+/17+ (27)	Excellent	1:1	>4	4
dcA28	3+/3+ (5)	Good	1:1	>8	>7
dcA30	4+/4+ (7)	Good	1:1	2	>2
dcA31	5+/6+ (9)[f]	Moderate	1:1	>5	>4
dcA74	4+/4+ (10)	Poor	<1:1	1	>2

[a] The first plus (+) indicates the number of plants that contain the CaMV 35S–*mudrA* transgene, and the second plus (+) indicates the subset of CaMV 35S–*mudrA* plants that are also Basta resistant. The number in parentheses indicates the total number of outcross plants tested in the T_1 generation.

[b] CaMV 35S–*mudrA* transgene inheritance tested by RT-PCR or genomic PCR.

[c] Resistance to Basta applied to the leaf surface.

[d] Blot probed with pUC18 DNA to detect the *Bar* transgene.

[e] Blot probed with CaMV 35S DNA to detect CaMV 35S–*mudrA* construct.

[f] Indicates possible transgene silencing. The presence of CaMV 35S–*mudrA* was tested by RT-PCR.

CaMV 35S–*mudrA* cDNA Programs Somatic Excision of *Mu1* Elements

The *a1-mum2* allele contains a *Mu1* element at the *a1* locus (Chomet et al., 1991). In the absence of transposase, *a1-mum2* kernels are colorless; as shown in Figure 4A, when crossed with a *MuDR* source, *Mu1* somatic excisions occur and are visualized as reddish purple spots on the kernel aleurone. To test for transgene *mudrA*-mediated somatic excision, we crossed lines expressing *mudrA* to *a1-mum2* stocks in which the single copy of *MuDR* originally present had been segregated out of the stock (Chomet et al., 1991; Qin et al., 1991). We asked if CaMV 35S–*mudrA* could activate *Mu1* excisions at the *a1-mum2* reporter gene.

For each transgenic line, we planted a family (kernels derived from the same ear) segregating 1:1 for the transgene (Figure 4A) and crossed all individuals reciprocally with a line homozygous for *a1-mum2* but lacking *MuDR.* As shown in Figure 4B and summarized in Table 2, all three herbicide-resistant full-length cDNA transgenic lines did catalyze excisions, with the best line, cA75B+, having activity in 22 of 22 ears. Negative control crosses using herbicide-sensitive parents resulted in no spotted kernels in the 50 progeny ears examined (~13,000 kernels). None of the transgenic lines containing deletions in CaMV 35S–*mudrA* yielded spotted kernels (0 out of ~7000 kernels).

When a single-copy *MuDR a1* line is crossed to an *a1-mum2* tester (*MuDR a1*/*a1* × *a1-mum2*/*a1-mum2*), 50% spotted kernels are expected and are typically observed (Lisch et al., 1995; Hsia and Schnable, 1996). The expectation for a subset of the transgene crosses (hemizygous CaMV 35S–*mudrA*, *a1-mum2*/*A1* × *a1*/*a1*) was that herbicide-resistant plants should produce 25% spotted kernels, 50% purple, and 25% colorless. As shown in Figure 4C, progeny of cA36+-derived ears exhibited a low excision frequency (26 of 1018 kernels, or 2.6%), whereas 11% of cA75A+ kernels (493 of 4458) and 18% of cA75B+ kernels (406 of 2222) were spotted. Two ears exhibited the expected 25% excision frequency.

We hypothesized that transgene silencing was responsible for the low excision frequency catalyzed by line cA36. In fact, all spotted and unspotted kernel progeny of a cA36+

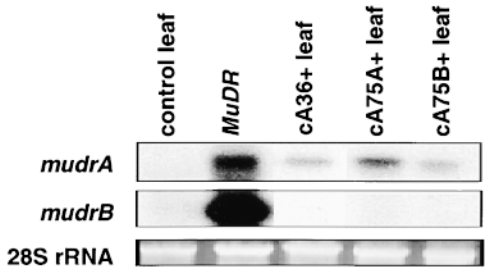

Figure 3. RNA Gel Blot Hybridization Analysis of *MuDR* and CaMV 35S–*mudrA* Full-Length cDNA Transgenic Lines.

Control total RNA is from a leaf of a standard inbred tester. *MuDR* RNA is from an immature ear of an active, high-copy *MuDR* line. The transgene samples are from mature leaves of T_0 plants. Both *mudrA* and *mudrB* are from the same blot, probed with the cross-hybridizing *mudrA+B* (BX1.0) probe. No *mudrB* transcript was detected in any cA lines. The 28S rRNA panel is from the ethidium bromide–stained agarose gel.

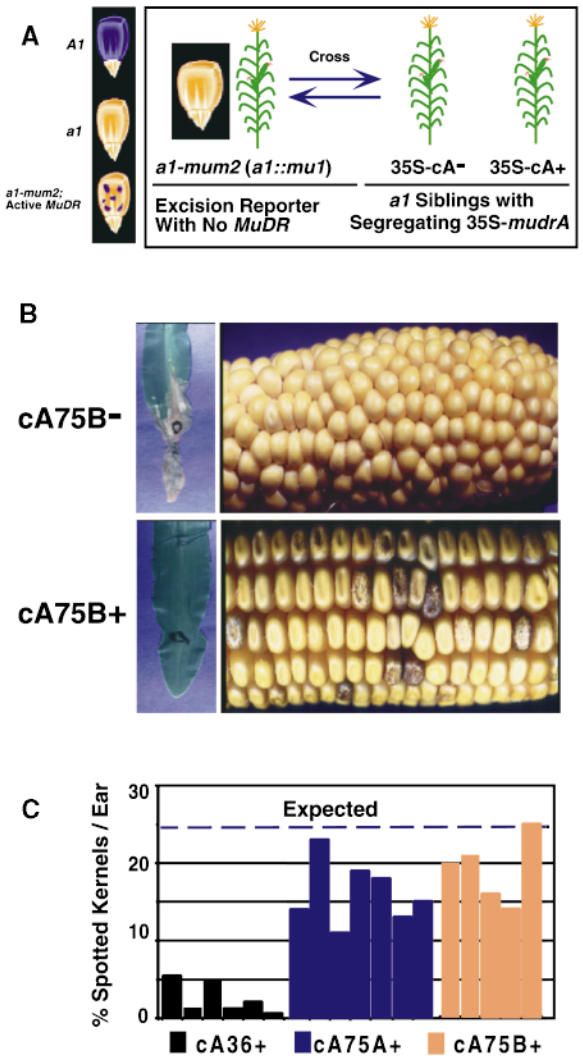

Figure 4. Genetic Test of the Ability of CaMV 35S–*mudrA* to Catalyze Excisions of *Mu1* Elements at the *a1* Locus in the Absence of Intact *MuDR* Elements.

