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A maize *MuDR* transposon promoter shows limited autoregulation

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Abstract Transgenic maize expressing luciferase under the control of the *mudrB* terminal inverted repeat promoter (TIRB) of the *MuDR* transposon was assayed for transgene expression in active and inactive *Mutator* lines. We find that active *MuDR* elements increase TIRB-luciferase expression by 2- to 10-fold, relative to non*MuDR* or silenced *MuDR* lines, in embryonic leaves in 75% of plants tested. However, this increase does not persist in juvenile and adult leaves. In pollen, TIRB-luciferase expression is up to 100-fold higher than in leaves but is unaffected by the presence or absence of active *MuDR*. Because the MURA transposase binds to a motif within TIRB, we hypothesize that MURA may act as a weak transcriptional activator of TIRB or may partly inhibit host-induced silencing of TIRB in active *Mutator* lines during the early stages of somatic growth. Our results contrast with those for the maize transposon *Spm*, in which the TNPA transposase acts as a repressor of the *Spm* promoter in active *Spm* lines.

Key words *Zea mays* · *Mutator* · *MuDR* promoter · DNA methylation

Introduction

Because transposons can increase mutation frequency, their activities are regulated by the host and by features intrinsic to each element system. Transcriptional controls include host-induced gene silencing and transposase-mediated autoregulation. Mobile elements have probably evolved mechanisms to inhibit or temporarily evade host-induced silencing, as suggested by the high copy numbers of retroelements in many organisms (SanMiguel et al. 1996; Wolffe and Matzke 1999). Transposase-mediated autoregulation may ensure transposon survival by protecting the host from dramatic increases in transposon copy number (reviewed in Fedoroff and Chandler 1994). For example, a programmed failure to splice intron 3 (IVS3) of the *Drosophila P* element results in the production of a truncated *P* transposase that acts as a repressor of *P* element transcription in somatic cells and oocytes (Lemaitre and Coen 1991). The TNPA transposase encoded by the maize *Spm* element inhibits transcription from its own promoter when the promoter is active, but acts as a transcriptional activator when the promoter is silent (Schläppi et al. 1994).

Mutator activity in maize (Robertson 1981) is caused by a high-copy-number, diverse family of *Mu* transposable elements. *MuDR* encodes proteins required for transposition of subfamilies of non-autonomous elements, *Mu1–Mu8* (Chomet et al. 1991; Hershberger et al. 1991; Qin et al. 1991; reviewed in Bennetzen et al. 1993). In most active *Mutator* plants, there are more than three copies of *MuDR* and up to 50 copies of various non-autonomous elements (reviewed in Bennetzen et al. 1993). *MuDR* encodes two genes, *mudrA* and *mudrB*, which are convergently transcribed from promoters located within their respective terminal inverted repeats, TIRA and TIRB (Fig. 1A); *MuDR* transcripts are very abundant in active Mutator lines (Hershberger et al. 1995). *mudrA* and *mudrB* transcripts each contain three introns (Fig. 1A); the two introns within each ORF are retained in some transcripts (Hershberger et al. 1995). Thus multiple polypeptides can potentially be encoded by each gene (summarized in Raizada and Walbot 2000).

A fully spliced *mudrA* cDNA encodes an 823-amino acid MURA protein that binds to *Mu* family TIRs within a 32-bp motif, the MURA binding site or MBS (Benito and Walbot 1997). The MBS sequence is highly conserved in the mobile *Mu* elements (*Mu1*, *Mu2*, *Mu3*,

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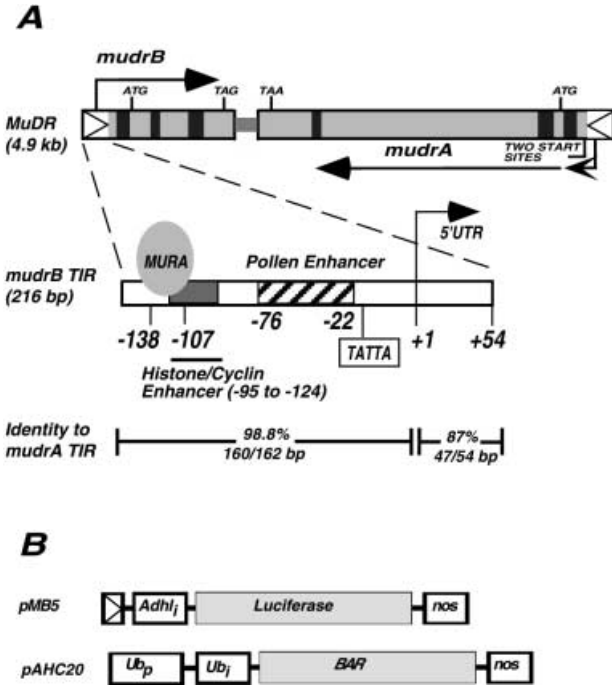

Fig. 1A, B Structures of the *MuDR* element, the *mudrB* terminal inverted repeat (TIR) promoter and the plasmids used to make transgenic maize plants. **A** Structure of the *MuDR* element and *mudrB* promoter. *Upper panel mudrA* and *mudrB* are encoded in antiparallel orientation and transcribed from promoters located in the terminal inverted repeats (*box with triangle*). The *gray* regions represent exons and *black boxes* represent introns. *Lower panel* The *mudrB* terminal inverted repeat consists of the promoter and part of the 5′ untranslated leader. Numbers indicate positions relative to the transcription start site. The putative TATA box is at position –16. Percentages represent the degree of nucleotide identity to the *mudrA* TIR. The MURA transposase is shown bound to the binding site defined in vitro (Benito and Walbot 1997). The region at –95 to –124 (indicated in *dark gray*) has strong homology to histone hexamer and nonamer motifs as well as the B cyclin (MSA) enhancer. The region at position –22 to –76 (*hatched box*) shows high nucleotide sequence identity to the pollen enhancers of the tomato genes *LAT59* and *LAT52*, and the maize gene *ZM13* (M. Raizada et al. 2001). **B** Plasmids used in this study. The 216-bp *mudrB* TIR is represented by a *box containing a triangle*. pAHC20 encodes resistance to the herbicide Basta. Abbreviations: i, intron; Ub, maize ubiquitin; p, promoter

Mu7, *Mu8* and *MuDR*) (Benito and Walbot 1994). The 823-amino acid MURA is also sufficient to program somatic excision of *Mu* elements (Raizada and Walbot 2000). This and other evidence (Eisen et al. 1994; Lisch et al. 1999) demonstrates that MURA is the *MuDR*/*Mu* transposase. The functions of other possible MURA proteins, if any, are unknown at present. The *mudrB* gene product, MURB, has been hypothesized to play a role in catalyzing *Mu* insertions, but no specific function in transposition has been demonstrated (Lisch et al. 1999; Raizada and Walbot 2000). Preliminary evidence indicates that MURB proteins exhibit non-specific DNA binding (G. Rudenko, M. Fitzgerald, S-H. Kim, A. Ono and V. Walbot, unpublished data).

In addition to MBS motifs (at –107 to –138, Fig. 1A), both TIRA and TIRB promoters contain a putative meristem transcriptional enhancer region (at –95 to –124) that partially overlaps the MBS. Downstream of the MBS is a strong pollen enhancer (at –22 to –76) (Raizada and Walbot, submitted). It is likely that these and perhaps other motifs are responsible for the high levels of *MuDR* transcripts measured by blot hybridization (Hershberger et al. 1995) and by in situ hybridization (Joanin et al. 1997).

We wanted to determine whether the polypeptides encoded by *MuDR* autoregulate their own promoters. One possible mechanism involves protection of the MBS region from host methylation, an activity that would have an impact on all *Mu* elements including *MuDR*. Protection could underlie the observation that in active Mutator lines, *Mu1* TIRs are hypomethylated. Loss of *MuDR* activity by segregation or epigenetic silencing results in the methylation of *Mu1* elements, in particular at the *Hin*fI site located within the MBS (Chandler and Walbot 1986; Bennetzen 1987). Introduction of transcriptionally active *MuDR* elements (Chandler et al. 1988) or a transgene encoding the 823-amino acid-MURA (Raizada and Walbot 2000) results in rapid demethylation of *Mu1* and *Mu2* TIRs. Because methylation is usually correlated with the loss of transcriptional activity, we hypothesized that the presence of an active *MuDR* element would enhance *MuDR* promoter activity by preventing epigenetic silencing.

Alternatively, we had reason to suspect that proteins encoded by *MuDR* would repress TIRA and TIRB promoter activities. Quantification of *MuDR* transcripts and proteins suggests that there is a non-linear correlation between element copy number and expression. Multicopy *MuDR* lines clearly have higher levels of *mudrA* and *mudrB* transcripts than single-copy lines. Joanin et al. (1997) noted, however, that *MuDR* transcript abundance in plants with 20 elements is only about two-fold higher than in lines with two copies. Secondly, MURA and MURB protein levels in diverse tissues are very similar in single-copy and several high-copy *MuDR* lines (G. Rudenko and V. Walbot, submitted). Thirdly, the frequency of somatic excision is similar in lines that express low and high levels of *mudrA* transcripts (Raizada and Walbot 2000). These data suggest that both *mudrA* and *mudrB* could be subject to feedback repression at the transcriptional and/or the translational level.

