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The *MuDR* transposon terminal inverted repeat contains a complex plant promoter directing distinct somatic and germinal programs

Manish N. Raizada, Maria-Ines Benito and Virginia Walbot*
Department of Biological Sciences, Stanford University, Stanford, CA 94305–5020, USA

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*For correspondence (fax +1 650 725 8221; e-mail walbot@stanford.edu).

Summary

The *Mu* transposons of maize are under stringent developmental control. Elements excise at high frequencies in terminally dividing somatic cells, but not in meristems. *Mu* elements in germinal cells amplify, without excision, and insert throughout the genome. All activities require *MuDR*, which encodes two genes, *mudrA* and *mudrB*, whose near-identical promoters are located in the transposon terminal inverted repeats (TIR). We have fused the 216 bp TIR of the *mudrB* gene to GUS and luciferase reporters. We demonstrate that TIRB programs reporter expression in diverse, meristematic somatic cells, paradoxically in those cells in which *Mu* excisions are repressed. In germinal cells, immature tassel and mature pollen, reporter expression increases up to 20-fold compared to leaf. By RNA blot hybridization, we demonstrate that endogenous *mudrB* and *mudrA* transcripts increase significantly in mature pollen; sequence comparisons demonstrate that the *MuDR* TIRs contain plant cell-cycle enhancer motifs and functionally defined pollen enhancers. Therefore, the *MuDR* TIR promoters are developmentally regulated in both somatic and germinal tissues. Because database sequence analysis suggests that the *MuDR* TIR enhancers should be functional in both monocots and dicots, we suggest that the native *MuDR* promoter be used in attempts to transfer the unique behavior of *Mu* transposition to heterologous hosts.

Keywords: *Zea mays*, mutator, pollen, cell cycle, transposon tagging.

Introduction

A distinct feature of maize *Mutator* (*Mu*) transposons is a switch in transposition outcome in somatic compared to germinal cells. In the soma, *Mu* elements excise in terminally dividing cells, but not in their meristematic progenitors (Levy and Walbot, 1990). In cell lineages that give rise to gametes, few excisions occur. Instead, replicative insertions occur at a high frequency in pre-meiotic, meiotic and postmeiotic haploid gametes (Robertson, 1981; Robertson, 1985). All of these activities require *MuDR* (Figure 1a); this element encodes two genes, *mudrA* and *mudrB*, whose protein products, MURA and MURB, catalyze excisions and insertions (Chomet *et al.*, 1991; Hershberger *et al.*, 1991; Qin *et al.*, 1991). MURA has homology to bacterial transposases (Eisen *et al.*, 1994), and MURA alone is sufficient to catalyze somatic excisions (Lisch *et al.*, 1999; Raizada and Walbot, 2000). No specific role has been assigned to MURB, but it is implicated as a helper protein for insertions because lines without *mudrB* lack new *Mu* insertions (Lisch *et al.*, 1999; Raizada and Walbot, 2000). *MuDR*-encoded proteins also catalyze the transposition of the nonautonomous *Mu* elements (*Mu1–Mu8*); these do not encode proteins (reviewed in Bennetzen *et al.*, 1993).

Because multiple subfamilies of *Mu* elements amplify late in germinal development, *Mu* elements are very useful for large-scale mutagenesis experiments in maize. One goal has been to transfer *MuDR/Mu* to other grasses with large genomes. Previously, we demonstrated that a CaMV 35S-*mudrA* transgene was able to cause somatic excisions, but not germinal insertions in maize (Raizada and Walbot, 2000). The CaMV 35S promoter is not active in maize microspores (Fennell and Hauptmann, 1992). In part, this result prompted us to examine the native *mudrA* and *mudrB* promoters located within their terminal inverted

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repeats (TIRs). Compared to other plant transposons, *Mu* elements have unusually long 210–220 bp TIRs (reviewed in Benito and Walbot, 1994). Both the left and right TIRs of mobile *Mu* elements contain the ~30 bp MURA binding site (Benito and Walbot, 1997). Surprisingly, there are several other stretches of high conservation among all *Mu* TIRs, although the overall nucleotide identity is only 77% (Benito and Walbot, 1994).

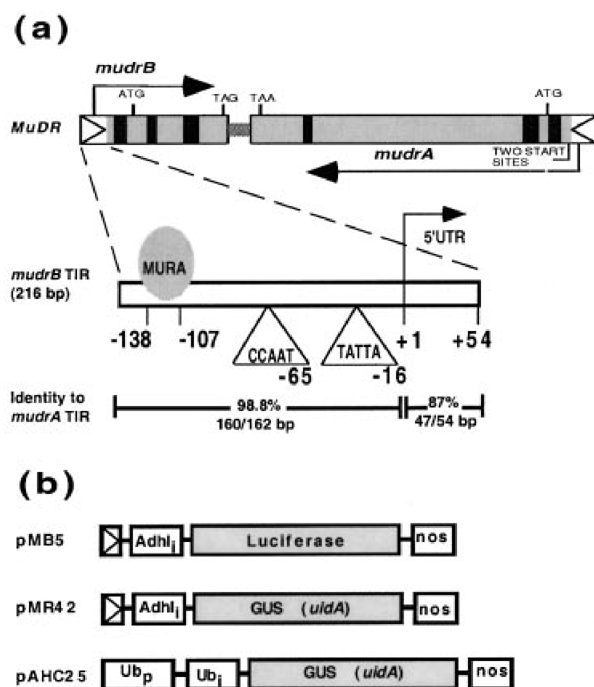


Figure 1. Structures of the *mudrB* terminal inverted repeat (TIR) promoter and of the promoter–reporter plasmids used to make transgenic maize plants.
(a) Structure of the *MuDR* element and *mudrB* promoter. *Top: mudrA and mudrB* are transcribed in antiparallel orientation from promoters contained in the TIRs (box with triangle). Grey regions represent exons and black boxes represent introns. *Bottom:* The *mudrB* TIR consists of the promoter, CAAT and TATA boxes, and part of the 5′ untranslated leader. Numbers shown are relative to the transcription start site. Percentages represent the nucleotide identity to the *mudrA* TIR. MURA transposase (shaded oval) is shown at its binding site (Benito and Walbot, 1997).
(b) Reporter plasmids used in this study. The 216 bp *mudrB* TIR is represented by a box containing a triangle. i, intron; Ub, maize ubiquitin; p, promoter.

Unlike the other *Mu* element TIRs, the TIRs of *MuDR* are 97% identical to each other and contain promoters (Figure 1a). Each TIR consists of ~160 bp of upstream promoter and ~50 bp of 5′ leader sequence (Hershberger *et al.*, 1995) (Figure 1a). In the promoter region, there are only two base polymorphisms between TIRA and TIRB, and RNA hybridization analysis has suggested that these genes are similarly regulated (Hershberger *et al.*, 1995; Joanin *et al.*, 1997).

In transient assays in maize protoplasts from a permanent tissue culture line, the *mudrB* TIR conferred only weak expression (Benito and Walbot, 1994). To elucidate promoter functions more fully, we now use transgenic maize to quantify the ability of TIRB to program GUS and luciferase expression during the somatic and germinal phases of the maize life cycle. Our purpose was to distinguish transcriptional and post-transcriptional mechanisms of regulation contributing to excision and insertion timing. In particular, reporter gene expression is compared in meristems versus non-dividing somatic cells and in vegetative tissues versus pollen. We also examined *mudrA* and *mudrB* transcripts in postmeiotic pollen, a cell type in which *MuDR* expression has not been previously reported, but in which new *Mu* insertions occur at a high frequency (Robertson and Stinard, 1993). Finally, we performed a database analysis of *mudrA* and *mudrB* TIRs to identify candidate tissue-specific enhancer motifs.