(A) At left is a diagram demonstrating the expected aleurone phenotypes of different *A1* genotypes. At right is the genetic experiment in which sibling plants carrying the *a1-mum2* excision reporter were crossed to or by transgenic T_2 generation plants segregating 1:1 for the transgene. cA+ indicates herbicide resistance and cA− indicates herbicide sensitivity.
(B) At left are the leaf phenotypes of transgenic line cA75B parents 5 to 7 days after application of Basta herbicide. At right are the phenotypes of progeny of crosses between *a1-mum2* and +/− transgene lines. The ears are the T_4 generation.
(C) Percentage of spotted kernels per ear of cA+ CaMV 35S–*mudrA* lines. All kernels have one copy of *a1-mum2* and one copy of the CaMV 35S–*mudrA* locus; both genes were transmitted through pollen in crosses onto *a1* tester ears. Twenty-five percent of kernels were expected to be spotted. Each bar represents an individual ear.

ear were herbicide sensitive (nine out of nine). The two most spotted of these progeny expressed only low levels of *mudrA* transcript (data not shown). In contrast, for both cA75A+ and cA75B+, all seven spotted kernels tested gave rise to herbicide-resistant seedlings, each of which was shown to also carry the CaMV 35S–*mudrA* transgene by PCR and have abundant *mudrA* expression on RNA gel blots (data not shown). This suggests that in the cA36-derived family shown in Table 2, the *bar* and *mudrA* transgenes were in the process of silencing.

To demonstrate conclusively that CaMV 35S–*mudrA* was directly responsible for the somatic excisions seen, we grew seedlings from spotted *a1-mum2*/*a1* cA75 kernels to maturity and crossed them with an *a1* non-*Mutator* stock. If a *MuDR* element separate from the CaMV 35S–*mudrA* transgene element were responsible for the excision phenotype, then independent assortment of an unlinked element or meiotic recombination would separate the two. As shown in Table 3, among 357 spotted progeny kernels tested, nearly all were herbicide resistant, indicating that they inherited the transgene locus. The nine partially herbicide-sensitive plants all inherited the CaMV 35S–*mudrA* transgene, based on PCR analysis; we conclude that the *bar* gene was simply silencing in these individuals. Consequently, any contaminating *MuDR* element would have to be located within 1 centimorgan ($P < 0.05$) of the transgene in cA75, which is highly unlikely. We consider that these results directly prove that the fully spliced *mudrA* cDNA is sufficient to program somatic excisions of *Mu* elements in maize.

Developmental Timing of Excisions

On quick visual inspection, the *mudrA* cDNA programmed somatic excisions with the same characteristically late developmental timing as active *Mutator* lines (Levy et al., 1989;

Table 2. Summary of *Mu1* Excision Activity at *a1-mum2*[a]

	Progeny		
Transgenic Parent	Spotted Ears	Unspotted Ears	Percentage of Ears Spotted
cA36−	0	13	0
cA36+	6	15	29
cA75A−	0	13	0
cA75A+	34	4	89
cA75B−	0	10	0
cA75B+	22	0	100
dcA28+	0	7	0
dcA30+	0	8	0
dcA31−	0	8	0
dcA31+	0	8	0
dcA74+	0	2	0

[a] The experiment is described in Figure 4A.

Table 3. Cosegregation of *a1-mum2*–Conferred Excision Phenotype with Inheritance of the CaMV 35S–*mudrA* Transgene in Line cA75+[a]

Kernel Phenotype of Progeny	Expected Transgene Inheritance (%)	Total Tested	Herbicide Resistant	Herbicide Sensitive	Observed Transgene Inheritance	Phenotype Linkage to Transgene
Random	50	13	7	6	7+/13	unlinked
Spotted only	100	357	348	9(0)[b]	357+/357	<1 centimorgan ($P < 0.05$)

[a] The cross performed was (spotted, *a1-mum2*/*a1*, hemizygous CaMV 35S–*mudrA*) × (unspotted, *a1*/*a1*).
[b] Partial herbicide sensitivity. Genomic PCR was performed using CaMV 35S–*mudrA*—specific primers; the resulting gel was DNA gel blotted and probed with the *mudrA* probe. All nine plants carried the CaMV 35S–*mudrA* transgene.

Levy and Walbot, 1990). Microscopic scoring of *Mu1* excisions at *a1-mum2* indicated that >80% of the sectors were composed of ≤10 cells programmed by either CaMV 35S–*mudrA* or a single copy of *MuDR.* As shown in Figure 5, the sector size distribution seen with line cA75A+ was nearly identical to the single-copy *MuDR* line, whereas a slightly broader distribution was observed in line cA75B+. Even if the latter pattern is a true representation of this line, it reflects a shift in timing by only one or two cell divisions. Slight differences in anthocyanin pigment diffusion could easily account for the small variation seen.

In *Mutator* lines, excision timing is not affected by varying *MuDR* or excision reporter copy number (Levy and Walbot, 1990), in contrast to the dosage responses of *Ac* (Brink and Nilan, 1952). It seemed possible that the more variably competent CaMV 35S–*mudrA* transgenes might exhibit dosage dependence; however, a change from two doses (maternal transmission) to one dose (paternal transmission) had no measurable impact on either excision timing or its frequency (Figures 5E and 5F).

In *Mutator* lines, excisions are largely limited to somatic cells; germinal revertants have either not been observed in large populations or occur with a frequency of $<10^{-4}$ (reviewed in Bennetzen, 1996). A germinal excision at *a1-mum2* that restores gene expression (a revertant) would be a solid purple kernel or sector of purple kernels on an ear. An initial experiment in which both the *a1-mum2* allele and the transgene were transmitted through the female lineage yielded only one out of ~6800 purple kernels, which we attribute to contamination, because no further instances of putative germinal excisions were observed in the next generation. These results suggest that at best, the CaMV 35S–*mudrA* transgene catalyzes germinal excisions at a frequency equal to or less than native *MuDR* stocks.

Excision Frequency Is Not Correlated to *mudrA* Transcript Abundance

In terms of average excision frequency per spotted kernel, it is visually clear that some ears with a cA75B+ transgene match the most spotted examples of single-copy *MuDR* lines, whereas the frequency in lines cA36+ and cA75A+ is much lower (data not shown). Nevertheless, it appears that the CaMV 35S–*mudrA* transgene can fully complement the *MuDR*-catalyzed excision intensity per kernel.

One difference, however, is that excision frequency is visually more variable in transgenic lines than in a single-copy *MuDR* line. We hypothesized that the variable levels of excision catalyzed by CaMV 35S–*mudrA* in some progeny could reflect differences in transgene expression. We compared excision frequency to *mudrA* transcript levels. Sector number was determined for eight sibling *a1-mum2* kernels for both cA75A+ and cA75B+. In line cA75B+, there is a 47-fold range in spot number, from nine spots per kernel to 439, but as shown in Figure 6, there is less than a twofold increase in seedling transcript abundance between them. Eight ranked kernels of line cA75A+ showed similar results (data not shown). Therefore, above the minimum threshold required for somatic excision, sector frequency is not positively correlated with transcript levels. These results suggest that CaMV 35S–*mudrA* excision activity is post-transcriptionally determined. The caveat in this experiment is that we compared endosperm excision frequency to transcript abundance using RNA prepared from the tips of leaf 4.

Mu Insertions Are Not Detected in the Germline of CaMV 35S–*mudrA* Transgenic Plants

To test the ability of the CaMV 35S–*mudrA* cDNA to catalyze germinal insertions, we grew spotted kernels of genotype CaMV 35S–*mudrA*/-;*a1-mum2*/*a1* and crossed them to the *a1* tester; these plants had three unlinked copies of *Mu1* and the molecularly similar element *Mu2.* Figure 7 exemplifies the DNA gel blot hybridization screen for new *Mu1* or *Mu2* insertion fragments in the progeny against the background of segregating parental fragments. No new fragments were detected in 38 progeny, drawn equally from maternal and paternal transmission of the transgene, or in a second test of 34 progeny grown from the most highly spotted kernels. If the CaMV 35S–*mudrA* transgene can catalyze germinal insertions of *Mu* elements, then the frequency must be <5% ($P < 0.05$) per plant or <2.5% per donor *Mu1* element per generation. This is less than the weakest active *Mu* line in which a single copy of *MuDR* in a poor chromosomal position catalyzes new insertions at a frequency of 6 to 14% per *Mu1* element per generation (Lisch et al., 1995).