Feedback repression of *MuDR* transcription could be mediated directly by the MURA transposase, by MURB or by other factors. The TIRs are the likely targets, because TIRA and TIRB are 97% identical (Fig. 1A), and they are represent the only extended sequence that is conserved between *mudrA* and *mudrB*. TIRA and TIRB include ~160 bp upstream promoter regions and encode the first ~50 bp of their respective trancripts (Hershberger et al. 1995). To determine if autoregulation contributes to *Mutator* biology, we constructed transgenic maize plants that express the firefly luciferase gene under the control of the entire 216-bp *mudrB* TIR (Fig. 1B). In this report, we quantify the autoregulatory effects of

active *MuDR* elements on TIRB-luciferase expression in successive leaves and in mature pollen.

Materials and methods

Vectors

Construction of pMB5 has been previously described (Benito and Walbot 1994). It consists of the complete 216-bp *mudrB* TIR fused to the maize *Adh1* intron 1 and the firefly luciferase cDNA. Plasmid pMR42 was constructed by removing the luciferase cDNA from pMB5 as a *Bgl*II fragment and replacing it with the *uidA* cDNA from pJB4 (Bodeau and Walbot 1992) as a *Bgl*II fragment. Plasmid pAHC20 was obtained from P. Quail (Christensen and Quail 1996).

Maize transformation

A detailed transformation protocol is available at http://www.standord.edu/~walbot/StableMaizeTransf.html. Briefly, embryogenic A188 × B73 (HiTypeII) calli (Armstrong and Green 1985; Armstrong 1994) were first osmotically treated (Vain et al. 1993), then transformed using the PDS 1000HE biolistic device (BioRad, Hercules, Calif.) at 650 psi, with a second treatment 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 1.0 cm, and from the mesh screen to the target 5.9 cm. For three bombardments, a total of 25 μg of plasmids pMB5, pMR42 and pAHC20 were coprecipitated in equimolar quantities onto 2 mg of 1 μM spherical gold particles (Alameda Scientific Instruments, Richmond, Calif.) following 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). To identify herbicide-resistant plants, an area of 5 cm diameter on the leaf surface was painted with a mixture of 0.75% glufosinate ammonium (Ignite 600, 50% solution, Hoescht, Montreal, Canada) and 0.1% Tween 20.

Plant material

Plasmids were transformed into embryogenic calli established from a cross between the inbred lines A188 and B73. The F1 hybrid line is called HiII (Armstrong and Green 1985). This line does not contain active copies of *MuDR*, judging from the lack of unmethylated *Hin*fI sites in its *Mu1* TIRs (Raizada and Walbot 2000) and the lack of full-length 4.9-kb *MuDR* elements revealed by Southern analysis (G. Rudenko and V. Walbot, unpublished results). Transformed callus line TIR45 was regenerated to produce plant TIR45.1. From transformed callus line TIR41.3, three clones were regenerated – plants TIR41.3, TIR41.7 and TIR41.9. Pollen from these T_0 generation transformants was crossed onto three types of female testers, termed here *MuDR*+, *MuDR*– and silenced *MuDR*. Female tester *MuDR*+ (family MrE11) has the following genotype: segregating *MuDR*, *a1-mum2*/*a1*, *R C1* (W23 inbred background); only ears grown from mutable (*a1-mum2*, *MuDR*+) kernels were crossed to transgenic pollen. Female tester *MuDR*- (family MrE10) was derived from the *MuDR*+ line by outcrossing of the *MuDR* element in family MrE11; it no longer contains a full-length 4.9-kb *MuDR* element, as revealed by Southern analysis (G. Rudenko and V. Walbot, unpublished results). The female tester line silenced *MuDR* (family MrE15) was a high-copy *MuDR* line that lost somatic instability in the previous generation and was then selfed. The *MuDR* TIR *Sac*I sites in this family are resistant to cleavage, which is indicative of epigenetic modification (G. Rudenko and V. Walbot, unpublished results). It has the following genotype: silenced *MuDR*, stable *bz2::mu1*, *R C1* (inbred background W23); only ears grown from non-spotted kernels were crossed to transgenic pollen. The plants analyzed in this report belong to the T_1 generation progeny of all these crosses.

Southern analysis

The *Mu1*-specific probe used was the 650-bp *Ava*I-*Bst*N1 internal fragment of *Mu1*; it was isolated as a *Sma*I fragment from plasmid pA/B5 (Chandler and Walbot 1986). pA/B5 cross-hybridizes with *Mu1* (1.4 kb), *Mu2* (1.75 kb) and *Mu1.0* (~1 kb) elements. To determine transgene copy number, a luciferase probe was isolated as a 1.2-kb *Eco*RI fragment from pMB5 (Benito and Walbot 1994) and hybridized to samples from transgenic plants hemizygous for the transgene locus. To determine the methylation status of TIRB, a probe flanking the TIRB promoter in the pUC19 vector was isolated as a 550-bp *Ssp*I-*Sph*I fragment from pMB5. Genomic DNA was isolated from leaves using the protocol of Dellaporta (1994), blotted and hybridized to ^{32}P-radiolabelled probes as previously described (Warren and Hershberger 1994).

Luciferase assays

Leaves were sampled by combining 4–8 0.5-cm punches from separated areas of each leaf blade. Tissues were frozen in liquid nitrogen, stored at –80°C, then homogenized on ice with sand in CCLR buffer (Luehrsen et al. 1993), using prechilled mortars, pestles, materials and buffer. The homogenate was centrifuged at 5000×*g* at 4°C. Extracts were immediately assayed for luciferase (Luehrsen et al. 1993). Non-transformed tissues were used to determine background levels. All values were normalized to total protein using Bradford Reagent (BioRad). Because the CCLR buffer reacts with this reagent, CCLR buffer was added to BSA protein standards, and the extract volume was kept to less than 0.05% of the total reagent volume. Because the data for Figs. 2 and 3 versus Fig. 4 were sampled 2 years apart, the absolute luciferase expression values cannot be compared, as a result of aging of the photomultiplier tube in the luminometer and possible differences in the luciferin assay buffer.

Results

Analysis of transgenic lines

Biolistic delivery was used to transform embryogenic calli of HiII (the F1 hybrid of inbreds A188 and B73) (Armstrong and Green 1985) with plasmids pMB5 (Benito and Walbot 1994) and pAHC20 (Christensen and Quail 1996). pMB5 encodes the 216-bp *mudrB* terminal inverted repeat fused to the maize *Adh1* intron 1 and the firefly luciferase cDNA (Fig. 1B). pAHC20 encodes resistance to Basta and was used to select stably transformed calli. From a total of 49 herbicide-resistant calli, five regenerated lines expressed luciferase. Two independent lines, TIR41 and TIR45, were selected for this study. These lines had the lowest numbers of luciferase transgenes (TIR41, three copies at one locus; TIR45, eight copies at one locus). In addition, these lines were the least prone to epigenetic transgene repeat silencing and expressed stable levels of luciferase for three generations (T_0–T_2).

Effect of active *MuDR* on TIRB-luciferase in leaves

The TIRB promoter results in a low but easily measured level of luciferase activity in transgenic maize (Fig. 2). To determine if active *MuDR* elements repressed or