Results

Characterization of transgenic lines

We co-bombarded maize embryogenic Hill callus (Armstrong and Green, 1985) with two plasmids, pMB5 and pMR42 (Figure 1b), in which reporter gene expression is programmed by the full-length *mudrB* 216 bp TIR (see Experimental procedures), and a third plasmid encoding

Table 1. Characterization of transgenic lines

Line	T_0 plant luciferase	T_0 plant GUS	T_1–T_2 transgene stability	Luciferase transgene copy number	GUS transgene copy number
TIR15	+	–	unstable	10	ND
TIR41	+	–	stable	>3	ND
TIR45	+	–	stable	8	ND
TIR49	++	++	silenced[b]	>9	>16
TIR18	–	+[a]	silenced	ND[c]	>12
TIR32	–	++	stable	ND[c]	4

[a]Some transgene silencing observed.
[b]GUS and luciferase co-silenced.
[c]ND, not determined.

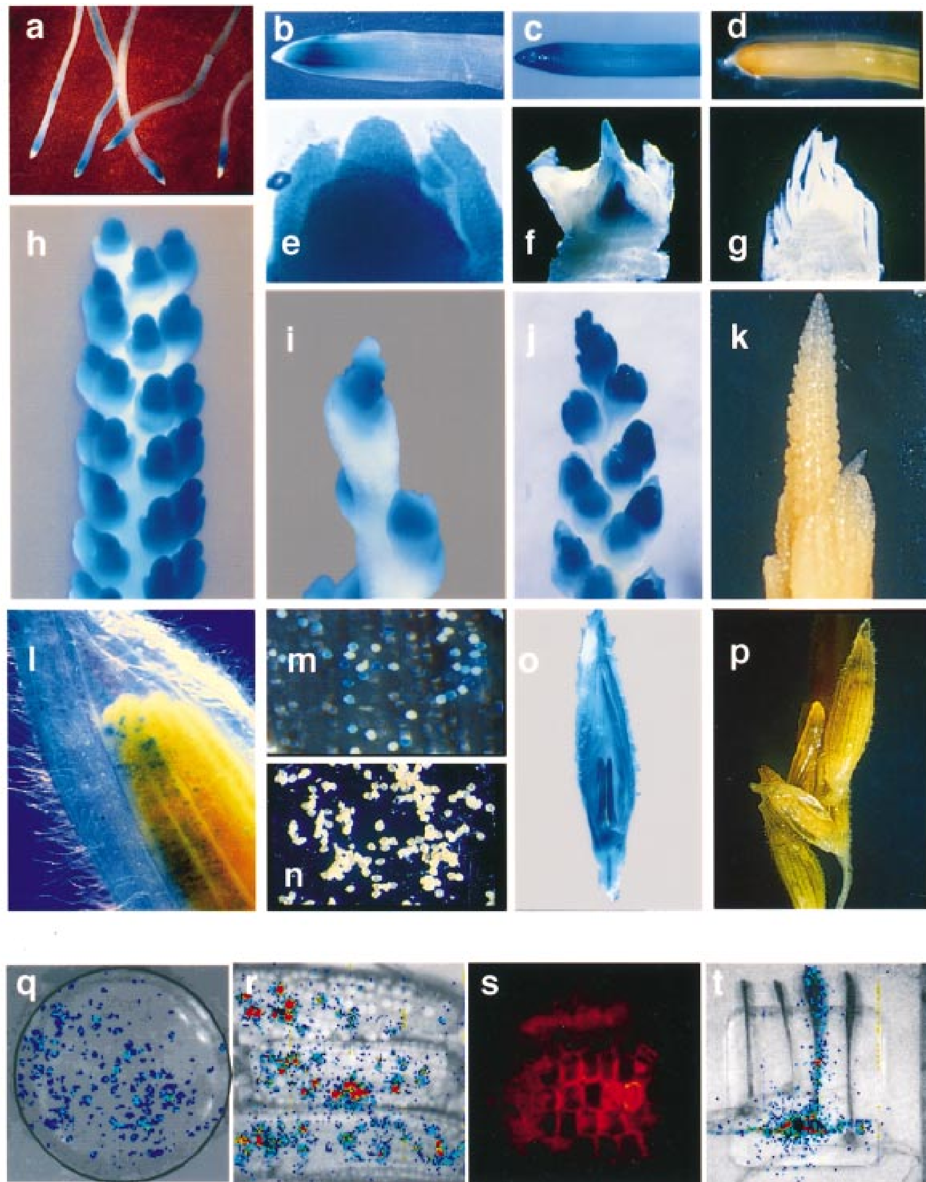


Figure 2. Reporter gene expression patterns.
(a–o) Tissues were stained with X-gluc substrate at 37°C. (a) TIRB-GUS seedling roots (transgenic line TIR32); (b) TIRB prop root (TIR49); (c) Ub-GUS prop root; (d) untransformed stained sibling prop root; (e) close up of TIRB-GUS shoot apical meristem of a 2-week-old seedling (TIR32); (f) shoot apex of TIRB-GUS, distal view of tissue shown in (e); (g) untransformed stained shoot apex of a 2-week-old seedling; (h) TIRB-GUS tassel branch taken from a 1.4 cm immature tassel from a 12-leaf plant (TIR49); (i) TIRB-GUS individual spikelet primordia (TIR49); (j) Ub-GUS immature tassel branch; (k) an untransformed sibling, stained 2 cm immature tassel; (l) TIRB-GUS whole spikelet containing anthers and stained pollen grains (TIR49); (m) TIRB-GUS pollen segregating 1 : 1 for the transgene (TIR32); (n) untranformed sibling pollen, stained; (o) Ub-GUS whole spikelet and stained developing anthers; (p) untransformed, stained spikelets. (q–t) Tissues were sprayed or soaked with luciferin substrate and video imaged; the signal was converted to a quantitative color scale in which orange indicates the regions of highest expression and blue indicates the regions of lowest expression. (q) TIRB-luciferase pollen; (r) TIRB-luciferase developing ear, segregating for the transgene; (s) TIRB-luciferase developing ear; (t) TIRB-luciferase 1-week-old seedlings segregating for the transgene. In (r) and (s), kernels were sliced to expose the underlying embryo and endosperm. In (r), the transgene was transmitted through the pollen, while in (s) it was transmitted through the megagametophyte.

resistance to Basta® (Christensen and Quail, 1996). Forty-nine herbicide-resistant callus lines were obtained (lines TIR 1–49), and a subset of these expressing luciferase or GUS were analyzed (Table 1). Only line TIR49 expressed high levels of both reporter genes. Southern blot hybridization analysis using luciferase and *uidA* probes demonstrated that each of the lines was the result of an independent plasmid integration event (Table 1). Line TIR18 (GUS) and line TIR49 were analyzed in primary (T_0) regenerants, but later epigenetically silenced. Lines TIR32,

41 and 45, and to a lesser extent line TIR15, have retained expression for up to three generations (T_0–T_2). Multiple transgenic lines expressing both GUS and luciferase reporters were analyzed to verify that developmental patterns resulted from the *mudrB* TIR promoter rather than a specific transgene position or plasmid rearrangement.