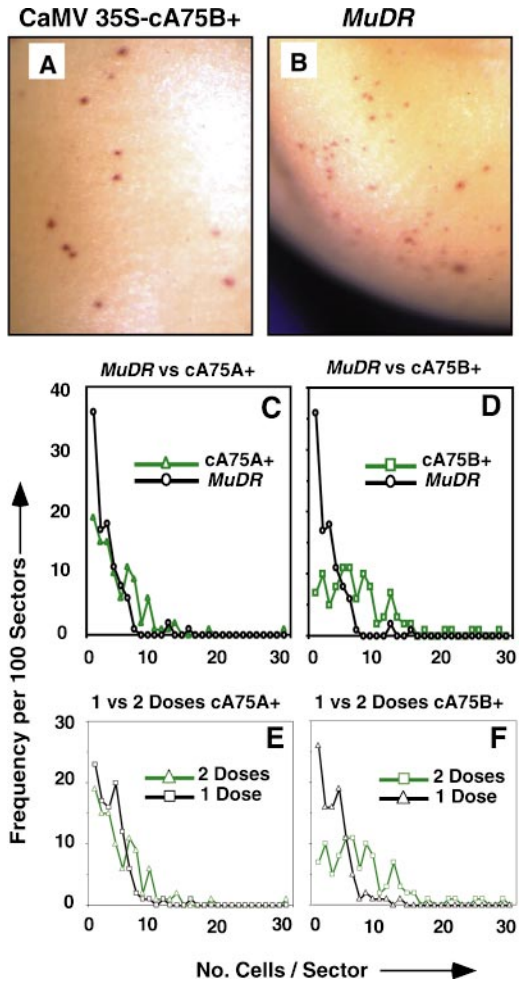

Figure 5. Comparative Analysis of the Developmental Timing of Excision from Aleurone Cells of *Mu1* from *a1-mum2* in *MuDR* and CaMV 35S–*mudrA* Lines.

(A) cA75B+ aleurone. Most of the excisions shown consist of single cells.

(B) One-copy *MuDR* aleurone. Most sectors consist of one or two cells.

(C) and **(D)** Sector size distribution comparison. One hundred randomly chosen sectors were scored per line. The *MuDR* source was selfed, and kernels contain one to three copies of *MuDR* plus one to three copies of *a1-mum2.* The cA75+ line contains two doses of CaMV 35S–*mudrA* and two to three copies of *a1-mum2.* The *MuDR* data in both **(C)** and **(D)** are the same. The *MuDR* kernel had one additional 48-cell sector (not shown on graph).

(E) and **(F)** Sector size distribution as a function of CaMV 35S–*mudrA* transgene locus dose. The two-dose data are from **(C)** and **(D)**, respectively. The one-dose kernels contain one dose of CaMV 35S–*mudrA* and two to three copies of *a1-mum2.* In the two-dose kernels, the transgene had been transmitted through the female. Single-dose kernel transgenes had been transmitted through male germinal cells.

CaMV 35S–*mudrA* Transgene Reactivates *Mu1* Excisions in an Epigenetically Silenced *MuDR* Background

Because the fully spliced *mudrA* cDNA and its deletion derivatives were sufficient to demethylate *Mu1* elements in callus (Figure 2), we asked if they were also sufficient to reactivate epigenetically silenced *Mutator* lines or whether the transgenes were susceptible to homology-dependent silencing (reviewed in Vaucheret et al., 1998) by silenced copies of *MuDR.* First, do CaMV 35S–*mudrA* transgenes restore somatic excision activity in silenced lines? We crossed the transgenes into 10 families containing multiple copies of silenced *MuDR* elements and visually scored reactivation of excision of a *Mu1* element at a pigment locus (*bronze2*) that had been somatically stable (zero spots per ear) or silencing (zero to 20 spots per kernel) in the previous generation. As illustrated in Figure 8A, the silenced *MuDR* sources were homozygous *bz2*::*mu1* and were crossed as pollen onto three types of *bz2* females: those hemizygous for the CaMV 35S–*mudrA* transgene (cA+ or dcA+), their herbicide-sensitive siblings (cA− or dcA−) (negative controls), and a multicopy active *MuDR* source (positive control). As shown in Table 4, 17 out of 20 ears hemizygous for the cA75A+ and cA75B+ transgenes showed reactivation of somatic excisions at *bz2*::*mu1* (1328 of 7071 kernels, or 19%), whereas none of the 18 herbicide-sensitive siblings produced any spotted kernels (zero of 5125 kernels).

On a per ear basis, the reactivation by cA75+ (85%) was comparable to reactivation by a source of multicopy, active *MuDR* (90%). On a per kernel basis, 21% of the maximum 50% cA75B+ kernels were spotted (934 of 4461), whereas 54% of the maximum 100% multicopy *MuDR* kernels were spotted (937 of 1740). In contrast, none of 13 ears carrying the epigenetically unstable cA36+ transgene reactivated (zero of 3812 kernels). In a small test, neither of two previously described truncated transgene lines reactivated somatic excision: dcA31+ (zero of 862 kernels) and dcA74+ (zero of 127 kernels). An additional *mudrA* transgenic line carrying a 1.8-kb 3′ truncation, dcA76+, also failed to reactivate excision at *bz2*::*mu1* (zero of 2811 kernels). Therefore, in this experiment, a stable, full-length *mudrA* transgene, but not deleted transgenes, was able to reactivate *Mu1* element excisions in epigenetically silenced *MuDR* lines.

Methylation of *Mu1* TIRs is a molecular correlate of *MuDR* epigenetic silencing. Therefore, by DNA gel blot hybridization analysis, we asked if expression of the full-length *mudrA* transgene could demethylate *Mu1* element TIR HinfI sites, which had become methylated after *MuDR* silencing. From one cA75B+ ear (segregating 1:1 for the transgene) that had inherited a pool of methylated *Mu1* and silenced *MuDR* elements, unspotted, herbicide-sensitive progeny showed a high degree of TIR methylation in leaf 4 DNA. In contrast, spotted, herbicide-resistant siblings showed partial to nearly complete DNA demethylation at the *Mu1*/*Mu2* TIRs in leaf 4 DNA samples (Figures 8B and 8C). Although

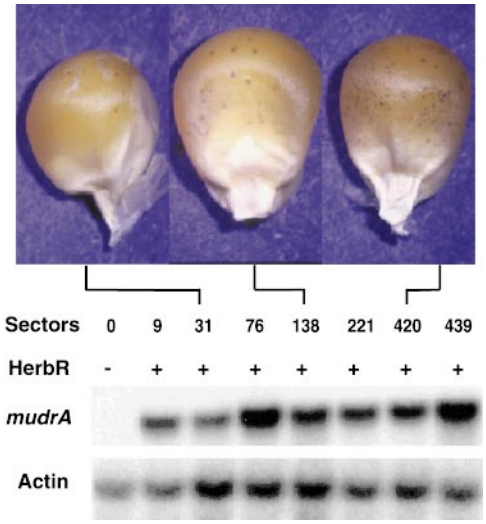

Figure 6. Aleurone *Mu1* Excision Frequency Compared with *mudrA* Transcript Abundance.