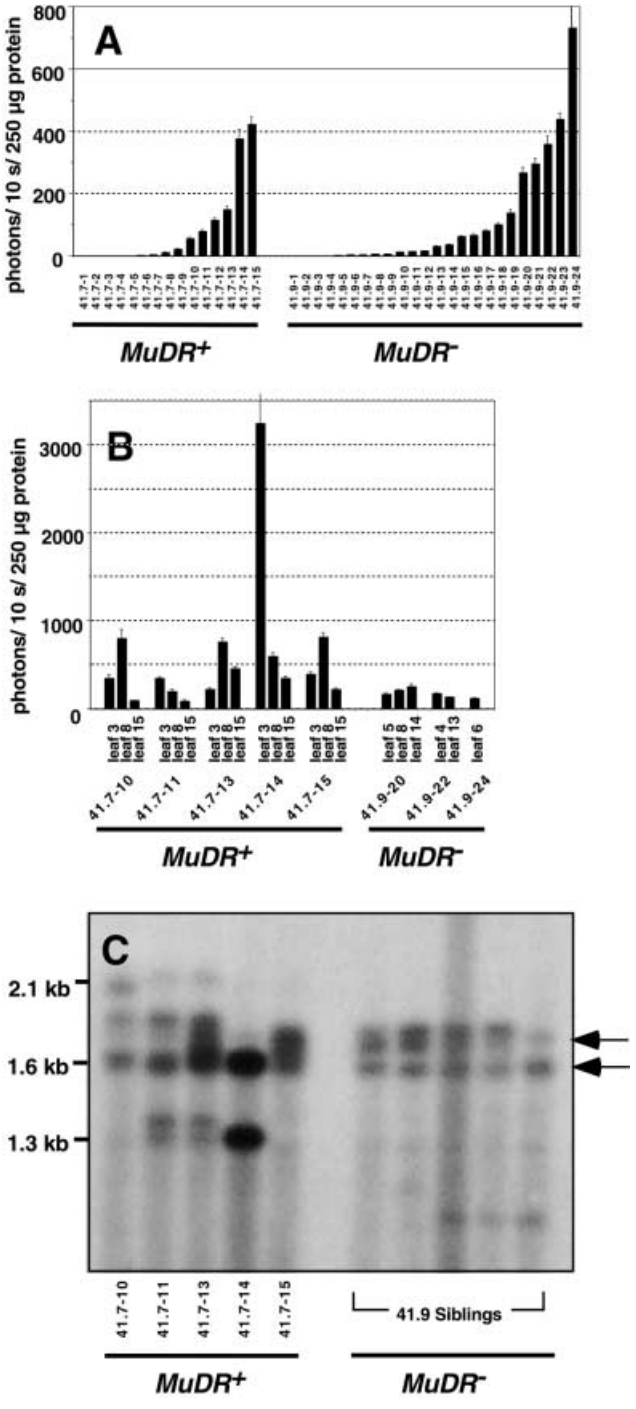

Fig. 2A–C Effect of *MuDR* activity on TIRB-luciferase expression in leaves of transgenic line TIR41 (plants TIR41.7 and TIR41.9). Pollen segregating for the TIRB-luciferase transgene was crossed onto female tester ears with or without active *MuDR* elements. **A** Luciferase expression in seedling leaf 1. **B** Luciferase expression in adult leaves. A subset of the best luciferase expressing seedlings were grown to maturity, and leaves along the shoot gradient were reassayed for luciferase expression. Leaf numbering is by order of emergence. **C** Southern analysis to determine *MuDR* activity status. When *MuDR* elements are active, the *Hin*fI sites in *Mu1* and *Mu2* elements are unmethylated and a *Hin*fI digest generates fragments of 1.3 kb and 1.6 kb, respectively, on Southern blots. When *MuDR* elements are inactive or absent, the *Hin*fI sites are methylated and resistant to cleavage, thus generating fragments of higher molecular weight. In the Southern blot shown, genomic DNA was isolated from leaf 15, digested with *Hin*fI, blotted and hybridized to the ^{32}P radiolabelled probe pA/B5, which recognizes *Mu1* and *Mu2* elements. The 1.6- and 1.8-kb fragments (*arrows*) are methylated *Mu* elements from the HiII line

enhanced TIRB-luciferase expression, we tested luciferase levels in the progeny of primary transformants after crosses to Minimal Line Mutator plants segregating for active *MuDR* elements (Chomet et al. 1991). We performed four pairwise comparisons to examine the effect of *MuDR* on TIRB-luciferase expression. The general protocol involved crossing pollen segregating 1:1 for the transgene to a non-transgenic ear parent. For example, pollen was crossed to a low-copy *MuDR*+/– tester, heterozygous for the *a1::mum2*/*a1* reporter allele but containing no other *Mu1* elements. Because the genotype of the transgenic parent was *A1*/*A1*, Mutator activity status could not be scored using somatic mutability; instead, Mutator status was confirmed by assessing the methylation of *Mu1* and *Mu2* elements. The TIRs of these closely related elements each have a *Hin*fI site. In plants with (a) transcriptionally active *MuDR* element(s), these *Hin*fI sites can be fully digested, liberating 1.3- and 1.6-kb fragments from *Mu1* and *Mu2*, respectively. After loss of *MuDR* activity, by segregation or by epigenetic silencing, the *Hin*fI sites become methylated and are poorly digested, resulting in higher molecular weight fragments (Chandler and Walbot 1986; Bennetzen 1987).

Inbred lines of maize typically contain several *Mu1* and/or *Mu2* elements, which are fully methylated until the line is crossed with a source of MURA transposase (Chandler et al. 1988; Raizada and Walbot 2000). The HiII transgenic plants contain two such elements: when methylated within the TIRs, fragments of 1.6 kb and 1.8 kb are produced. Methylation of the TIRs of the *Mu1* element in the *a1::mum2* reporter allele yields a 2.1-kb fragment (Lisch et al. 1995).

TIR41.7/*TIR41.9* progeny

In the first comparison, pollen segregating for the TIRB-luciferase transgene was utilized from two primary transformants, plants TIR41.7 and TIR41.9, regenerated from the same transformed callus. Pollen from plant TIR41.7 was crossed onto an active *MuDR* tester line (*MuDR*+) that contains only one or two transcriptionally active *MuDR* elements. Pollen from plant TIR41.9 was crossed to a zero copy *MuDR* line (*MuDR*–), a sibling of the active *MuDR* tester line in which the original active *MuDR* element had segregated away in a previous generation.

We initially analyzed transgene expression in leaf 1, one of five embryonic leaves preformed in the seed. As shown in Fig. 2A, half of the TIR41.7 X *MuDR*+/–

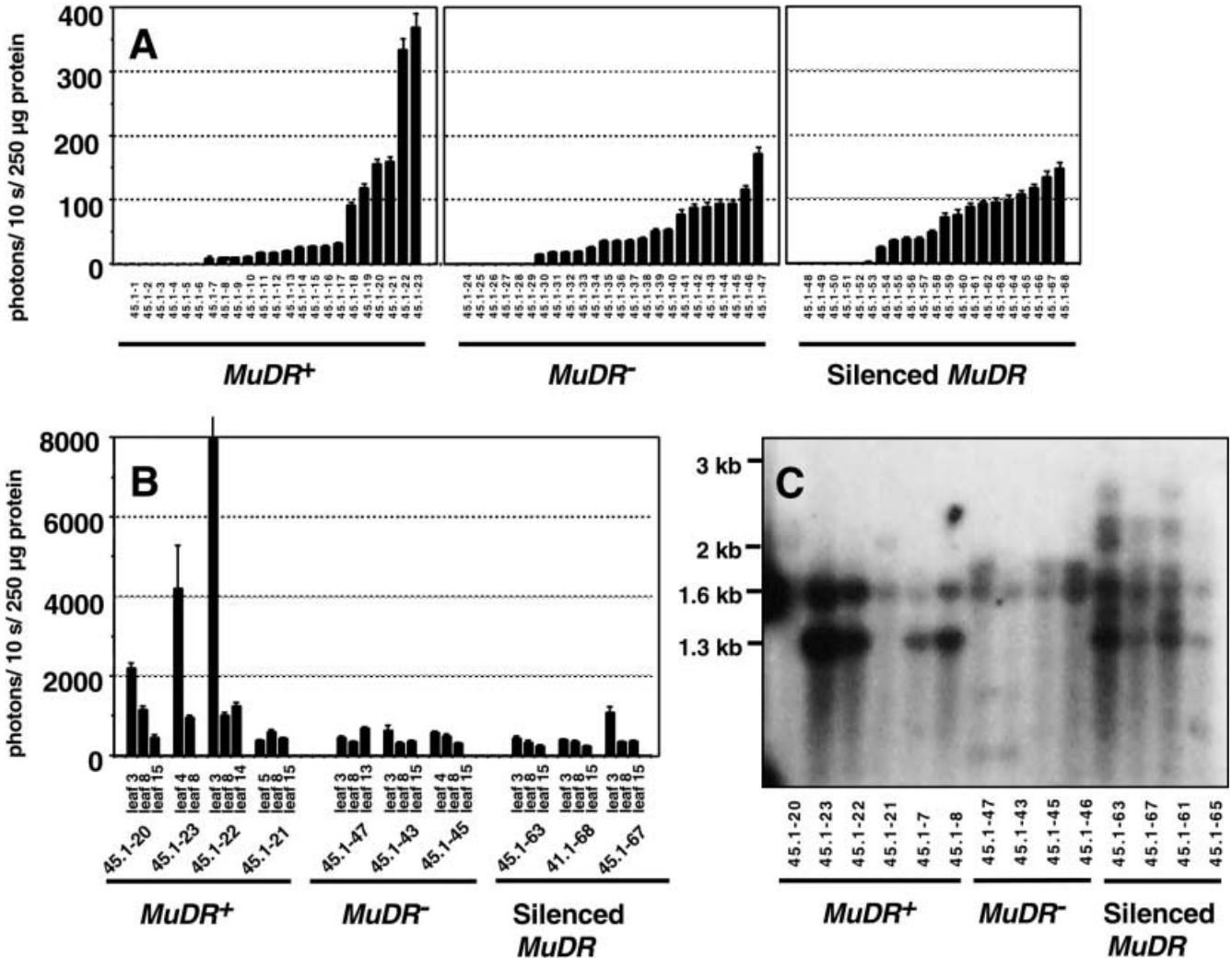

Fig. 3A–C Effect of *MuDR* activity on TIRB-luciferase expression in leaves of the progeny of plant TIR45.1 (*test A*). Pollen segregating for the TIRB-luciferase transgene was crossed onto three types of female testers, and the progeny were analyzed. **A** Luciferase expression in seedling leaf 1. **B** Luciferase expression in adult leaves. A subset of the best luciferse expressing seedlings were grown to maturity and leaves along the shoot gradient were reassayed for luciferase expression. **C** Southern blot analysis to determine *MuDR* activity status. Details are given in the legend to Fig. 2

seedlings expressed luciferase above background, as expected for the 1:1 segregation of the luciferase transgene locus. Surprisingly, a similar distribution of luciferase expression was observed in progeny crossed to the *MuDR*-strain, with the exception of one *MuDR*-plant, TIR41.9-24, which expressed a two-fold higher level of luciferase than the best expressing plants. The means of the two populations were similar (*MuDR*+, 83 photons/10 s/250 µg protein; *MuDR*–, 111 photons/10 s/250 µg protein), and the two populations were not significantly different when analyzed by the Wilcoxon rank sum test ($P = 0.42$). Therefore, the presence of a transcriptionally active *MuDR* element neither increased nor decreased luciferase expression in leaf 1.