TIRB-GUS survey of promoter expression

We surveyed a variety of vegetative, floral and germinal tissues for GUS reporter expression programmed by TIRB. No expression was observed in any non-transformed sibling tissues (Figure 2d,g,k,n,p). In primary root (Figure 2a) and aerial prop root (Figure 2b), TIRB-GUS expression was restricted to mitotic cells at the root tip, and was absent from the root cap (Jensen and Kavaljian, 1958) even after 20 h of staining initiated by vacuum infiltration. In mature roots, this pattern was observed in transformants TIR32 (7/7 plants), TIR18 (4/5) and TIR49 (3/3). Some TIR32 primary roots exhibited a 'zebra stripe' pattern of expression in the more mature zones; GUS expression could mirror regions of secondary root initiation (Figure 2a). In fact, at lateral root bifurcations, GUS expression increased (data not shown). A transgenic maize line (line P4) containing the maize ubiquitin promoter-GUS fusion plasmid pAHC25 (Figure 1b) (Christensen and Quail, 1996) served as the GUS-staining control for this study and as a reference marker. Ubiquitin-GUS expression first appeared in the protoderm within 1 h of staining and uniformly stained the root within 4 h with no preference for the meristem (Figure 2c).

At the shoot apex, GUS expression was very strong in the meristem and in newly initiating leaf primordia, but dramatically decreased in more mature primordia (Figure 2f). In fact, the shoot apex could be located simply by staining a whole maize seedling without removing the surrounding layers of leaf sheath tissue. This pattern of expression was observed in all three transformants examined: TIR18 (2/2 plants), TIR49 (11/11) and TIR32 (15/15). In TIR32 apices (Figure 2e), expression was present throughout the apical dome (3/4 seedlings). In green seedling leaves, patchy GUS expression could be observed, sometimes in single guard cells and trichomes, but there was no consistent GUS staining in mature leaf tissue (data not shown).

Strong TIRB-GUS expression in male inflorescence primordia appeared within 4 h of vacuum-infiltrated staining. Expression was highly specific to presumed meristematic regions (Figure 2h,i). This pattern was observed in both TIR49 (2/2 seedlings) and TIR32 (9/9 seedlings). A very similar, although slightly wider zone of GUS expression was exhibited in ubiquitin-GUS transformants (Figure 2j). Because GUS staining was not observed in the central portion of tassels with either the ubiquitin or TIRB promoter constructs, it is difficult to make any conclusions about TIRB expression in these cells based solely on GUS staining. There is no reason to suspect that the striking inflorescence patterns are artifacts of GUS substrate penetration, because RNA *in situ* hybridization using a ubiquitin probe exhibited a similar pattern (Donlin *et al.*, 1995). Ubiquitin is expected to be upregulated in highly metabolic, growing and dividing cells.

In mature male spikelets of both TIR49 and TIR32, TIRB promoted weak GUS expression in glumes and very high expression in mature pollen (Figure 2l). Figure 2m shows pollen grains segregating 1 : 1 for GUS expression, as expected (hemizygous transgenote X tester line). All three independent transformants exhibited strong pollen expression, detectable within 20 min of substrate staining. The ubiquitin-GUS transformant exhibited very high expression throughout the spikelet, including developing anthers and pollen (Figure 2o). Unlike TIRB-GUS, Ub-GUS was also strongly expressed in the anther wall (Figure 2o).

All the GUS patterns were observed in two generations (T_0, T_1), and therefore represented heritable patterns of expression. In addition, there was no difference in the localization of expression in the presence or absence of active *MuDR*. The panels shown in Figure 2 represent a mixture of non-*Mutator* and *Mutator* backgrounds.

Using video imaging, luciferase expression could be detected in pollen grains of TIRB-luciferase transformants (Figure 2q). Strong expression was also observed in developing embryo, aleurone and endosperm 14 days after pollination (Figure 2r,s), consistent with TIRB-GUS expression (data not shown). Weak luciferase expression was observed in immature developing leaf and in the region of newly forming secondary roots (Figure 2t). Thus, luciferase is a useful visual marker in maize, in particular in pollen and kernel tissues.

TIRB-luciferase whole adult plant survey

Whole plant quantitative surveys were performed for three independent TIRB-luciferase transformants and one TIRB-GUS transformant (Figure 3). Expression was constitutively low in all vegetative tissues examined (leaf sheath, leaf blade, husk leaf, tiller leaf, glumes). Immature whole ears from the lower nodes of the plant also exhibited low expression; these ears are developmentally arrested unless the dominant, upper ears are lost. By contrast, immature tassel exhibited a 28-fold increase in luciferase expression compared to leaf tissue (Figure 3c). The highest expression was observed in whole male spikelets, which exhibited a 10–40-fold increase in expression compared to leaves (Figure 3a,b,e). Expression in pollen was 20–22-fold higher compared to average leaf expression in TIR45 and TIR32 (Figure 3a,e); pollen expression ranged from twofold

to 32-fold higher in TIR15, depending on the leaf being compared (Figure 3d). The low expression observed in somatic ear tissues (glumes, silks, husks) and high expression observed in spikelets appeared to be organ- and tissue-dependent, not position-dependent. For example, tissues of an ectopic second ear located beside the main tassel at the top of the plant expressed GUS at similarly low levels as a lower ear, whereas male spikelets emerging from the ectopic ear expressed GUS at high levels (Figure 3e).

We conclude from this initial survey that the *mudrB* TIR programs weak, constitutive expression of reporter genes in maize. It exhibits a specific and dramatic upregulation in expression in immature tassel, and this enhanced expression is also observed in mature pollen.