All kernels were from a single cA75B+ ear of the T_3 generation, and they are ranked by the number of excision sectors in the aleurone, from zero at left to 439 at right. Seedlings were scored for herbicide resistance, and RNA was isolated from the tips of leaf 4. The same RNA gel blot was probed with the 1.3-kb *mudrA* probe and subsequently with a maize actin probe used as an RNA loading control.

demethylation by the transgene was weaker than by the active *MuDR* source, expression of the transgene clearly resulted in the demethylation of multiple *Mu* elements. Thus, based on excision and demethylation, CaMV 35S–*mudrA* does reactivate epigenetically silenced *Mutator* lines.

Does the transgene first reactivate *MuDR* and then become silenced by homology-dependent transgene silencing? We asked if the CaMV 35S–*mudrA* transgene had reactivated transcription of epigenetically silenced *MuDR* elements in the same spotted or control kernel siblings used in the demethylation test. As shown in Figure 8D, we performed RNA gel blot analysis on these plants using total RNA from pooled immature ears, a tissue previously shown to express very high levels of *MuDR* transcript in active *Mutator* lines (Hershberger et al., 1995). As expected, immature ears from cA75B+ but not cA75B− plants expressed high levels of *mudrA* transcript. RT-PCR demonstrated, however, that the source of all transcript was the CaMV 35S–*mudrA* transgene and not reactivated *MuDR* elements (Figure 8E). In addition, RNA gel blot analysis and RT-PCR experiments using total RNA from immature ear tissue failed to detect *mudrB* transcript, which would be expected from restoration of *MuDR* transcription (Figures 8D and 8E). Therefore, the CaMV 35S–*mudrA* transgene does not appear to transcriptionally reactivate the resident epigenetically silenced *MuDR* elements during the somatic growth of the plant, although the transgene itself remains transcriptionally active.

The continued transcriptional silencing of *MuDR* elements implies that much of the excision and demethylation activity seen in these progeny was programmed directly by the CaMV 35S–*mudrA* transgene and not by reactivating *MuDR* elements. Further support for this comes from the result that the deleted transgenes were unable to cause excisions in inactive *Mutator* lines (Table 4), even though they could demethylate *Mu1* elements (Figure 2B). Because the full-length *mudrA* transgene did not reactivate transcription of silenced *MuDR* elements but did cause the demethylation and excision of *Mu1*/*Mu2* elements, we conclude that the transgene remains functional in nuclei containing homologous silenced copies of *MuDR*. Therefore, CaMV 35S–*mudrA* is not measurably silenced, transcriptionally or post-transcriptionally, by these homologs.

DISCUSSION

We have successfully generated transgenic maize expressing the *Mutator* transposase MURA. Experiments were made possible by the discovery that whereas the *mudrA* gene is toxic to bacteria in plasmid form, it is stable in M13. Direct DNA transfer of M13 clones may finally permit *Mu* transposon tagging in other species and determination of whether maize-specific host factors are required for the regulation of *Mutator* phenomena.

MuDR encodes two genes, *mudrA* and *mudrB*, both of which produce multiple transcripts (Figure 1B). We have demonstrated that the fully spliced *mudrA* cDNA coding region (Figure 1C), predicted to encode 823 amino acids, is

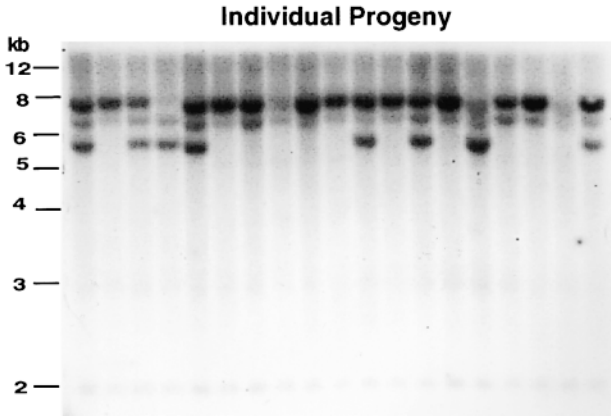

Figure 7. DNA Gel Blot Screen for *Mu1* and *Mu2* Germinal Insertions in the Progeny of CaMV 35S–*mudrA* Plants.

Line cA75B+ progeny were used, and all kernels were spotted. DNA was digested with HindIII and probed with the *Mu1*/*Mu2*–specific probe pA/B5. Only parental fragments, visualized as segregating bands in the progeny, are seen. DNA gel migration size standards are indicated at left.

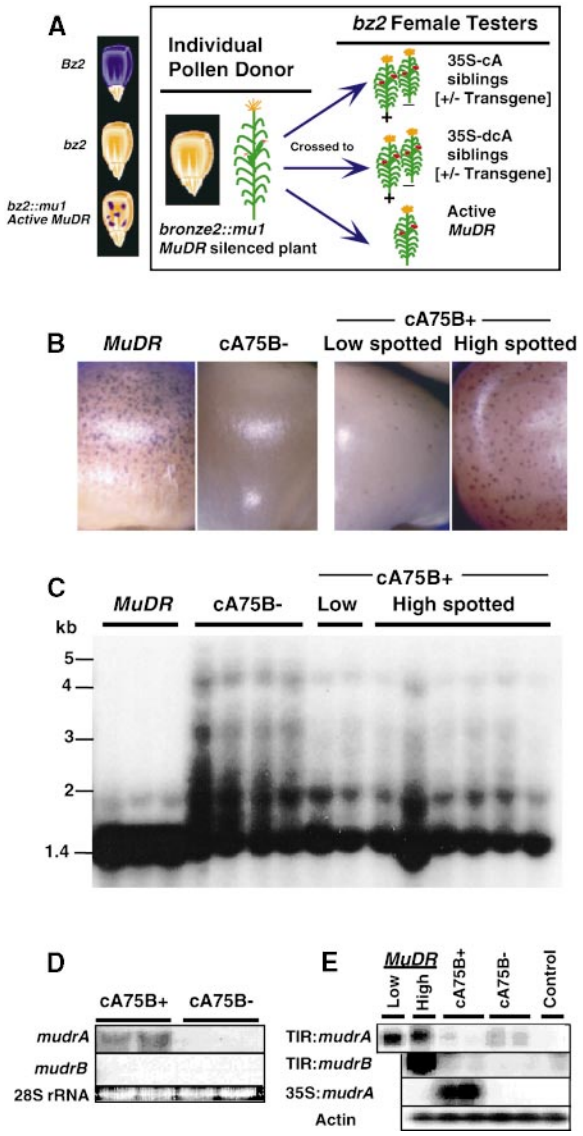

Figure 8. Stability of the CaMV 35S–*mudrA* Transgene in the Presence of Multiple Silencing *MuDR* Elements.

(A) At left is a cartoon of aleurone genotypes and phenotypes at the *Bz2* locus. At right is a schematic of the genetic experiment.

(B) Kernel progeny showing reactivation of excisions at *bz*::*mu1* from a previously silenced *MuDR* line. Pollen from a silenced *bz2*::*mu1* individual was crossed onto *bz2* testers containing either high-copy active *MuDR* or the cA75B+ transgene. The cA75B+/− kernels are siblings from the same ear.