We transplanted a subset of the best expressing seedlings of both test groups and assayed luciferase expression in successive leaves (Fig. 2B). In subsequent embryonic (leaf 3, 4 or 5) and juvenile leaves (leaf 6 or 8), nearly all *MuDR*+ and *MuDR*– plants had luciferase values in the range of 121–388 photons/10 s/250 µg protein. The *MuDR*– plant that previously exhibited the highest luciferase expression in seedling leaf 1, plant TIR41.9-24, now expressed luciferase at levels comparable to, or lower than, those in any of the other *MuDR*+/– plants by adult leaf 6 (Fig. 2B). One *MuDR*+ plant, plant TIR41.7-14, expressed more than ten times as much luciferase activity as the mean of its siblings. Interestingly, Southern analysis subsequently demonstrated that this was the only plant that contained fully unmethylated *Mu1* and *Mu2* elements indicative of sustained *MuDR* activity (Fig. 2C).

In upper leaves, no high luciferase values were detected The range of values for the *MuDR*+ and *MuDR*– families overlapped in adult leaves 13–15 (*MuDR*+, 90–451 photons/10 s/250 µg protein; *MuDR*–, 130–254 photons/10 s/250 µg protein). Therefore, this initial, albeit small, comparison suggested that the

presence of an active *MuDR* element(s) enhanced TIRB-luciferase expression, rather than repressing it, but that this effect occurred only transiently in the fully expanded, embryonic leaves.

Overall, luciferase expression decreased 10-fold in adult leaves (leaves 13–15) compared to embryonic and juvenile leaves (3–8) in four of the seven plants examined (Fig. 2B). In an analysis of additional primary transformants lacking *MuDR*, luciferase expression was typically found to be 1.3- to 10.5-fold higher in lower leaves than in upper leaves (data not shown). One hypothesis to account for this is that this gradient might be altered by the presence of an active *MuDR* element. As clearly shown in Fig. 2B and later in Fig. 3B, this gradient in transgene expression appears to be independent of the *MuDR* status of the plant.

TIR45.1 progeny test A

In the second comparison, we used pollen segregating for the TIRB-luciferase transgene from an independent primary transformant, plant TIR45.1. We crossed pollen from this plant onto three female testers, the low-copy *MuDR* stock (*MuDR*+), the zero-copy *MuDR* stock (by outcross segregation, *MuDR*–), and an epigenetically silenced high-copy *MuDR* stock (silenced *MuDR*). Fig. 3A shows the normalized luciferase values for seedling leaf 1 in all three sets of progeny. In all families, luciferase values were low (< 200 photons/10 s/250 μg protein) with the exception of two seedling progeny belonging to the *MuDR*+ family, plants TIR45.1-22 and TIR45.1-23, that expressed luciferase ~7-fold above background.

We transplanted 3–4 of the best expressing seedlings from each group and then sampled one embryonic (leaf 3), one juvenile (leaf 8) and one adult (leaf 14 or 15) organ from each plant. As shown in Fig. 3B, luciferase levels in the *MuDR*– and silenced *MuDR* plants were similarly low. Confirming previous observations, in two of the four *MuDR*+ plants, TIR45.1-23 and TIR45.1-22, luciferase levels were 4- and 8-fold higher, respectively, in embryonic leaves than in the best expressing *MuDR*- or silenced *MuDR* plant (plant TIR45.1-67). As in line TIR41 (Fig. 2), luciferase expression in the upper leaves was low in all families.

As shown in Fig. 3C, we then analyzed the *MuDR* activity state of all plants by examining the extent of DNA methylation at the *Hin*fI site of *Mu1*/*Mu2* elements in all plants scored for luciferase activity. The two best expressing plants, TIR45.1-23 and TIR45.1-22, showed dramatic increases in the number of unmethylated *Mu1* elements compared to their siblings, indicative of strong Mutator activity. The two *MuDR*+ family siblings that expressed lower levels, plants TIR45.1-20 and TIR45.1-21, contained methylated *Mu1*/*Mu2* element(s); in embryonic leaves these plants expressed 2- to 20-fold lower luciferase activities than their unmethylated siblings. We conclude that progeny of plant TIR45.1 express much higher levels of luciferase in their lower leaves when they contain active *MuDR* elements.

In comparing the progeny of the *MuDR*- and silenced *MuDR* families, we draw a second conclusion from Fig. 3 concerning the possible existence of a dominant negative inhibitor of *MuDR*/*Mu* activities. Parental lines that lacked *MuDR* activity after segregation (*MuDR*–) or after epigenetic silencing of many *MuDR* elements were crossed to the same transgenic line; progeny of both types of crosses had the same luciferase expression. This observation suggests that there is no inhibitory factor that accumulates in the epigenetically silent lines that can silence a TIRB promoter. Furthermore, the higher copy number of methylated *Mu1* and *Mu2* elements in the silenced *MuDR* individuals compared to the *MuDR*– plants (Fig. 3C) had no impact on TIRB-luciferase expression in leaves; all were similarly low. Therefore, there is no reason to suspect epigenetic cross-talk at the DNA level between endogenous *Mu* element TIRs and its TIRB promoter homolog in the luciferase transgene.

TIR41.3 progeny

In the third comparison, the progeny of plant TIR41.3, a primary transformant regenerated from the same callus as plants TIR41.7 and TIR41.9, were examined (Fig. 4A–C). Pollen segregating for the TIRB transgene was crossed to the low-copy *MuDR* stock (*MuDR*+) and zero-copy *MuDR* stock (*MuDR*–). Because the first two tests demonstrated that the TIRB-luciferase transgene was only responsive to *MuDR* in lower leaves, we restricted our leaf sampling to these leaves and to herbicide-resistant, transgenic progeny. In contrast to the previous two tests, both *MuDR*+ and *MuDR*– family levels were similarly distributed. In seedling leaf 1, shown in Fig. 4A, all plants expressed luciferase at or near background levels (< 35 photons/10 s/100 μg protein). In leaf 3, the population means were similar: *MuDR*+ at 610 and *MuDR*– at 429 photons/10 s/250 μg protein, respectively. By leaf 5, however, mean expression in the *MuDR*+ family (615 photons/10 s/250 μg protein) was nearly two-fold higher than in the *MuDR*– family (326 photons/10 s/250 μg protein). In leaf 5, luciferase expression in the two families was also found to be significantly different by the Wilcoxon rank sum test ($P = 0.006$).

When we examined the *MuDR* activity status of these plants, 7 of 12 *MuDR*+ plants had mostly demethylated *Mu1* and *Mu2* elements and showed a higher *Mu1*/*Mu2* copy number (Fig. 4D). However, within the *MuDR*+ family, there was a poor correlation between the methylation status of *Mu1* and *Mu2* elements and expression of the TIRB-luciferase transgene in either leaf 3 or leaf 5. In fact, of four *MuDR*+ family plants containing methylated *Mu* elements (plants TIR41.3-3, TIR41.3-5, TIR41.3-8 and TIR41.3-11), luciferase levels were both the highest and lowest of the population (Fig. 4B, C).