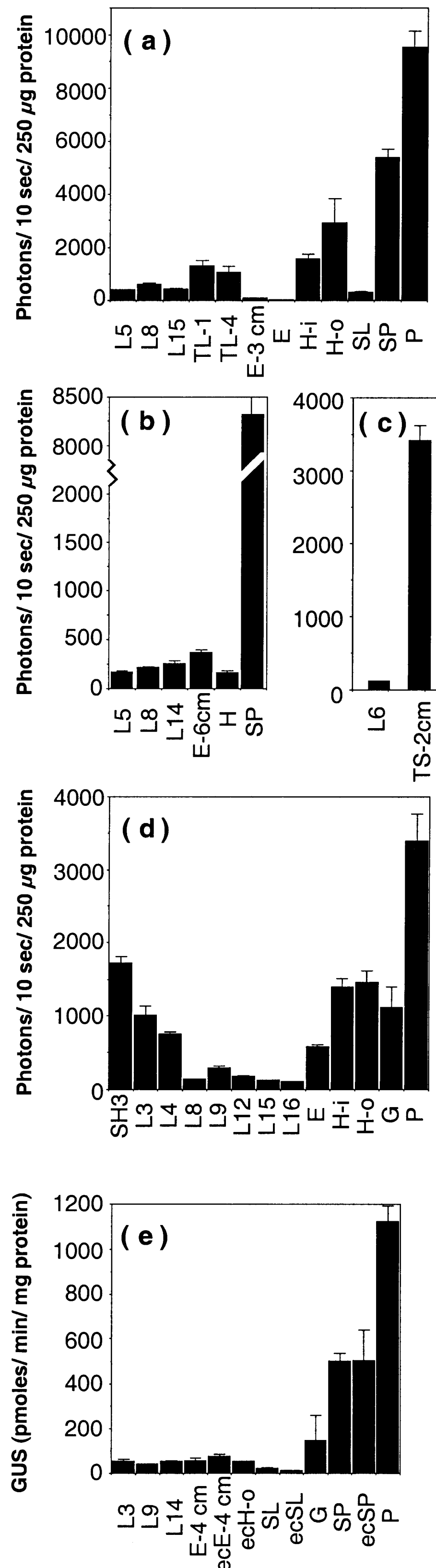
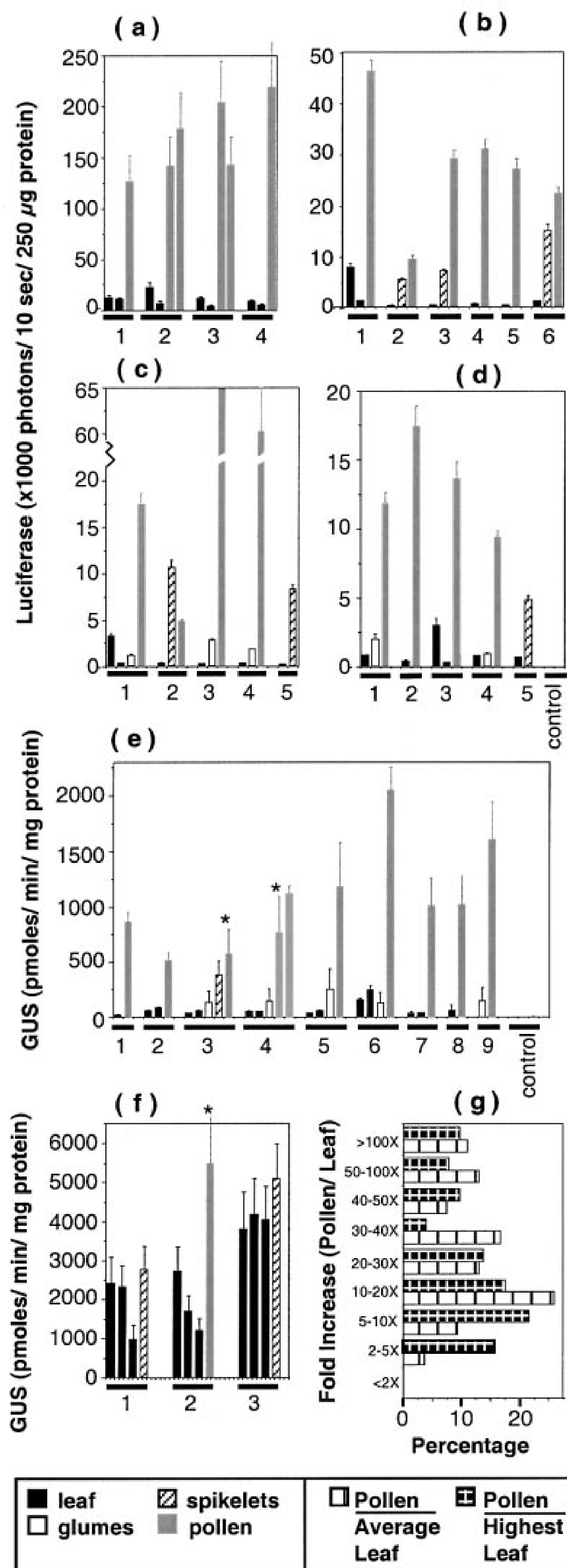


Figure 3. Quantitative whole plant survey of *mudrB* TIR-reporter expression.
(a) TIR45 (luciferase), T_1 generation (progeny of low copy *MuDR* × TIR45 plant 1). (b) TIR41 (luciferase), T_1 generation (progeny of non-*MuDR* tester × TIR41 plant 9). (c) TIR41 (luciferase), T_1 generation (progeny of non-*MuDR* tester × TIR41 plant 9). (d) TIR15 (luciferase), T_1 generation (progeny of non-*MuDR* tester × TIR15 plant 4). (e) TIR32 (GUS), T_1 generation (progeny of TIR32 × medium copy *MuDR*). This plant contained a second ectopic (ec) ear, located at the top of the plant, from which both silks and male spikelets emerged. Each graph represents an individual plant. Luciferase and GUS values were normalized to total protein levels. Leaf numbering is from the bottom of the plant. SH, leaf 3 sheath; L, leaf; E, immature ear; H-i, inner husk leaf; H-o, outer husk leaf; G, glumes; P, pollen; TL, tiller leaf; SL, silk; SP, spikelet; TS, immature tassel; ec, ectopic (ear located next to tassel).

Specific upregulation by MuDR TIRs in pollen

The whole plant level survey indicated a potential regulated increase in *mudrB* TIR-driven expression in pollen. To confirm this, we performed a more detailed analysis using 4–18 plants per transformant to compare leaf, glume, spikelet and pollen expression (Figure 4). Combining data from 55 plants, at both the T_0 and T_1 generations, there was a 33.4-fold higher level of expression in pollen than average leaf levels. Compared to the highest leaf expression on each plant, pollen expression was 23.4-fold higher. As shown in Figure 4(g), there was a wide distribution in pollen-enhancement, from two- to > 100-fold. It is likely that transgene silencing, in either leaf or pollen samples, contributed to the distribution extremes. Non-transformed leaf, glume, spikelet and pollen had negligible levels of background GUS and luciferase expression (Figure 4d,e and data not shown).

The pollen enhancement was independent of transgene chromosomal location or integration pattern. In the T_1 generation, luciferase transformant TIR15 had an average increase in expression in pollen over averaged leaf values of 31.5-fold (18 plants), compared to TIR41 at 42.6-fold (7 plants) and TIR45 at 42.2-fold (12 plants). T_0 expression of TIR49 was 20.5-fold higher in pollen than leaf (4 plants). In whole spikelets, luciferase expression from all 3 transformants was > 9.6-fold higher than in leaves (19 plants)



(Figure 4 and data not shown). Representative plants from different crosses are shown in Figure 4(a–d).

The increase in pollen expression was also independent of the reporter gene used. In the T_1 generation, GUS transformant TIR32 showed an average increase in expression of 20.6-fold over averaged leaf expression (8 plants) with a distribution range similar to the luciferase transformants (Figure 4e). Whereas *mudrB* TIR-GUS levels increased by four- to 39-fold in whole spikelets (Figure 4e, plant 3), anthers (plants 3 and 4) or mature pollen (plants 1–9), the maize ubiquitin promoter-GUS transformant showed an average increase of only 1.9-fold in spikelets or whole anthers compared to average leaf levels; there was no significant difference in 2 of the 3 plants examined.

The *mudrB* TIR region appeared to enhance reporter gene expression specifically in pollen, not in floral glumes and other ancillary tissues. In transformant TIR15, luciferase expression in glumes increased by an average of only 1.3-fold compared to the most highly expressed leaf, whereas pollen expression increased by 9.5-fold (6 plants, Figure 4d). Similarly, in the four plants analyzed of transformant TIR41, glume expression increased by 2.9-fold, while pollen expression increased by 71.1-fold (Figure 4c). For GUS transformant TIR32, glume expression increased by threefold, while pollen expression increased by 14.5-fold (4 plants). This suggests that the *mudrB* terminal inverted repeat contains pollen or gamete-specific enhancer sequences, not general floral enhancers.