(C) DNA gel blot analysis of the methylation status at HinfI sites within the MURA binding sites in the TIRs of *Mu1* and *Mu2* elements (see Figure 2 for assay description). Kernels pictured in **(B)** were planted; DNA from leaf 4 was digested with HinfI, and DNA gel blots were probed with the *Mu1*/*Mu2*–specific probe pA/B5. DNA gel migration size standards are indicated at left.

(D) RNA gel blot analysis of immature ear tissue from plants derived from cA75B+/− kernels shown in **(B)** and analyzed in **(C)**. The *mudrA*- or *mudrB*-specific probes were hybridized against 15 μg of total RNA. Each lane represents ear RNA pooled from two plants.

(E) RT-PCR analysis of cA75B+/− immature ears. Samples are from the plants analyzed in **(B)** through **(D)**. This analysis was designed to detect the presence of reactivated *mudrB* (TIR:*mudrB*) transcripts from the silenced *MuDR* elements and to distinguish between *mudrA* transcripts originating from the CaMV 35S–*mudrA* transgene or from the silenced *MuDR* (TIR:*mudrA*) elements. For RT-PCR, we used promoter-specific 5′ PCR primers. PCR products were DNA gel blotted and probed with the *mudrA*-, *mudrB*-, or actin-specfic probes. The *MuDR* sample is from a high-copy *MuDR* line. The control is a non-*MuDR* tester. Actin primers were included during RT-PCR as an internal control.

sufficient to catalyze a high frequency of developmentally late somatic excisions in transgenic maize (Figures 4 and 5). Consequently, transcriptional and post-transcriptional regulation acting through the 5′ or 3′ UTRs and intron retention are not required to set excision timing. The 2.7-kb cDNA clone complements two other known functions of an active 4.9-kb *MuDR* element: demethylation of *Mu* TIRs (Figure 2B) and partial reactivation of epigenetically silenced *Mutator* lines (Figure 8 and Table 4). We find no evidence that the cDNA clone under the control of a CaMV 35S promoter catalyzes germinal insertions (Figure 7).

CaMV 35S–*mudrA* Is Sufficient to Catalyze Somatic Excisions

We have demonstrated that the CaMV 35S–*mudrA* transgene programs a high frequency of *Mu1* somatic excisions from the anthocyanin pigment locus *a1* (Figure 4 and Table 2) and, more recently, from two maize *Lc*::*mu1* transgenic loci (M.N. Raizada and V. Walbot, unpublished results). By a genetic test, excisions at *a1-mum2* appear to be directly caused by the CaMV 35S–*mudrA* cDNA transgene and not by a contaminating or cryptic *MuDR* element (Table 3). The cDNA corresponds to the fully spliced mRNA, which represents ~61% of *mudrA* transcript in multicopy *MuDR* seedlings (Hershberger et al., 1995). The transgene is predicted to encode an 823–amino acid protein, but we cannot rule out the involvement of smaller polypeptides resulting from internal initiation. By design, the alternative 736–amino acid protein predicted to be translated when *mudrA* intron 3 is retained could not be produced in our transgenic lines. Intron 3 has an intriguingly rare 5′ splice site consensus sequence (Jackson, 1991) and fails to splice in ~20% of seedling transcripts (Hershberger et al., 1995).

Somatic excision frequency from *a1-mum2* is similar when the transposase is supplied by CaMV 35S–*mudrA* or a single copy of *MuDR*. Therefore, *MuDR*-encoded MURB is not required for frequent somatic excision nor for its timing. The caveat in our conclusion is that all maize lines tested

Table 4. Reactivation of Somatic Excisions at *bz2*::*mu1*[a]

Parent	Progeny		
Female Tester	Spotted Ears	Unspotted Ears	Percentage of Ears Reactivated
cA75A−	0	9	0
cA75A+	3	3	50
cA75B−	0	9	0
cA75B+	14	0	100
cA36−	0	10	0
cA36+	0	13	0
dcA31−	0	1	0
dcA31+	0	2	0
dcA74−	0	3	0
dcA74+	0	1	0
MuDR	13	1	90

[a] The experiment is described in Figure 8A.

contain MURB polypeptides expressed from *mudrB* homologs (*hmudrB*); because some homologs contain only a single amino acid substitution, they could conceivably substitute for wild-type MURB (G. Rudenko and V. Walbot, unpublished results).

Plants Expressing the *mudrA* cDNA Retain Late Excision Timing

A striking feature of *Mutator* activity is that excisions are restricted to the final cell divisions of somatic tissues. This developmental control was hypothesized to result from programmed retention of intron 3, use of alternative transcription or polyadenylation sites, or translational control exerted through the 5′ leader sequence (Hershberger et al., 1995). These possibilities are ruled out by our results, because these features are missing from the expression vector. In maize, the CaMV 35S promoter has strong activity in both mature tissues and meristematic cells, such as at the root apex (Omirulleh et al., 1993), and yet it programs the late excision timing characteristic of *Mutator.*

Other hypotheses to explain late excision timing are that *mudrA* is not translated in meristematic cells or that MURA does not interact with its target chromatin site in these cells. For example, during human V(D)J recombination to generate immunoglobin genes, recombinase activity is controlled by changes in chromatin structure (Stanhope-Baker et al., 1996). Benito and Walbot (1997) previously demonstrated that the 823–amino acid MURA polypeptide is a DNA binding protein in vitro, specific to the TIRs. In this study, we have demonstrated in vivo that *mudrA* expression results in the demethylation of the MURA binding site in both embryogenic callus cells and differentiated leaf cells, implying that MURA is in fact translated and interacts with its DNA target in both cell types (Figure 2). It appears, therefore, that *Mu* excision timing is regulated post-translationally.

Our callus result is consistent with two previous studies (James and Stadler, 1989; Planckaert and Walbot, 1989) that found that *Mu1* elements in calli derived from active *Mutator* plants can remain stably unmethylated at the HinfI TIR sites for many months in tissue culture. Furthermore, plants regenerated from these calli have unmethylated TIRs. Cultures derived from an inactive *Mutator* line retain largely methylated TIRs in culture and during plant regeneration. Collectively, all results suggest that the methylation status at the TIR HinfI sites reflects the presence or absence of *MuDR* proteins, even in meristematic cells.

The control of transposition timing in plants is poorly understood (reviewed in Fedoroff, 1989). An increase in copy number leads to later excisions in the case of *Ac* (Brink and Nilan, 1952; Scofield et al., 1993) but earlier excisions by *Dotted* (Coe and Neuffer, 1977). We speculated that our transgenic MURA source might be present at subthreshold levels. When we altered the parental transmission and hence gene dosage of the CaMV 35S–*mudrA* transgene, we found no change in excision timing (Figures 5E and 5F). This result is in accordance with all previous observations in which excision timing is independent of *MuDR* copy number. With a reliable and active MURA transgene (line cA75B+) in maize, which is incapable of self-transposition, it may now be possible to select *trans*-acting excision timing mutants. To date, the only alteration in *Mu* timing is a single line with the *bz1*::*mu1* reporter gene in which excision is much earlier than normal in the aleurone (Walbot, 1992).