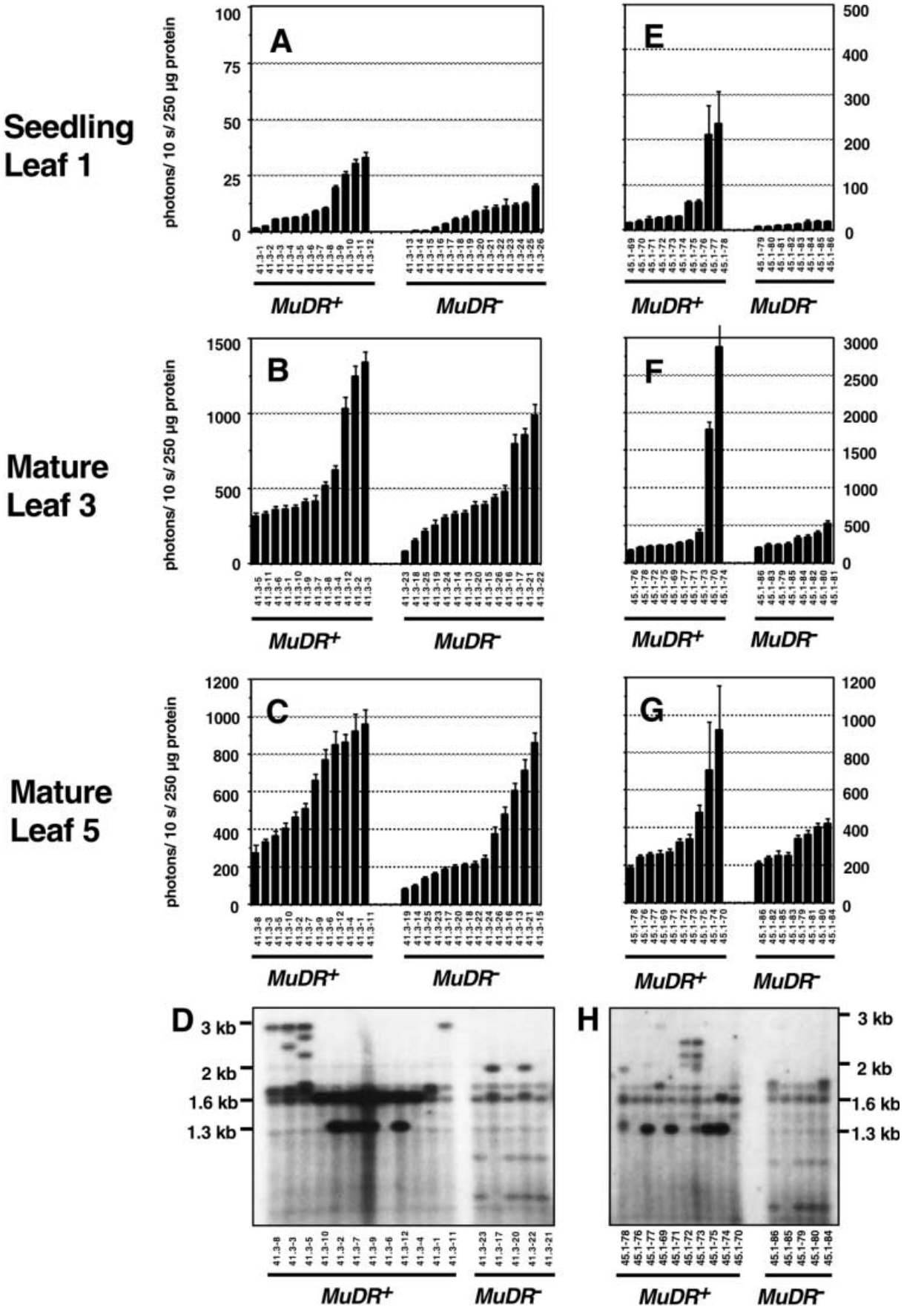
Seedling Leaf 1
Mature Leaf 3
Mature Leaf 5
photons/ 10 s/ 250 µg protein
A
B
C
D
E
F
G
H
MuDR+
MuDR-
3 kb
2 kb
1.6 kb
1.3 kb

Fig. 4A–H Effect of *MuDR* activity on TIRB-luciferase expression in leaves of the progeny of plant TIR41.3 (**A**–**D**) and plant TIR45.1, *test B* (**E**–**H**). Pollen segregating for the TIRB-luciferase transgene was crossed onto female testers either possessing or not possessing segregating active *MuDR* elements as described *below* each graph. All progeny were analyzed, but only data for herbicide-resistant plants are shown. **A–C** Luciferase expression in the progeny of plant TIR41.3 is shown for seedling leaf 1 (**A**), and fully expanded leaves 3 (**B**) and 5 (**C**). **D** Southern blot to determine *MuDR* activity status in plant TIR41.3 progeny. Genomic DNA was isolated from leaf 12. Further details are described in the legend to Fig. 2. **E–G** Luciferase expression in the progeny of plant TIR45.1 is shown for seedling leaf 1 (**E**), and fully expanded leaves 3 (**F**) and 5 (**G**). **H** Southern blot to determine *MuDR* activity status in plant TIR45.1 *test B* progeny. Genomic DNA was isolated from leaf 12

It is difficult to explain why the means of the *MuDR*+ and *MuDR*– families were significantly different at leaf 5, even though luciferase activity did not correlate with Mutator activity within the *MuDR*+ family. One unusual feature of the TIR41.3 progeny is that luciferase expression varied dramatically from leaf to leaf within each plant, even though embryonic leaves 2–5 share cell lineages that can be traced back into the shoot apex (Poethig et al. 1986). For example, whereas three plants from each *MuDR*+ and *MuDR*– family expressed luciferase at levels about two-fold higher than siblings at leaf 3 (Fig. 4B), their expression decreased by up to fivefold at leaf 5 (for example, plants TIR41.3-22 and TIR41.3-3, Fig. 4C). In contrast, the expression of other siblings increased up to three-fold between leaf 3 and leaf 5 (plants TIR41.3-1, TIR41.3-11, TIR41.3-15) (Fig. 4B, C). These results suggest that luciferase expression in these individuals was unstable, perhaps changing dramatically in adjacent leaf sectors. If each plant was a mosaic of high and low expressing luciferase sectors, then the values we obtained would depend on where the leaf punch was obtained within a leaf; we therefore consider it possible that the sampling in this family was unreliable.

We conclude from this third pairwise comparison that there was a poor correlation between expression of the TIR41.3 transgene and *MuDR* activity. We found no evidence for *MuDR* acting as a repressor of TIRB-luciferase expression, and the evidence for an enhancer function was weak.

TIR45.1 progeny test B

Because the progeny of plant TIR45.1 appeared to be responsive to the presence of *MuDR*, we tested progeny from a cross between TIR45.1 pollen and a sibling *MuDR*+ female; the *MuDR*– progeny were from the same ear used for the initial *test A*. As shown in Fig. 4E–G, out of 18 herbicide-resistant plants, five showed luciferase activities significantly above those in other *MuDR*+ or *MuDR*– siblings. All five plants were in the *MuDR*+ family. In seedling leaf 1, *MuDR*+ plants TIR45.1-77 and TIR45.1-78 expressed luciferase at levels ~10-fold higher than the mean for the other 16 plants (Fig. 4E). At leaf 3, *MuDR*+ plants TIR45.1-70 and TIR45.1-74 expressed 6.3-fold and 10.2-fold more luciferase, respectively, than the mean of the other 16 plants (Fig. 4F). Finally, at leaf 5, *MuDR*+ plants TIR45.1-75, TIR45.1-74 and TIR45.1-70 expressed 1.7- to 3.2-fold higher amounts of luciferase than the mean of the other 15 plants (Fig. 4G).

When we checked plants for Mutator activity, five out of ten *MuDR*+ plants possessed some unmethylated *Mu1* elements (Fig. 4H); of the five best expressing luciferase plants, four belonged to this active Mutator subclass. Plant TIR45.1-70 was a notable exception; it showed strong luciferase expression in leaves 3 and 5, but possessed no unmethylated *Mu1* elements (Fig. 4H). We also note the reciprocal exception, *MuDR*+ plant TIR45.1-71, which had unmethylated *Mu1* elements, but failed to enhance luciferase expression in any leaf (Figs. 4E–H).

Unlike the TIR41.3 progeny, the rank order in luciferase expression among TIR45.1 progeny was very similar between leaves 3 and 5 in both *MuDR*+ and *MuDR*– families; this transgene allele is particularly stable in expression profile (Fig. 4F and G). Nevertheless, whereas *MuDR*+ plants TIR45.1-77 and TIR45.1-78 had ~10-fold higher expression in leaf 1 than their siblings, they failed to sustain this expression differential in leaves 3 and 5. These individuals contain some unmethylated *Mu1* elements, but they also contain faint ~2-kb methylated bands, suggesting that these individuals may have been a mosaic for Mutator activity.

In the TIR45.1 *test B* experiment, a repressor function for silencing or silenced *MuDR* elements can be ruled out because plants that experienced silencing (for example, TIR45.1-72 and TIR45.1-73) never show enhanced transgene expression. Therefore, we conclude from these tests that there is a strong, though not complete, positive correlation between TIRB-luciferase expression and *MuDR* activity in lower maize leaves. All the evidence contradicts the hypothesis that *MuDR*-encoded proteins act as repressors of TIRB-luciferase expression.

Effect of active *MuDR* on TIRB-luciferase in pollen

We then asked if the presence of active *MuDR* elements enhanced or repressed expression of the TIRB-luciferase transgene in a germinal cell type, mature pollen. We collected pollen from a subset of TIR45.1 *test A* plants previously used in the leaf expression study. As shown in Fig. 5A, expression in pollen is 20–100 fold higher than is typical of leaves. As in leaves, the mean expression levels of the *MuDR*– and silenced *MuDR* pollen were similar (22810 vs. 24194 photons/10 s/250 μg protein). The mean of the four *MuDR*+ family plants was higher, ~40000 photons. Plant 45.1-21 exhibited partial *Mu1* TIR methylation and no *Mu1* amplification (Fig. 3C);