Finally, the increase in *mudrB* TIR-mediated pollen enhancement was not affected by the presence or absence of transcriptionally active *MuDR* elements. For example, in transformant TIR45, pollen samples of sibling progeny crossed to different *MuDR* backgrounds all displayed dramatic increases in pollen expression. As shown in Figure 4(b), the transgene in a low copy *MuDR* background (plants 1 and 2), 0 copy *MuDR* background (plants 3 and 4), or epigenetically silenced high copy *MuDR* background (plants 5 and 6) always displayed pollen enhancement. Plants 1–6 were all derived from the same transgenic male parent. We conclude that binding of MURA to the terminal

Figure 4. A comparison of *mudrB* TIR reporter gene and ubiquitin-GUS expression in leaf, glume, male spikelet and pollen.
(a–f) Each *x*-axis number refers to an individual plant. The asterisk (*) denotes that an entire, fertile anther was used. GUS and luciferase levels were normalized to total protein levels. (a) TIR49 (luciferase), T_0 generation; (b) TIR45 (luciferase), T_1 generation; (c) TIR41 (luciferase), T_1 generation; (d) TIR15 (luciferase), T_1 generation. Control, untransformed sibling, leaf and pollen. (e) TIR32 (GUS), plant 1, T_0 generation; plants 2–9, T_1 generation. Control, untransformed leaf and pollen samples. (f) Ubiquitin promoter – GUS, T_0 generation. (g) A summary of reporter gene expression enhancement in pollen compared to leaf. Data from all transformants are shown. Fifty-five plants were used in the distribution of pollen/average leaf expression. Fifty-one plants were used to compare the ratio of pollen to highest leaf luciferase value.

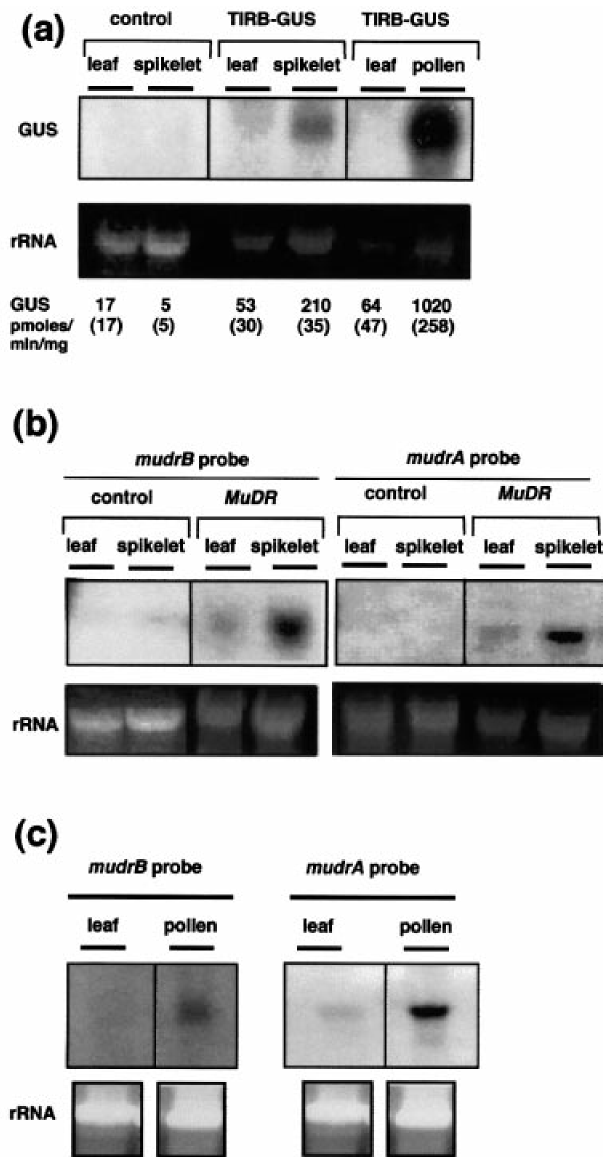


Figure 5. RNA gel blot analysis of *mudrB* TIR-GUS, endogenous *mudrA* and *mudrB* in leaf compared to spikelet and pollen tissues.
(a) The *uidA* probe was used. GUS refers to enzymatic expression. Numbers in parentheses represent the standard errors. The control used was an untransformed sibling. (b) The *mudrB*- and *mudrA*-specific probes were used. The Mutator source is a low-copy *MuDR* line. The control is a non-*Mutator* line. (c) The *mudrB- and mudrA*-specific probes were used. The Mutator source is an active, high copy *MuDR* line.

inverted repeat neither enhances nor interferes with promoter expression in pollen.

Pollen enhancement occurs at the RNA level

Because the *mudrB* TIR region contains both promoter and 5′ untranslated leader sequences, we asked if reporter gene enhancement in pollen was mediated at the transcriptional or translational level. As shown in Figure 5(a), there is a good correlation between *uidA* transcript levels and increased GUS activity in whole spikelets and pollen when compared to leaf. In spikelets, GUS expression increased fourfold, while transcript levels increased 4.3-fold. In pollen, GUS expression increased > 16-fold, while *uidA* RNA levels increased > 22-fold compared to leaf tissue. We conclude that the *mudrB* TIR region contains transcriptional pollen enhancers or that the *mudrB* 5′ UTR region promotes transcript stability in pollen cells.

We then asked if endogenous *mudrB* transcript levels in a low copy *MuDR* line were enhanced in whole spikelets and isolated pollen. As shown in Figure 5(b), *mudrB* transcript levels increased 4.7-fold in spikelets compared to leaves, parallel to the 4.3-fold increase of *uidA* transcripts in spikelets (Figure 5a). As shown in Figure 5(c), *mudrB* transcript levels increased 9.2-fold in pollen compared to leaves. Therefore, the increase in expression observed in spikelets and pollen using the isolated TIRB promoter with a reporter gene parallels the increase in endogenous *mudrB* transcript levels.

mudrA encodes the *Mu* transposase. As shown in Figure 5(b), RNA blot hybridization indicates that endogenous *mudrA* transcript levels also increased > 3.2-fold in spikelets, which is lower but similar to *mudrB* levels from the same tissue samples. Similarly, *mudrA* transcript levels increased 12.3-fold in pollen compared to leaves (Figure 5c). These data suggest that the nine nucleotide polymorphisms (two in the promoter and seven in the 5′ untranslated region) between TIRA and TIRB do not significantly alter *mudrA* leaf or spikelet/pollen regulation at the transcript level. We conclude that both *mudrB* and *mudrA* transcripts are upregulated in pollen and whole spikelets and that the ~216 bp terminal inverted repeats are sufficient to confer this enhancement.

Discussion

We designed a promoter–reporter fusion approach in an effort to understand how the pattern of *MuDR* expression could explain the timing, frequency and type of transposition activity of *Mu* elements. We considered that data derived from a transposon promoter–reporter study using independent integration events would be biologically relevant for two reasons. First, transposon promoters have evolved to function in the context of constantly changing flanking host sequences. Second, unlike stable genes, the boundaries of a transposon promoter are delineated by the outer edge of the terminal inverted repeats. We used the entire *mudrB* TIR in our study, which should contain all of the promoter elements required for *mudrB* expression. These features of transposon biology overcome difficulties in transgene promoter fusion studies

in which nearby host regulatory sequences or omission of an important upstream regulatory sequence compromise interpretation. Furthermore, because the promoters within TIRA and TIRB differ by only two nucleotides and program similar patterns of transcript accumulation (Hershberger *et al.*, 1995; Joanin *et al.*, 1997) we expect that results with TIRB are applicable to TIRA as well.

Mu elements are differentially regulated in somatic and germinal tissues. *Mu* excisions in somatic cells are frequent but occur exceptionally late during development (Levy and Walbot, 1990; McCarty *et al.*, 1989). In contrast, insertions, but not excisions, occur at a high frequency in developing microspores (Robertson, 1981; Robertson, 1985). In this report, we have discovered that the transposase-encoding *MuDR* element TIR contains a promoter region that confers specific although unexpected developmental regulation in both somatic and germinal cells.