Excision Frequency Is Not Correlated to *mudrA* Transcript Levels

Although *MuDR* transcript levels vary widely, most *Mutator* lines catalyze the excision of *Mu* elements in the aleurone at comparable frequencies. In this report, we have directly demonstrated that there is not a correlation between *mudrA* transcript levels and excision frequency. We propose that an additional post-transcriptional, rate-limiting step, conferred by a host-encoded factor or a feature of the *mudrA* coding region, controls excision frequency. One possibility is the third base codon usage of *MuDR* exons, which is very biased toward A or U, as found in rarely expressed maize genes (Fennoy and Bailey-Serres, 1993); it is possible that this rare codon usage contributes to the lack of correlation observed between *MuDR* transcript and activity levels. *Ac* and *Spm* also exhibit biased codon usage (Fennoy and Bailey-Serres, 1993). The poor translation of transposase transcripts may be an adaptive feature to buffer the maize host from quantitative changes in transcript levels resulting from transposon duplications.

No Evidence for Germinal Insertions

We detected no *Mu* germinal insertions in 72 progeny tested (Figure 7). This result could be interpreted as a requirement for MURB or another form of MURA. It is interesting that the d201 *MuDR* deletion line reported by Lisch et al. (1999), which has a complete *mudrA* gene but lacks *mudrB*, also failed to cause germinal insertions of *Mu* elements in 193 plants tested. However, *Mu1* elements in line d201 retain HinfI site methylation, and somatic excision is less robust than in our CaMV 35S–*mudrA* lines. Therefore, we propose that silencing rather than the lack of *mudrB* per se might be responsible for the lack of germinal insertions observed by Lisch et al. (1999).

In our transgenic lines, the lack of germinal insertions may also be due to poor expression of the transgene in germinal cells. Whereas the CaMV 35S promoter has strong expression in maize leaves, there is almost no expression in maize microspores in transient assays (Fennell and Hauptmann, 1992; Jardinaud et al., 1995). Furthermore, there is no expression in mature pollen of stably transformed *Arabidopsis* and only very weak expression in transgenic tobacco pollen (Wilkinson et al., 1997). By contrast, the *MuDR* TIR promoters have weak activity in suspension cells (Benito and Walbot, 1994) but produce abundant transcripts in pollen (M.N. Raizada and V. Walbot, manuscript submitted for publication).

The CaMV 35S–*mudrA* transgene may also be translated at levels less than those required for germinal insertion but not for somatic excision. The construct has an altered translation start sequence and possesses no introns, which are known to boost expression of transgenes in maize (Callis et al., 1987). In addition, the translation start sequence of *mudrA* in the transgene has been changed from its native CCAUGG to UCAUGG; in an analysis of 85 maize genes, this first-position cytosine was found in 58% of AUG start codons, whereas uracil, the rarest base used, was found only 8% of the time (Luehrsen and Walbot, 1994).

Aside from germinal insertions, we recently demonstrated that *MuDR* catalyzes a high frequency of somatic insertions by using a transgenic *Mu1* element that permits us to plasmid rescue new *Mu* insertions (*RescueMu*::*Lc*; M.N. Raizada and V. Walbot, manuscript in preparation). After crossing CaMV 35S–*RescueMu*::*Lc* to CaMV 35S–*mudrA*, however, we were unable to detect somatic insertions in leaves with this assay. This leaves open the possibility that wild-type *mudrB* may be required for any *Mu* insertion activity.

mudrA Transgene Is Active in the Presence of Methylated, Silencing Copies of *MuDR*

Host-induced silencing of viruses and transgenes is seen as part of an endogenous defense mechanism against pathogens and transposons (reviewed in Martienssen, 1996; Vaucheret et al., 1998). A viral transgene can silence its homolog in a subsequently introduced virus (Ratcliff et al., 1997). In this report, we expressed a plant transposase as a transgene in its native host, in a background containing endogenous copies of the transposon. We predicted dramatic transgene silencing for several reasons. First, although the mechanisms of silencing are not understood, it is certain that sequence homology recognition is a key component (Napoli et al., 1990; Meyer et al., 1993; Park et al., 1996). It is also hypothesized that transgene repeats and high RNA expression can trigger silencing (reviewed in Vaucheret et al., 1998). Finally, multicopy *MuDR* lines also produce abundant transcript (Hershberger et al., 1995), and unlinked *MuDR* elements undergo simultaneous methylation and cosilencing even in the absence of a homologous transgene (reviewed in Walbot, 1991; Fedoroff and Chandler, 1994).

We therefore speculated that a multicopy *mudrA* cDNA, with nearly 100% identity to the long coding regions of endogenous, presumably methylated copies of *MuDR*, would additively result in “super-silencing” of the transgene. However, when we introduced the *mudrA* cDNA into a background containing multiple copies of actively silencing and recently silenced copies of *MuDR* (Figure 8A), the transgene retained excision activity (Figure 8B and Table 4), TIR demethylation activity (Figure 8C), and expression on RNA gel blots (Figures 8D and 8E). Therefore, we found no evidence of homology-induced transgene silencing transcriptionally or, by inference, post-transcriptionally.

One hypothesis to explain the apparent lack of epigenetic interaction of the CaMV 35S–*mudrA* with endogenous *MuDR* elements is that *MuDR* silencing may require the TIRs. The *mudrA* and *mudrB* promoters are nearly identical (166 of 168 bp) and are located within the TIRs. When silencing occurs, both *mudrA* and *mudrB* transcripts are undetectable (Hershberger et al., 1991), and methylation of the TIRs is associated with this silencing. The TIRs also encode the 5′ UTRs of all *mudrB* transcripts and half of the *mudrA* transcripts (those initiating at position +169). These 5′ UTRs share 40 of 47 bases of identity (Hershberger et al., 1995). Consequently, simultaneous silencing of *mudrA* and *mudrB* is likely accomplished through transcriptional and/or post-transcriptional mechanisms acting through the TIRs. If this is true, our transgene, devoid of its native TIR, would not be affected by the silencing process. As a practical implication, such an epigenetically stable source of MURA might prevent silencing of *Mu* elements during future transposon-tagging experiments by preventing maintenance methylation of the TIRs.

Prospects for Gene Tagging and Understanding *MuDR* in Transgenic Plants

Although the CaMV 35S–*mudrA* construct used in this experiment does not program a high frequency of germinal insertions, we are very optimistic that it will be possible to transfer *Mutator* activities to heterologous hosts. In maize, we know that a single, full-length *MuDR* element programs

germinal insertions (Lisch et al., 1995). With the use of M13, it should now be possible to clone and deliver genomic *mudrA* and even entire *MuDR* elements to other organisms by biolistic transformation. Versions of maize transposons *Ac* and *Spm* are used in efficient transposon-tagging lines in Arabidopsis and tomato (Osborne and Baker, 1995; Wisman et al., 1998). It will be intriguing to assay the developmental timing of *Mu* excisions and insertions in these or other hosts. In fact, both Arabidopsis (GenBank) and rice (Eisen et al., 1994; Yoshida et al.,1998) contain *mudrA*-like sequences, suggesting that *Mu* elements might have been active previously in diverse dicot and monocot species.