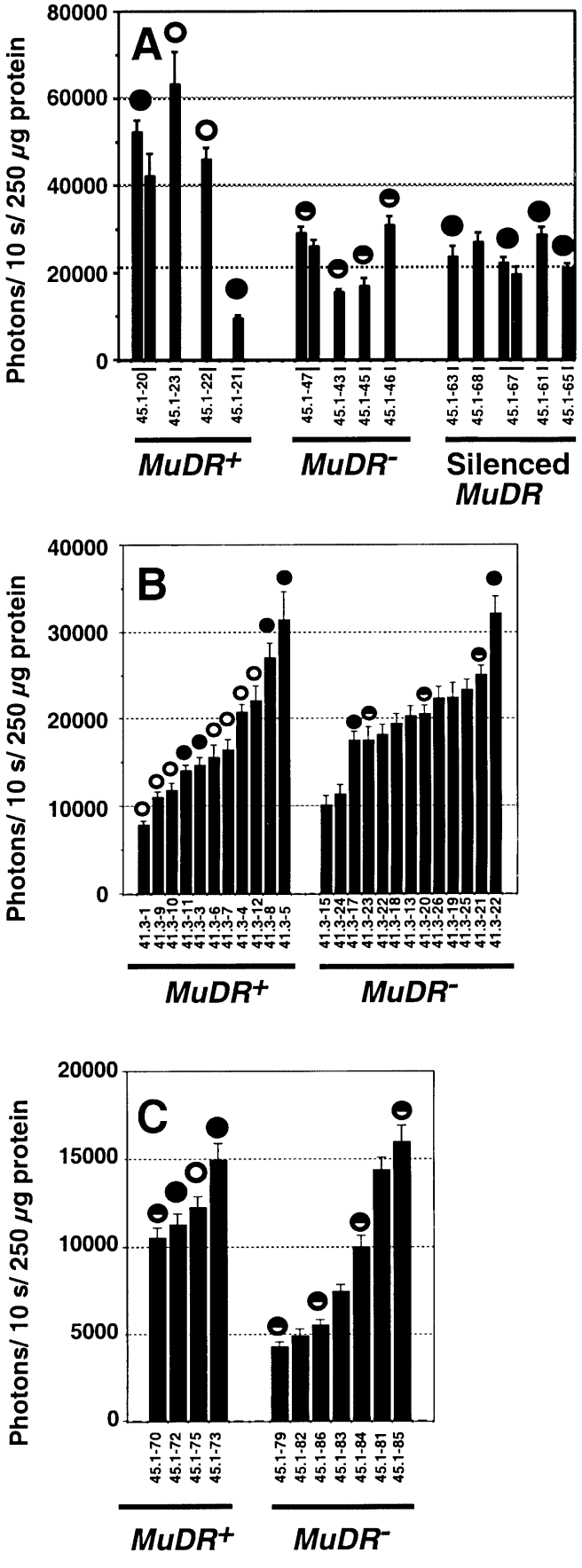

Fig. 5A–C Effect of *MuDR* activity on TIRB-luciferase expression in pollen obtained from the progeny of plants TIR45.1 *test A* (**A**), TIR41.3 (**B**) and plant TIR45.1 *test B* (**C**). The female tester receiving TIRB-luciferase pollen is listed *below* each graph. *Above* each histogram is a symbol to denote the *MuDR* activity status of each plant, determined by Southern analysis at leaves 12–15; the details of this assay are described in the legend to Fig. 2. Symbols: *closed circle*, inactive *MuDR*, indicating that these plants possess methylated *Mu1* and *Mu2* element terminal inverted repeats (TIRs); *open circle*, active *MuDR*, indicating that these plants possess unmethylated *Mu1* and *Mu2* element TIRs; *half circle*, ambiguous *MuDR* status, indicating that these plants possess neither completely methylated *Mu1* and *Mu2* TIRs nor unmethylated *Mu1* element TIRs

these are hallmarks for the loss of Mutator activity. If data from plant 45.1-21 are eliminated because it was no longer *MuDR*+, the mean is about two-fold higher (52170 photons/10 s/250 μg protein) than in the *MuDR*– lines.

Importantly, within the *MuDR*+ family, there was no correlation between pollen luciferase expression and *MuDR* activity as assayed by leaf *Mu1* DNA *Hin*fI methylation levels. To test this relationship further, we examined luciferase expression in pollen of plants TIR41.3, a family that showed a poor correlation between leaf luciferase expression and *MuDR* activity (Fig. 4A–D). As shown in Fig. 5B, in TIR41.3 individuals, both the distribution and mean of pollen luciferase values in the *MuDR*+ and *MuDR*– families were similar (mean, 17524 vs. 20026 photons/10 s/250 μg protein, respectively). Within the *MuDR*+ family, there was no correlation between luciferase expression and leaf *MuDR* activity. Finally, in Fig. 5C, we examined the pollen of TIR45.1 *test B* progeny. The peak luciferase values in both *MuDR*+ and *MuDR*- values were nearly identical; within the *MuDR*+ family, there was again no correlation between leaf *MuDR* activity and pollen luciferase expression. We conclude from these three tests that the presence of *MuDR* neither enhances nor represses TIRB-luciferase expression in mature pollen.

Methylation levels in the TIRB transgene

The variation in luciferase expression levels might reflect changes in the methylation of the TIRB-transgene. There is a strong correlation between DNA methylation at *Sac*I sites in the *MuDR* TIR and active epigenetic silencing in high-copy *MuDR* lines (Walbot 1992; Martienssen and Baron 1994). As summarized in Fig. 6, we have not observed differences in methylation levels at *Sac*I sites in TIRB between TIR45.1 *test A* siblings segregating for active *MuDR* elements. Although some methylation is observed, the *Sac*I sites in the TIRB transgene arrays remain significantly unmethylated even after two generations (T_0 and T_1) without *MuDR* activity. We have observed no significant changes at the *Hin*fI, *Pst*I, *Nco*I and *Bgl*I sites in the TIRB transgene

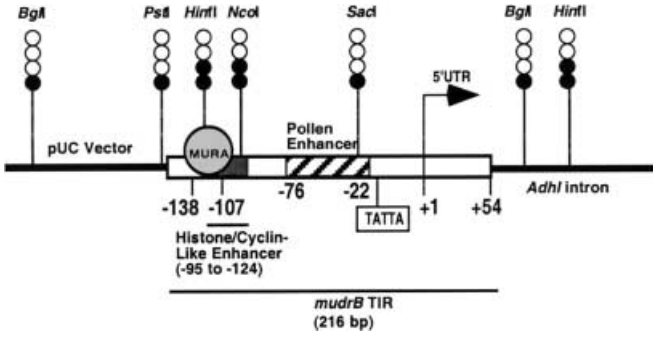

Fig. 6 Summary of methylation levels at the TIRB-luciferase transgene from TIR45.1 plants. Genomic DNAs from upper leaves of *MuDR*+ and *MuDR*– plants were digested with methylation-sensitive restriction enzymes, blotted and probed with a flanking pUC probe (see Materials and methods). The degree of inhibition of cleavage is indicated by the *filled circles below* each enzyme symbol. There was no significant difference in cleavage between *MuDR*+ and *MuDR*– individuals

promoter in the presence versus absence of active *MuDR* elements; all these sites remain only partly methylated, as revealed by Southern analysis. We have only checked methylation levels at a single generation (T_1); complete methylation of restriction sites in *Mu1* elements can require multiple generations after loss of Mutator activity (Bennetzen 1987). In other studies, de novo methylation of *Sac*I sites appears not to occur or to occur less efficiently when *MuDR* activity is lost by internal deletion in a single-element line or after segregation of a single element, as was the case in this study (Lisch and Freeling 1994; Lisch et al. 1995, 1999). In addition, because the transgene locus measured was multicopy, there may be a mixture of unmethylated and methylated TIRB promoters within the transgene array.

Discussion

Competition between transcription factors and truncated or full-length transposase has been suggested to occur at the promoters of *Drosophila P* elements and maize *Spm* and *Ac* elements (Lemaitre and Coen 1991; Fedoroff and Chandler 1994; Schläppi et al. 1994). *MuDR* transcripts could encode multiple full-length and truncated versions of the transposase, MURA, as well as MURB, a protein of unknown function. Of the predicted *MuDR* proteins, the longest MURA binds to a 32-bp site located approximately 100 bp upstream of the transcriptional start site (Benito and Walbot 1997). Consequently, the MURA transposase or a truncated derivative could contribute to the autoregulation of *MuDR* transcription in somatic and germinal cells.

The terminal inverted repeats (TIRs) of *MuDR* elements contain the promoters, the MURA binding site and part of the 5′ UTRs of the *mudrA* and *mudrB* transcripts (Hershberger et al. 1995). The TIRs could mediate transcriptional or translational autoregulation. In transgenic maize, we have examined the quantitative effects of an active *MuDR* element(s) on the *MuDR* TIRB promoter and 5′ untranslated leader of *mudrB* using a firefly luciferase reporter gene. We measured 273 leaf samples and 51 pollen samples representing 136 plants and two independent TIRB-luciferase transformants. We have three key findings.

First, active *MuDR* is not required to maintain TIRB promoter function in leaves or germinal cells (Figs. 2, 3, 4 and 5). The TIRB promoter-luciferase construct has maintained its expression for at least three generations in lines lacking active *MuDR* elements (Figs. 2, 3, 4 and 5, and unpublished results). Second, an active *MuDR* element acts as a weak (2- to 10-fold) enhancer of TIRB-luciferase expression in embryonic leaves 2 to 5. This enhancer effect was observed in 75% of the lineages examined, but in no case was it sustained in the juvenile or adult leaves or in pollen. Embryonic leaves may be a mosaic of high- and low-expressing luciferase sectors (Figs. 2, 3, 4 and 5). Third, we found no evidence that either full-length or truncated *MuDR*-encoded products or epigenetically silent *MuDR* elements act as repressors of TIRB-luciferase expression in leaves or in pollen (Figs. 2, 3, 4 and 5). Because TIRA is nearly identical to TIRB, it is likely that this conclusion holds true for both TIRs of *MuDR*.