MuDR *expression is upregulated in meristematic somatic cells*

Using several independent transformants, we observed heritable GUS upregulation by the *mudrB* TIR in all shoot apical meristems, root apical meristems and tassel primordia examined; meristematic activity was uniformly much stronger than neighboring tissues (Figure 2). We observed low GUS expression in the non-replicating cells of the root quiescent center and root cap. These results are consistent with the immunolocalization study by Donlin *et al.* (1995) in which MURB staining intensity was high in both the shoot apex and inflorescence meristems. By *in situ* hybridization, Joanin *et al.* (1997) observed the highest accumulation of both *mudrA* and *mudrB* transcripts in endothelial cells surrounding the embryo sac (tapetum). The cells at this stage are actively synthesizing DNA without cell division. We tentatively conclude from these

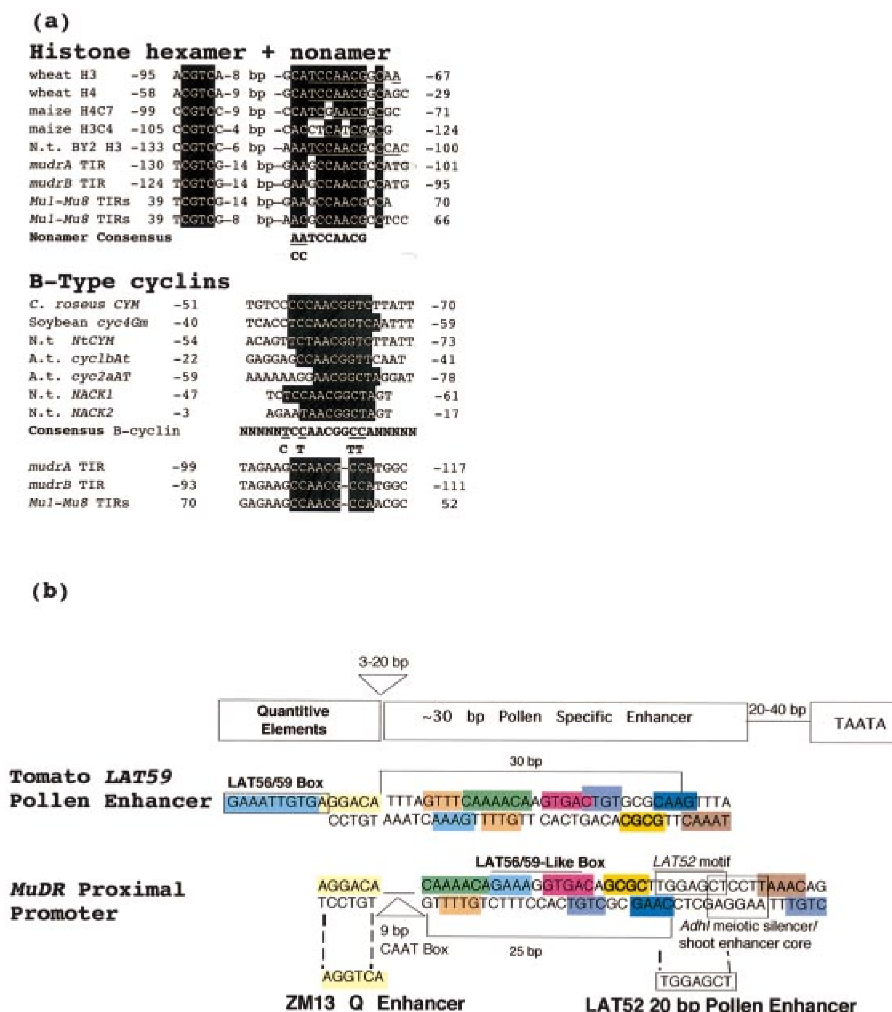


Figure 6. Legend on facing page.

independent observations that both *MuDR* promoters may be upregulated in cells synthesizing DNA. Experiments using synchronized cell cultures will be useful to further test this hypothesis. If true, this result is surprising because *Mu* element excisions are preferentially excluded from pluripotent actively dividing somatic cells; instead, excisions are frequent just before or after the last division of somatic cells as scored using cell-autonomous excision markers (McCarty *et al.*, 1989; M. Raizada and V. Walbot, manuscript in preparation).

All Mu *TIRs contain motifs similar to functionally defined plant enhancers operating in rapidly dividing cells*

In an effort to predict if the *MuDR* promoters would function in other hosts, we performed a database search to look for candidate plant enhancer motifs in the *MuDR* TIRB region. First, we searched for cell-cycle enhancer motifs as shown in Figure 6(a). In the –95 to –124 region, we found high similarity to motifs previously identified as important for H3 histone, H4 histone, kinesin and cyclin gene expression in dividing cells of both monocots and dicots (Brignon and Chaubet, 1993; Chaubet *et al.*, 1996; Kawata *et al.*, 1988; Lepetit *et al.*, 1993; Shen and Gigot, 1997; Terada *et al.*, 1993) (Figure 6a). Not only does *MuDR* TIRB contain these motifs, but they are conserved in TIRA and the eight nonautonomous *Mu* elements (data not shown). Furthermore, 7/9 *Mu* elements contain a second overlapping direct repeat of one of the motifs, the homologous M-phase specific activator (MSA) (Ito *et al.*, 1998) and histone nonamer motif (Reichheld *et al.*, 1998), from positions –104 to –112 in both the left and right TIRs. We note that the first MSA/nonamer repeat overlaps the MURA transposase binding site (at –107 to –138) by 1 bp, while the duplicated motif and histone hexamer surprisingly occupy 12/32 bp of the MURA binding site (data not shown). One possibility is that in meristematic cells, transcription factors bind to not only *MuDR* TIR histone/cyclin motifs, but also to the same motifs within the TIRs of *Mu1-Mu8*, thus preventing MURA transposase binding or transposition in dividing cells. Transcription factor and transposase competition for DNA binding sites have been proposed to occur within the promoters of *Spm* and *Ac* elements (Fedoroff and Chandler, 1994). It will be useful to perform site-directed mutagenesis of the *MuDR* TIR cyclin/histone motifs to confirm their identity and of the *Mu1* motifs to determine if *Mu1* excision timing becomes altered. Being able to control *Mu* excision timing would be very useful in characterizing *Mu*-tagged alleles.

MudrA *and* mudrB *are highly expressed in pollen and their TIRs contain candidate pollen enhancer motifs*

Our most dramatic result was that the TIRB region programmed a 30-fold average increase in luciferase and GUS expression in mature pollen compared to the average mature leaf. This conclusion is based on a survey of 55 plants representing five independent TIRB transformants

Figure 6. Nucleotide comparison of *Mu* element terminal inverted repeats to functionally defined enhancers found in the promoters of monocot and dicot genes.