METHODS

Plasmid Vectors

phMR53 (cA transgene) is the *mudrA* cDNA clone that was reconstructed in vitro and stabilized in a low-copy yeast vector, as previously described (Benito and Walbot, 1997). The yeast plasmid was then used as the template for polymerase chain reaction (PCR) amplification. The 5′ primer sequence was GCGAATTCATGGACTTGACGCCCAG and the 3′ primer sequence was CGGAATTCCTACATAACAGTCTTACAAC. Amplification conditions were 95°C for 45 sec, 51°C for 1 min, and 74°C for 3 min for 28 cycles using Pfu polymerase (Stratagene, La Jolla, CA). The cDNA clone starts at position +449 of the published *MuDR* sequence (Hershberger et al., 1991) at the putative ATG translational start site, changing the sequence context from CCATGG to TCATGG. The clone ends at position +3209 at the end of cDNA clone C4 and includes 63 bp of the native 3′ untranslated region (UTR) but none of the native polyadenylation signals (Hershberger et al., 1995). The amplified product was subcloned into the EcoRI site of Phagescript SK (Stratagene) to give clone phMR49. All cDNA ligation products checked inserted in the same orientation in the Phagescript vector, as did a separate cloning of a nearly full-length genomic *mudrA* clone. Attempts made to ligate the cDNA or genomic clones in the opposite orientation were unsuccessful. The double-stranded replicative form of M13 was first in vitro transcribed using T3 RNA polymerase and in vitro translated using rabbit reticulocyte extract to detect the presence of frameshifted clones. All clones gave full-length protein products. The cDNA was then subcloned as an EcoRI fragment from phMR49 into the same sites in phMR52 (M13) to give phage MR53. The cDNA thus contains a new 130-bp cauliflower mosaic virus (CaMV) 35S leader sequence. Large amounts of double-stranded DNA were isolated 4 hr after transfection of XL-1 Blue cells (OD_{600nm} = 0.4; Stratagene). Both the transfection time and the number of times the clone was transfected were minimized to prevent the selection of internal deletions in M13. The final clone was fully sequenced as double-stranded DNA and found to be intact. The same sample of DNA used for sequencing served as the source for biolistic transformation.

phMR52 is a potentially useful M13 cassette vector for the cloning and expression of toxic genes in plants. The nopaline synthase (nos) terminator was subcloned from plasmid pR as an XbaI-EcoRI fragment, filled in with the Klenow fragment of DNA polymerase I, and then blunt-ligated to the HincII site of pBluescript KS+ (Stratagene) to give clone pMR50. The CaMV 35S promoter from +7072 to +7565 (Franck et al., 1980) from pJD255 was then added as a SstI-PstI subclone into the same sites of pMR50 to give clone pMR51, a new CaMV 35S–nos cassette in pBluescript KS+. This clone includes 130 bases of the native CaMV 35S leader sequence. The CaMV 35S–nos sequence was then subcloned as a XhoI-SacI fragment into the same sites in Phagescript SK to give phage MR52.

pAHC20 is the ubiquitin promoter–*Bar* herbicide resistance plasmid that was kindly provided by P. Quail (Plant Gene Expression Center, Albany, CA) (Christensen and Quail, 1996).

Stable Maize Transformation

A detailed protocol can be found at http://www.stanford.edu/~walbot/StableMaizeTransf.html. Briefly, embryogenic A188 × B73 (HiTypeII) calli (Armstrong and Green, 1985; Armstrong, 1994) were osmotically treated (Vain et al., 1993) and then transformed using the PDS 1000HE biolistic device (BioRad, Hercules, CA) at 650 psi; this was repeated at 1100 psi in a vacuum of 27 psi (Gordon- Kamm et al., 1990; Sanford et al., 1993). The distance from the rupture disc to the macrocarrier was 1 cm, and that from the mesh screen to the target was 5.9 cm. For three bombardments, 25 μg total of phMR53 and pAHC20 was coprecipitated in equimolar quantities onto 2 mg of 1 μM spherical gold particles (Alameda Scientific Instruments, Richmond, CA) using the procedure of Wan et al. (1994). Transformed calli were selected on 3 mg/mL bialaphos (Meiji Seika Kaisha Ltd., Yokohama, Japan) (Spencer et al., 1990). Individual resistant callus lines (designated cA1, cA2, cA3, etc.) were checked by RNA gel blot hybridization for the presence of transgene expression and selectively regenerated. The initial regenerated plants were called T_0, whereas the first seed belonged to the T_1 generation.

Leaf Herbicide Test

To test for bialaphos resistance, a 5-cm-diameter marked leaf surface was painted with 0.75% glufosinate ammonium (Ignite 600, 50% solution; Hoescht, Montreal, Canada) with 0.1% Tween 20 using a Q-tip. The area was visually scored for the presence or absence of necrosis 5 to 7 days later.

Herbicide and *MuDR* Transgene Cosegregation and Stability Tests

Transgenic lines were chosen that exhibited a 1:1 cosegregation pattern of leaf herbicide resistance to stable *mudrA* transgene expression in 8 to 20 T_1 progeny of an outcross. Thereafter, herbicide resistance was used to identify plants carrying the *mudrA* transgene. To examine transgene expression, we performed reverse transcription (RT)–PCR analysis on total leaf RNA isolated using Trizol (Gibco BRL, Rockville, MD). Two micrograms of total RNA was added to 2 μL of 5 × First Strand Buffer (Gibco BRL), 0.5 μL of 100 mM DTT, and 50 ng of *mudrA* RT-primer GATATGCATGGACCAAAGGCAC at *MuDR* position +1530 (*MuDR* numbering according to Hershberger et al., 1991) in a volume of 8 μL. The mix was heated for 5 min at 70°C and chilled on ice for 30 sec. Then a 2-μL cocktail was added consisting of 0.5 μL of 10 mM deoxynucleotide triphosphates (dNTPs), 0.5 μL RNasin (40 units per μL; Promega, Madison, WI), and 1 μL of Superscript II RT (200 units per μL; Gibco BRL), and the mixture was incubated at 42 to 50°C for 1 hr. For internal verification or to detect dcA line transcripts, two PCR products were generated.

Two and a half microliters of the first-strand cDNA was directly added to a 25-μL reaction consisting of AmpliTaqII buffer (Perkin-Elmer, Foster City, CA), 0.2 mM dNTPs, 2 mM $MgCl_2$, 100 ng of 5′ primer CaMV 35S +99 −122 (CGCTCATGTGTTGAGCATATAAG), 100 ng of 3′ primer *mudrA* +1412 (GCTCGAGTACAAGAGCTG-GAAGCT), and 100 ng of a second 3′ primer *mudrA* +1090 (CTTACA-GTATCCAACGTATC) with 1 unit of AmpliTaq. Cycle conditions were 3 cycles of 95°C for 1 min, 42°C for 1 min, and 72°C for 2 min, and 32 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C 2 min.

Verification of Kernel Genotype

Ears exhibiting somatic excisions at *a1-mum2* and *bz2*::*mu1* nearly always cosegregated with herbicide resistance. With a few exceptions, susceptible plants gave rise to spotted kernels. To check if these kernels still inherited the CaMV 35S–*mudrA* transgene but had experienced *Bar* transgene silencing, we performed PCR analysis on seedlings derived from these spotted kernels. PCR conditions were identical to those listed above, with 5 ng of genomic DNA used per 25-μL reaction. As an internal PCR control, 100 ng of the following maize actin primers were added to the reaction: 5′ primer pMAcI 5′ +464 (GCCTACGTAGGTGATGAGGCTCAGGC) and 3′ primer pMAcI 3′ +867 (CTCACACCATCACCTGAATCCATCAC) (Shah et al., 1983). The PCR products were electrophoresed, and a DNA gel blot was made and hybridized with either the *mudrA*-specific probe or maize actin probe.