TIRB promoter expression is not dependent on *MuDR*

Intact *MuDR* elements can undergo epigenetic silencing (Walbot 1992). This type of silencing of unlinked *MuDR* elements correlates with methylation of *Sac*I sites in the *MuDR* TIR (Walbot 1992; Martienssen and Baron 1994). Similarly, though *Mu1–Mu8* TIRs are hypomethylated relative to other host DNA when *MuDR* elements are active, they become methylated after *MuDR* activity is lost (Chandler and Walbot 1986; Bennetzen 1987; Chandler et al. 1988). This suggests that *MuDR*-encoded products protect the TIRs from methylation following DNA replication (Chandler et al. 1988; reviewed in Fedoroff and Chandler 1994). Because we introduced the TIRB-luciferase transgene into a background that lacked an active *MuDR* element, we expected dramatic silencing and DNA methylation of the TIRB promoter during months of callus culture and regeneration; instead, TIRB-luciferase transgenes have remained stably active for an additional three generations in the absence of active *MuDR* elements (Fig. 2, 3, 4 and 5, and data not shown). In agreement with this observation, a TIRB transgene allele has retained only a low level of DNA methylation at *Sac*I and other sites after two generations following its introduction in tissue culture (Fig. 6). Thus, our result appears to be consistent with conclusions drawn from examples of non-epigenetic mediated loss of *mudrA*; when *mudrA* is lost by internal deletion of a *MuDR* element in a single-copy *MuDR* line, the flanking *Sac*I sites remain generally unmethylated (Lisch and Freeling 1994; Lisch et al.

1995, 1999). These data suggest that the epigenetic silencing of multiple *MuDR* elements must involve an active de novo methylation process and that the TIRA/TIRB *Sac*I sites are an important target.

In the case of genetic loss of *MuDR*, why do *Mu1–Mu8* TIRs become methylated (Chandler and Walbot 1986; Bennetzen 1987), but not the *MuDR* TIRs? In Fig. 7, we present a model to explain this surprising difference. Unlike *Mu1–Mu8* element TIRs, the *MuDR* TIRs are transcriptionally active in all tissues, especially in pollen where expression is enhanced over 20-fold relative to that in leaf cells (Raizada and Walbot, submitted). We propose that transcription factor complexes present at TIRA and TIRB can partially or fully substitute for MURA in preventing methylation. Similarly, in mammalian imprinting, it has been hypothesized that transcription of the *H19* locus in oocytes but not sperm prevents methylation of the maternal but not the paternal allele (reviewed in Surani 1999). The *Sac*I site in TIRA and TIRB is located within the *MuDR* pollen enhancer and overlaps 5 bp of a 7-bp motif found in 20-bp pollen enhancer of the tomato gene *LAT52* (Raizada and Walbot, submitted). Because TIRB-luciferase has remained very active in pollen (Fig. 5), the lack of *Sac*I TIRB methylation should not come as a surprise.

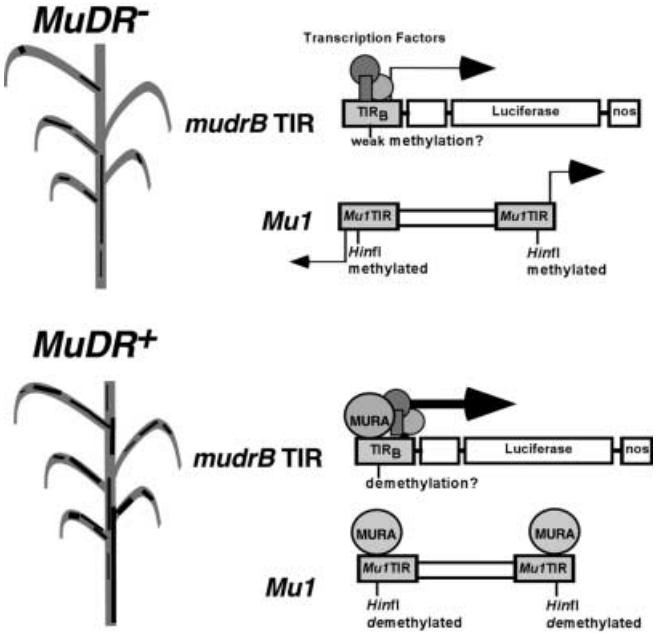

Fig. 7A, B Model to summarize and explain the effect of *MuDR* activity on the expression of TIRB-luciferase and the *Mu1* cryptic outward-reading promoter. **A** In the absence of the *Mutator* transposase, MURA is not present. Consequently, the *Mu1* terminal inverted repeat (TIR) becomes methylated at *Hin*fI and other sites by host maintenance methylase. The absence of MURA permits transcription factors to bind to the methylated *Mu1* TIRs to activate a cryptic outward reading promoter (summarized from Martienssen et al. 1990; Barkan and Martienssen 1991). At the *Mu1* cryptic promoter, we propose that there is direct physical competition between MURA and transcription factors, which prevents outward transcription in an active *Mutator* plant. In contrast, the inward reading *mudrB* promoter and the MURA binding site do not overlap. We propose that transcription factors required for *mudrB* promoter activity partially substitute for MURA in both leaf cells and pollen to partially inhibit maintenance methylase activity and prevent gene silencing. In the absence of MURA, we propose that the *mudrB* promoter (TIRB) becomes stochastically methylated, leading to decreased transcription and fewer high luciferase expressing leaf sectors (shown as black stripes on plant). **B** In the presence of the MURA, MURA outcompetes transcription factors at the *Mu1* TIR, resulting in loss of expression of the cryptic outward-reading *Mu1* promoter. The *Mu1* TIR is also demethylated as an indirect consequence of binding of the MURA bound nearby, which protects the TIR from maintenance methylase activity. We propose that both inward reading *mudrB* and *mudrA* promoters (TIRB and TIRA) also become demethylated, or that MURA acts as a weak transcriptional activator, resulting in greater expression in leaf sectors

Active *MuDR* enhances expression of the TIRB-luciferase transgene

We note that *MuDR*-mediated enhancement is a developmentally transient phenomenon, which is consistent with the lack of demethylation observed at TIRB-luciferase in the presence of active *MuDR*. This enhancement is not mitotically heritable, as would be expected if the promoter was chemically modified by the presence or absence of active *MuDR* elements. Instead, there is a dramatic decreasing expression gradient along the shoot axis even in the presence of active *MuDR* (Fig. 3 and data not shown). Further studies will be required to understand the cause of this gradient and to determine if it is part of an endogenous developmental program.

In the absence of a mitotically stable change in DNA methylation at TIRB upon *MuDR* introduction, we must conclude that a *MuDR*-encoded product acts directly as a weak transcriptional activator of its own expression in the young shoot apex rather than as an epigenetic modifier. The *Spm* TNPA transposase has similarly been shown to be an activator of *Spm* promoters – though only of previously silent ones (Schläppi et al. 1994). Because cells in the shoot apex give rise to the tassel and ear, transient protection from silencing in the young and pre-floral meristem may be an adaptive transposon defense mechanism against stochastic host-induced gene silencing of *MuDR*.

Our conclusion disagrees with that derived from the results of transient assays in maize protoplasts, which have demonstrated that neither MURB nor MURA enhances transcription of TIRB-luciferase (Benito and Walbot 1994; A. Ono and V. Walbot, unpublished results). In these experiments, however, MURA or MURB expression was not verified.

Active *MuDR* does not repress it own expression

Our finding that *MuDR* acts only as an enhancer, not a repressor of its own transcription and/or translation, distinguishes it from other well-characterized transpo-

sons. In the *Spm* system, the TNPA transposase binding site overlaps the *Spm* promoter, suggesting that transcriptional repression is mediated by direct physical competition between the transposase and the transcriptional machinery (Schläppi et al. 1994). TNPA only acts as a repressor when its promoter is unmethylated and active, not when it is methylated and silenced (Schläppi et al. 1994). In our transgenes, the TIRB promoter was clearly active, but no repression was observed upon introduction of active MURA transposase. Indeed, Benito and Walbot (1997) demonstrated that in vitro the MURA transposase binds to its target site efficiently, whether it is unmethylated, hemimethylated or homomethylated.

Why is there no competition between MURA transposase and the transcriptional machinery at the TIRB promoter? In TIRB, the MURA binding site is located 87 bp upstream from the putative TATA box and 31 bp from the pollen enhancer (Hershberger et al. 1995; Benito and Walbot 1997; M. Raizada and V. Walbot, submitted). Therefore, the extended physical distance between the transposase binding site and transcription factor binding sites is likely to explain the lack of transcriptional repression by *MuDR* elements that we observed in pollen. On the other hand, the relatively constitutive expression of *MuDR* in plants (Joanin et al. 1997) may depend on the histone and cyclin-type motifs that are clustered within and near the MBS (Fig. 1). The observations in this paper suggest that the MURA transposase does not interfere with transcription factor activities in or near the MURA binding site in differentiated cells – and may even enhance their activities. In contrast, as shown in Fig. 7, at both *Mu1* and *Mu8* TIRs, MURA may compete directly with transcription factors that otherwise activate a cryptic outward reading promoter (Martienssen et al. 1990). In this case, the transposase binding site overlaps some of the transcription start sites (Barkan and Martienssen 1991).