Sequences homologous to the consensus are shaded (see text for references). Locations in promoters are relative to the start of transcription. In *Mu1–Mu8*, locations are relative to transposon termini. (a) *Mu* TIRs contain motifs required for transcriptional enhancement in fast-dividing cells. Two well-defined histone motifs (Brignon and Chaubet, 1993; Kawata *et al.*, 1988; Lepetit *et al.*, 1993; Terada *et al.*, 1993), a hexamer containing the conserved CGTC nucleotide core and a nonamer consensus [(AA/CC)TCCAACG], separated by 4–14 bp, are present; these elements help confer S-phase expression to histone genes (reviewed in Chaubet *et al.*, 1996; Shen and Gigot, 1997). Both *MuDR* TIRs have a 8/9 bp identity (–96 to –104) to the nonamer consensus [(AA/CC)TCCAACG] and a 10/11 bp match to the tobacco BY2 H3 nonamer region (Reichheld *et al.*, 1998). The match always includes the sequence CCAACG, an myb-like motif. Underlined bases are those conserved with the B-type cyclin mitosis-specific activator (MSA) consensus motif which we note both contain the core sequence CCAACG. Ito *et al.* (1998) have demonstrated that MSAs of the *Catharanthus roseus* CYM gene act as orientation-independent G2 and M phase enhancer of transcription in synchronized tobacco BY2 cells when placed upstream of a minimal CaMV 35S promoter. TIRB and TIRA each have a 9/11 bp match (CCAACG-CCA) to the MSA from –99 to –107. *C. roseus*, *Catharanthus roseus*; A.t., *Arabidopsis thaliana*; N.t., *Nicotiana tabacum*. (b) Sequence comparison of the *MuDR* TIR promoter to the 30 bp pollen enhancer of the tomato *LAT59* gene promoter and other maize (*ZM13*) and tomato (*LAT52,LAT56*) pollen enhancers. The overall structure of pollen enhancers is shown on top (summarized from Hamilton *et al.*, 1998). The sequence shown of *MuDR* corresponds to the –22 to –76 region, relative to the *mudrB* transcription initiation site, which is the same as position 87–141 of the published *mudrA* TIR sequence (Hershberger *et al.*, 1991). All *MuDR* sequences shown are identical in both *mudrA* and *mudrB* TIRs. Several shaded regions common to *MuDR* and the *LAT59* pollen enhancer (for example, the LAT52/56 box: (A)GAAA associated with GTGA/GTGG; motif ACTGT) have been previously shown to be important in conferring pollen-specific expression to minimal promoters (Eyal *et al.*, 1995; Twell *et al.*, 1991). In each case, base substitution or deletion was shown to reduce pollen-specific expression. The LAT52/56 box is found in TIRB starting at position –54. The sequence ACTGT is found at positions –43 and –69 of TIRB. The motif AGGACA, located 10 bp upstream of the *MuDR-LAT59* pollen specificity sequence starting at position –76, has a 5/6 bp match to the ZM13 Q enhancer. This sequence does not specify pollen expression, but rather acts as a pollen-specific quantitative enhancer of a downstream basal pollen promoter (Hamilton *et al.*, 1998). We note that the *MuDR* homolog to the Q enhancer, AGGACA, has a 5/6 bp match with the motif TGGACA located in the LAT59 promoter. Both motifs are located just upstream of the shared 30 bp enhancer region. Interrupting the region of *MuDR* homology to the *LAT59* enhancer at –28 to –38 are two overlapping sequences, TGGAGCT and CTCCTT, which have not previously been defined as pollen enhancer motifs. The former 7 bp sequence is completely conserved in the *LAT52* pollen 20 bp enhancer region (– 52 to – 71) (Bate and Twell, 1998) which is sufficient to specifically enhance pollen expression ninefold when located upstream of a minimal promoter (Bate and Twell, 1998). A linker replacement overlapping a 7 bp motif (TGGAGCT) within this region (called Domain C) reduced pollen expression fourfold compared to the –100 *LAT52* promoter (Bate and Twell, 1998). The second *MuDR* sequence (CTCCTT) is located in the *Adh*I promoter, 45–50 bp upstream of the TATA box, in a region previously identified as acting as a shoot enhancer and/or meiotic repressor of transcription (Kyozuka *et al.*, 1994).

monitored over two generations (Figure 4). RNA hybridization blots of native *MuDR* transcripts confirmed that enhancement was at the RNA level and included both *mudrA* and *mudrB* (Figure 5). By database analysis, we have identified a nested set of candidate motifs in the TIR promoters that match the suite of pollen enhancer motifs in well-studied genes, particularly the promoter of tomato *LAT59* (Figure 6b) (Twell *et al.*, 1991). Previously, linker-replacement mutagenesis of the tomato *LAT59* promoter has defined a 30 bp region essential for pollen-specific enhancement (Eyal *et al.*, 1995). When placed upstream of a minimal promoter in either orientation, these 30 bp alone enhanced pollen expression >25-fold, but had no effect in mesophyll-derived cells (Eyal *et al.*, 1995). As shown in Figure 6(b), both *mudrA* and *mudrB* TIRs have a 25 bp sequence located 17 bp upstream of the putative TATA box that contains seven motifs shared with the *LAT59* 30 bp pollen enhancer. Immediately upstream and downstream of this region are two additional conserved motifs defined as pollen enhancers in tomato *LAT59* and *LAT52* and in maize *ZM13* genes (Bate and Twell, 1998; Hamilton *et al.*, 1998; Hanson *et al.*, 1989; Twell *et al.*, 1990). Not only do the *MuDR* promoters share motifs common to other pollen enhancers, they also share a common promoter structure of three parts in defined spacing (Hamilton *et al.*, 1998). Most distal is a set of short quantitative enhancer elements located 3–20 bp upstream of the second element, a ~30 bp region which confers pollen specificity. The specificity elements are located 20–40 bp upstream of the third element, the TATA box. As shown in Figure 6(b), *MuDR* shares this tripartite structure relative to the downstream TATA box. Future experiments will be required to confirm the identity of the predicted *MuDR* pollen enhancer. Nevertheless, the striking sequence similarity and arrangement of a well-studied tomato pollen-enhancer to a monocot transposon promoter, in combination with TIRB expression studies (Figures 4 and 5), strongly suggest that the *MuDR* promoters contain pollen enhancers that we predict will be active in both dicots and monocots.

Unlike the case with the MSA and histone motifs, however, none of the other *Mu* element termini contain the full suite of pollen enhancer motifs. Point substitutions are numerous in this region in the nonautonomous *Mu* elements (data not shown).

MuDR promoter expression timing corresponds to the timing of Mu insertions during pollen ontogeny

Transcriptional enhancement of the *LAT* genes begins with the onset of microspore mitosis (Hanson *et al.*, 1989; Twell *et al.*, 1990) consistent with the timing of the final *Mu* insertions in individual sperm. At least 20% of new germinal insertions at the *Y1* locus occur after microspore mitosis II in the generative nuclei, resulting in brother sperm carrying different insertions (Robertson and Stinard, 1993). However, peak *LAT* gene enhancement occurs in the vegetative nuclei (Twell *et al.*, 1990), not the generative nucleus. It will be interesting to know if peak *MuDR* expression occurs in the generative or vegetative nucleus and whether or not the sequence differences between the *MuDR* and *LAT* promoters direct differential nuclear expression. The majority of new germinal *Mu* insertions occur before meiosis and in generative nuclei (Robertson, 1981; Robertson, 1985), hence expression is likely to occur throughout microsporogenesis. In a preliminary experiment, we have recently observed that TIRB programs a 2.3- to 33-fold increase in luciferase expression in whole immature anthers compared to leaves. Five pre-emergent tassels were analyzed and most microspores were in the postmeiotic tetrad and uninucleate stages, strongly suggesting that *MuDR* transcription is upregulated prior to microspore mitosis, although it may peak at that stage. In these same individuals, luciferase expression in mature pollen ranged from 12.5- to 52-fold higher compared to leaves (data not shown). We observed a > 20-fold enhancement in luciferase expression in a premeiotic immature tassel (Figure 3c). This result agrees with an immunolocalization study that reported high MURB accumulation in developing tassel primordia (Donlin *et al.*, 1995). Perhaps a candidate meiotic repressor sequence (CTCCTT; Kyozuka *et al.*, 1994) in the *MuDR* promoters could explain an additional observation of Donlin *et al.* (1995) that MURB was much less abundant in premeiotic mother cells; postmeiotic pollen were not analyzed. We conclude that programmed expression of the *MuDR* promoters during the germinal phase of the maize life cycle partly explains the high frequency of *Mu* germinal insertions. The complex nature of the *MuDR* TIRs, which include the MURA binding site (Benito and Walbot, 1997), CAAT and TATA boxes (Benito and Walbot, 1994), candidate somatic histone/cyclin motifs and pollen enhancers (Figure 6), explains why *Mu* TIRs are so unusually long when compared to other maize transposons; these motifs account for 100/162 bp of the *MuDR* TIRs preceding the transcriptional start site.