Verification That Cryptic *MuDRs* Did Not Cause Excisions

To ensure that the presence of excisions on kernels was caused by the *mudrA*-823 transgene and not by the reactivation of a tightly linked cryptic or contaminating *MuDR*, a total of 357 heavily spotted *a1-mum2* kernels derived from outcrosses to *a1* tester were pooled from ~20 ears of line cA75B, germinated, and tested for herbicide resistance (Table 3). All but nine seedlings displayed full herbicide resistance. The nine partially sensitive seedlings were tested for the presence of the *mudrA* transgene by PCR analysis, using the 5′ primer CaMV 35S +99 −122 and 3′ primer *mudrA* +1412, as described above; the presence of CaMV 35S–*mudrA* was verified by DNA gel blotting.

Nucleic Acid Probes

BX1.0 cross-hybridizes to both *mudrA* and *mudrB* transcripts and extends from the BamHI (+2865) to the XbaI (+3945) site in *MuDR* (Hershberger et al., 1995). The 1.3-kb *mudrA*-specific probe extends from *MuDR* sites +450 to +1790 (SphI site) and was isolated as a 1.3-kb PstI-SphI fragment from phage phMR49. The *mudrB*-specific probe extends from the StuI (+3630) to StuI (+4310) sites of *MuDR* and was isolated from plasmid pMR29. The *Mu1*-specific probe is the 650-bp AvaI-BstN1 internal fragment of *Mu1* and was isolated as a SmaI fragment from plasmid pA/B5 (Chandler and Walbot, 1986). It cross-hybridizes to *Mu1* (1.4 kb), *Mu2* (1.75 kb), and *Mu1.0* (~1 kb). The pBluescript KS+ probe is the entire 2.9-kb plasmid (Stratagene) that hybridizes to the pUC18 backbone of the *Bar* plasmid pAHC20 (Christensen and Quail, 1996). The CaMV 35S probe extends from +7072 to +7565 (Franck et al., 1980) and was isolated as a XbaI-PstI fragment from plasmid pR (Ludwig et al., 1990). The maize actin probe extends from +362 to +1338 (Shah et al., 1983) and was generated by PCR from plasmid pMAc1 obtained from R. Meagher (University of Georgia), using the PCR conditions listed above. Actin primer sequences were 5′-GCCGGTTTCGCTGGTGATGATGCGCC-3′ (5′ primer) and 5′-GTGATCTCCTTGCTCATACGATCGGC-3′ (3′ primer). Ten to 50 ng of probe DNA was prepared using a Deca-PrimeII random primer kit (Promega) and ^{32}P-dCTP (Amersham, Little Chalfont, UK), incubated at 37°C for >3 hr, and then purified on a NucTrap push column (Stratagene).

Identification of CaMV 35S–*mudrA*—Catalyzed *MuDR* Reactivated Transcripts

To identify endogenous *mudrA* transcripts, we performed RT-PCR as described above. The *mudrA* RT primer was GATATGCATGGACCA-AAGGCAC at +1530, and PCR primers were 5′ *mudrA* +210 CTC-CTCTAAATGCTCTCTGG and 3′ *mudrA* +1412 GCTCGAGTACAA-GAGCTGGAAGCT (numbering according to Hershberger et al., 1991). To identify reactivated *mudrB* transcripts, we used oligo-dT as the RT primer, and PCR primers were 5′ *mudrB* +4730 CTTGTA-CAGATCTTGTGACCAGTCGCA and 3′ *mudrB* +3780 GTCCAC-AAATCGATGTTACGGTCGTT. CaMV 35S–*mudrA* transcripts were identified as described above. As an internal control, actin was amplified using RT primer oligo-dT and PCR primers pMAcI 5′ +464 and pMAcI 3′ +867 (above).

Sector Size Scoring

To score the *a1-mum2* excision sector size distribution, we made close-up slides of randomly chosen kernels and projected them onto a wall surface. The number of cells in each sector were either scored visually if cell walls were clearly visible or scored by comparing the area of a single cell sector with the area of larger sectors in the transect by using a ruler on the projected surface. All sectors in focus were selected for scoring. On the curved aleurone surface, peripheral cells containing diffused anthocyanin pigment were subjectively excluded. For each distribution series, 20 to 25 sectors from each of four to six randomly chosen kernels were scored. All scoring was performed in blind test by undergraduate assistants.

Plant Materials

Kernel aleurone anthocyanin pigmentation requires the presence of the structural genes of the anthyocyanin pigment pathway, including *A1* and *Bz2*, in addition to its transcription factors *R* and *C1*. The T_0 transgenic plants were in an A188/B73 background (non-*MuDR*; *Bz2 A1 r*−/*r*− *C1*/*c1*) and were outcrossed to a W23 inbred *bz2* tester line (non-*MuDR*; *bz2*/*bz2 A1 R*− *C1*) (T_1 progeny).

a1-mum2 *Somatic Excision Material*

T_1 herbicide-resistant plants were then outcrossed either to an inbred *a1* tester line (non-*MuDR*; *a1*/*a1 Bz2 R*− *C1*) or to a line containing a *Mu1* element at the *a1* locus in the *a1-mum2* tester line (zero copy *MuDR*; *a1-mum2*/*a1-mum2 Bz2 R*− *C1*) to generate a non-*MuDR*, transgene-containing line in an *A1*/*a1* or *A1*/*a1-mum2* background (T_2 progeny). To look for somatic excisions in the T_3 generation (Figure 4 and Table 2), we crossed both herbicide-resistant and herbicide-sensitive sibling plants of the *A1*/*a1* genotype (T_2 genera-

tion) reciprocally to the *a1-mum2*/*a1-mum2* tester line to generate the genotype *R C1 Bz2*/− *A1*/*a1-mum2* or *a1*/*a1-mum2.*

For *A1*/*a1-mum2* T_2 plants, a subset (herbicide-resistant and herbicide-sensitive siblings) was crossed reciprocally to the *a1*/*a1* tester to generate T_3 progeny with the genotype *R C1 Bz2*/− *A1*/*a1* and *a1*/*a1-mum2* to look for somatic excisions. These ears were scored to determine w hether the expected 25% of kernels were spotted. To verify that excisions required the transgene source, we selected these *a1-mum2* spotted kernels *(R C1 Bz2 a1*/*a1-mum2*), and the resulting plants were outcrossed reciprocally to the inbred *a1* tester line. Spotted-kernel T_4 generation progeny (*R C1 Bz2 a1*/*a1-mum2*) were randomly chosen and assayed for herbicide resistance (Table 3). The other subset of *A1*/*a1-mum2* T_2 generation plants (both herbicide-resistant and herbicide-sensitive siblings) was crossed reciprocally to the *a1-mum2*/*a1-mum2* tester to generate T_3 progeny with the genotype *R C1 Bz2*/− *A1*/*a1-mum2* and *a1-mum2*/*a1-mum2.*

bz2::mu1 *Silenced Mutator Material*

T_1 herbicide-resistant plants were outcrossed again to a W23 inbred *bz2* tester line (non-*MuDR; bz2*/*bz2*) to generate a non-*MuDR* but transgene-containing T_2 line in a *bz2*/*bz2 A1 R*− *C1* background. To study the effects of the transgene on reactivating an epigenetically silenced high-copy *MuDR* line in the T_3 generation, we crossed both herbicide-resistant and herbicide-sensitive sibling T_2 females in pairs by pollen from an individual silenced *MuDR* donor containing a silenced *Mu1* element at the *bz2* locus in the *bz2*::*mu1* allele (*bz2*::*mu1*/*bz2*::*mu1, A1 R*− *C1*; high-copy *MuDR* "off" line) (Figure 8 and Table 4). Excision from *bz2*::*mu1* could create a wild-type *Bz2*-expressing pigmented sector.

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