In addition to a lack of transcriptional repression, we also found no evidence for translational repression. The only extended transcribed sequence shared by both *mudrA* and *mudrB* is the first ~50 bp of the 5′ untranslated leader located in the TIRs (Hershberger et al. 1995). This region contains the previously identified Site I (–2 to +14) and Site II (+17 to +24) protein binding sites identified in *Mu1* (Zhao and Sundaresan 1991). The binding to Site II is abolished in silenced Mutator lines, demonstrating a correlation between protein binding to this motif sequence and Mutator activity. Because our TIRB-luciferase transgene contained the 50-bp leader, our results suggest that this sequence is not sufficient to mediate feedback repression.

A caveat to this study is that we only examined the effect of a low number of copies of *MuDR* elements upon TIRB expression. We used a low-copy *MuDR* line segregating for active *MuDR* elements, so that we could use *MuDR*– siblings as near-isogenic negative controls. It is possible that repression only occurs when a plant contains a higher number of *MuDR* elements (Robertson 1983). Our preliminary results show that in both leaves and pollen, TIRB-luciferase transgene expression in high-copy *MuDR* backgrounds is similar to levels in primary transformants lacking *MuDR* (M. Raizada and V. Walbot, unpublished results). We tested two different standard, high-copy *MuDR* stocks, Robertson and *bz2::mu1*, and observed high luciferase expression in both leaves and pollen.

Conclusions

These experiments indirectly examined the interplay between DNA-binding proteins encoded by the maize transposon *MuDR* and its host at the *MuDR* promoter in TIRB. To this 216-bp region, transcription factors and DNA methylases encoded by maize, and transposase proteins encoded by *MuDR*, are expected to bind, perhaps competitively. We have discovered that one or more proteins encoded by *MuDR* enhances its own expression in maize embryonic somatic tissue, but neither enhances nor represses its own expression in germinal cells (pollen). We propose that the combination of embryonic enhancement and the lack of feedback transcriptional repression by *MuDR*-encoded proteins or RNA contributes to the ability of *MuDR* elements to attain high copy numbers in many maize lines.

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References

Armstrong CL (1994) Regeneration of plants from somatic cell cultures: applications for in vitro genetic manipulation. In: Freeling M, Walbot V (eds) The Maize handbook. Springer-Verlag, New York, pp 663–671

Armstrong CL, Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207–214

Barkan A, Martienssen RA (1991) Inactivation of maize transposon *Mu* suppresses a mutant phenotype by activating an outward-reading promoter near the end of *Mu1*. Proc Natl Acad Sci USA 88:3502–3506

Benito M-I, Walbot V (1994) The terminal inverted repeat sequences of *MuDR* are functionally active promoters in maize cells. Maydica 39:255–264

Benito M-I, Walbot V (1997) Characterization of the maize *Mutator* transposable element MURA transposase as a DNA-binding protein. Mol Cell Biol 17:5165–5175

Bennetzen JL (1987) Covalent DNA modification and the regulation of *Mutator* element transposition in maize. Mol Gen Genet 208:45–51

Bennetzen JL, Springer PS, Cresse AD, Hendrickx M (1993) Specificity and regulation of the *Mutator* transposable element system in maize. Crit Rev Plant Sci 12:57–95

Bodeau JP, Walbot V (1992) Regulated transcription of the maize *Bronze-2* promoter in electroporated protoplasts requires the *C1* and *R* gene products. Mol Gen Genet 233:379–387

Chandler VL, Walbot V (1986) DNA modification of a maize transposable element correlates with loss of activity. Proc Natl Acad Sci USA 83:1767–1771

Chandler VL, Talbert LE, Raymond F (1988) Sequence, genomic distribution and DNA modification of a *Mu1* element from non-*Mutator* maize stocks. Genetics 119:951–958

Chomet P, Lisch D, Hardeman KJ, Chandler VL, Freeling M (1991) Identification of a regulatory transposon that controls the *Mutator* transposable element system in maize. Genetics 129:261–270

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Dellaporta S (1994) Plant DNA miniprep and microprep: versions 2.1–2.3. In: Freeling M, Walbot V (eds) The Maize handbook. Springer-Verlag, New York, pp 522–525

Eisen JA, Benito M-I, Walbot V (1994) Sequence similarity of putative transposases links the maize *Mutator* autonomous element and a group of bacterial insertion sequences. Nucleic Acids Res 22:2634–2636

Fedoroff NV, Chandler V (1994) Inactivation of maize transposable elements. In: Paszkowski J (ed) Homologous recombination and gene silencing in plants. Kluwer Academic, Dordrecht, The Netherlands, pp 349–385

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR Jr, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Hershberger RJ, Warren CA, Walbot V (1991) *Mutator* activity in maize correlates with the presence and expression of the *Mu* transposable element *Mu9*. Proc Natl Acad Sci USA 88:10198–10202

Hershberger RJ, Benito M-I, Hardeman KJ, Warren C, Chandler VL, Walbot V (1995) Characterization of the major transcripts encoded by the regulatory *MuDR* transposable element of maize. Genetics 140:1087–1098

Joanin P, Hershberger RJ, Benito M-I, Walbot V (1997) Sense and antisense transcripts of the maize *MuDR* regulatory transposon localized by in situ hybridization. Plant Mol Biol 33:23–36

Lemaitre B, Coen D (1991) *P*-regulatory products repress in vivo the *P*-promoter activity in *P*-LacZ fusion genes. Proc Natl Acad Sci USA 88:4419–4423

Lisch D, Freeling M (1994) Loss of *Mutator* activity in a minimal line. Maydica 39:289–300

Lisch D, Chomet P, Freeling M (1995) Genetic characterization of the *Mutator* system in maize: behavior and regulation of *Mu* transposons in a minimal line. Genetics 139:1777–1796

Lisch D, Girard L, Donlin M, Freeling M (1999) Functional analysis of deletion derivatives of the maize transposon *MuDR* delineates roles for the MURA and MURB proteins. Genetics 151:331–341

Luehrsen KR, De Wet JR, Walbot V (1993) Transient expression analysis in plants using firefly luciferase reporter gene. Methods Enzymol 216:397–414

Martienssen R, Baron A (1994) Coordinate suppression of mutations caused by Robertson's *Mutator* transposons in maize. Genetics 136:1157–1170

Martienssen R, Barkan A, Taylor WC, Freeling M (1990) Somatically heritable switches in the DNA modification of *Mu* transposable elements monitored with a suppressible mutant in maize. Genes Dev 4:331–343

Poethig RS, Coe EH Jr, Johri MM (1986) Cell lineage patterns in maize embryogenesis: a clonal analysis. Dev Biol 117:392–404

Qin M, Robertson DS, Ellingboe AH (1991) Cloning of the *Mutator* transposable element *MuA2*, a putative regulator of somatic mutability of the *a1-Mum2* allele in maize. Genetics 129:845–854

Raizada MN, Walbot V (2000) The late developmental pattern of *Mu* transposon excision is conferred by a CaMV 35S-driven MURA cDNA in transgenic maize. Plant Cell 12:5–21

Raizada MN, Benito MI, Walbot V (2001) The *MuDR* transposon terminal inverted repeat contains a complex plant promoter directing distinc somatic and germinal programs. January issue of Plant J

Robertson DS (1981) *Mutator* activity in maize: timing of its activation in ontogeny. Science 213:1515–1517

Robertson DS (1983) A possible dose-dependent inactivation of *Mutator* (*Mu*) in maize. Mol Gen Genet 191:86–90

Sanford JC, Smith FD, Russell JA (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol 217:483–509

SanMiguel P, Tikhonov A, Jin YK, Motchoulskaia N, Zakharov D, Melakeberhan A, Springer PS, Edwards KJ, Lee M, Avramova Z, Bennetzen JL (1996) Nested retrotransposons in the intergenic regions of the maize genome. Science 274:765–768

Schläppi M, Raina R, Fedoroff N (1994) Epigenetic regulation of the maize *Spm* transposable element: novel activation of a methylated promoter by TnpA. Cell 77:427–437

Spencer TM, Gordon-Kamm WJ, Daines RJ, Start WG, Lemaux PG (1990) Bialaphos selection of stable transformants from maize cell culture. Theor Appl Genet 79:625–631

Surani MA (1999) Imprinting and the initiation of gene silencing in the germ line. Cell 93:309–312

Vain P, McMullen MD, Finer JJ (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep 12:84–88

Walbot V (1992) Reactivation of *Mutator* transposable elements of maize by ultraviolet light. Mol Gen Genet 234:353–360

Wan YC, Widholm JM, Lemaux PG (1994) Type I callus as a bombardment target for generating fertile, transgenic maize (*Zea mays* L.). Planta 196:7–14

Warren CA, Hershberger J (1994) Southern blots of maize genomic DNA. In: Freeling M, Walbot V (eds) The Maize handbook. Springer-Verlag, New York, pp 566–568

Wolffe AP, Matzke MA (1999) Epigenetics: regulation through repression. Science 286:481–486

Zhao ZY, Sundaresan V (1991) Binding sites for maize nuclear proteins in the terminal inverted repeats of the *Mu1* transposable element. Mol Gen Genet 229:17–26