Implications

Mu element behavior is distinct in somatic versus germinal cells. *Mutator* has two developmental programs that make it attractive for transfer to heterologous plants for transposon tagging: *Mu* elements rarely excise early in somatic tissues such as the shoot apex (Levy and Walbot, 1990), thus permitting the isolation of stable, tagged mutant alleles. Second, *Mu* elements insert at a high frequency late during germinal development (Robertson, 1981, 1985), causing the production of independent,

heritable mutants. In this report, we have demonstrated that the *MuDR* TIRs contain a promoter that programs distinct expression in somatic and germinal cells. We therefore suggest that it may be important to use the endogenous *MuDR* promoters when transferring *MuDR* to other hosts. In fact, we recently demonstrated that a CaMV 35S-driven *mudrA* construct was unable to catalyze germinal *Mu* insertions, even though it caused a high frequency of somatic *Mu* excisions in transgenic maize (Raizada and Walbot, 2000). In transgenic tobacco and *Arabidopsis* and transient assays in maize, the CaMV 35S promoter is not expressed in pollen (Fennell and Hauptmann, 1992; Jardinaud *et al.*, 1995; Wilkinson *et al.*, 1997). It will be useful to retransform plants with the *mudrA* cDNA under the control of its native promoter to determine if it is sufficient to cause insertions or if *mudrB* is required. It may even be useful to re-engineer the *Ac* and *Spm* transposases with the *LAT59/MuDR* pollen enhancers to try to increase the frequency of late germinal insertions of *Ds and dSpm* elements for transposon tagging experiments.

Experimental procedures

Vectors

Construction of pMB5 has been described previously (Benito and Walbot, 1994). It consists of the complete 216 bp *mudrB* TIR fused to the maize *AdhI* intron and 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. Plasmids pAHC20 and pAHC25 were obtained from P. Quail (Christensen and Quail, 1996).

Maize transformation

Embryogenic A188 X B73 (HiTypeII) calli (Armstrong and Green, 1985; Armstrong, 1994) were osmotically treated (Vain *et al.*, 1993) then biolistically transformed using the PDS 1000HE device (BioRad, Hercules, CA, USA) at 650 psi and 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 1.0 cm and from the mesh screen to the target, 5.9 cm. For three bombardments, 25 µg total of plasmids pMB5, pMR42 and pAHC20 were co-precipitated in equimolar quantities onto 2 mg of 1 µM spherical gold particles (Alameda Scientific Instruments, Richmond, CA, USA) following the procedure of Wan *et al.* (1994). Transformed calli were selected on 3 mg ml^{-1} bialaphos (Meiji Seika Kaisha Ltd, Yokohama, Japan) (Spencer *et al.*, 1990), which is the active ingredient in Basta herbicide. Herbicide resistant plants were selected by painting a 5 cm diameter leaf surface with 0.75% glufosinate ammonium (Ignite 600, 50% solution, Hoescht, Montreal, Canada) with 0.1% Tween 20. Transgenic line ubi:GUS was provided by J.C. Carle-Urioste, Stanford University, USA.

Nucleic acid probes

All *MuDR* numbering is according to Hershberger *et al.* (1991). The 1.3 kb *mudrA*-specific probe extends from *MuDR* sites +450 to +1790 (*Sph*I site) and was isolated as a 1.3 kb *Pst*I to *Sph*I fragment from phage phMR49. The *mudrB*-specific probe extends from the *Stu*I (+3630) to *Stu*I (+4310) sites of *MuDR*, and was isolated from plasmid pMR29. The luciferase probe was isolated as a 1.2 kb *EcoR*I fragment from pMB5 (Benito and Walbot, 1994). The *uidA* probe was isolated as a 1.8 kb *Sal*I–*BamH*I fragment from pJB4 (Bodeau and Walbot, 1992).

Transgene copy number determination

Genomic DNA was isolated from leaves using the protocol of Dellaporta (1994). To determine transgene copy number, Southern blots were hybridized with luciferase or *uidA* probes as previously described (Warren and Hershberger, 1994).

RNA hybridization analysis

Total RNA was isolated using Trizol (BRL) according to the manufacturer's instructions. For RNA gel blot analysis, 15 µg of total RNA was electrophoresed and transferred to Hybond N (Amersham, Piscataway, NJ, USA) and UV cross-linked. Membranes were pre-hybridized at 42°C for > 2 h in 50% formamide, 1 × P Buffer (0.2% BSA, 0.2% polyvinyl-pyrrolidone 40000, 0.2% Ficoll 400 000, 50 mM Tris–HCl pH 7.5, 0.1% sodium pyrophosphate, 1% SDS), 10% dextran sulfate, 0.58 g/10 ml NaCl, and 100 µg ml^{-1} denatured salmon sperm DNA. Hybridization was continued in the same solution after addition of the denatured probe for 12–20 h at 42°C. Filters were washed twice for 5 min at 25°C in 2 × SSC, 0.1% SDS, and then twice at 65°C for 30 min each in 0.1 × SSC, 0.1% SDS, and autoradiographed. Original X-rays were scanned and RNA bands were quantified using the BioRad Multi-Analyst Software program, version 1.0.2., then normalized to ribosomal RNA.

Histochemical analysis of β-glucuronidase activity

Fresh tissues were vacuum-infiltrated in 1 mM X-gluc substrate (Biosynth, A.G., Naperville, IL, USA) solution for 1–4 h at room temperature and then incubated at 37°C for 1–24 h as described previously (Jefferson, 1987) with the addition of 20% methanol.

Luciferase imaging

Fresh tissues were sprayed with 1 mM K-luciferin (Analytical Luminescence Laboratory, San Diego, CA, USA), incubated for 10–20 min at room temperature and then imaged using a Hamamatsu CCD camera with a Nikon AF MicroNikkor 60 mM lens and Argus 50 image processor software (Hamamatsu Photonics, Enfield, UK).

GUS and luciferase enzymatic assays

Leaves were sampled by combining 4–6 paper hole punches from a wide tip to base area of each leaf blade. All tissues were frozen in liquid nitrogen, stored at –80°C, then homogenized on ice in CCLR buffer (Luehrsen *et al.*, 1993) with sand using pre-chilled mortars, pestles, materials and buffer, and centrifuged at 4°C.

Extracts were kept on ice and immediately assayed. Assays were performed as described previously (Luehrsen *et al.*, 1993). GUS assays were performed in the presence of 20% methanol to reduce endogenous GUS activity (Kosugi *et al.*, 1990). No significant GUS or luciferase expression was ever observed in any non-transformed tissue including pollen. 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 < 1/200 of the total reagent volume.

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