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Mutator-Suppressible Alleles of *rough sheath1* and *liguleless3* in Maize Reveal Multiple Mechanisms for Suppression

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Manuscript received August 24, 1999
Accepted for publication September 28, 1999

ABSTRACT

Insertions of *Mutator* transposons into maize genes can generate suppressible alleles. *Mu* suppression is when, in the absence of *Mu* activity, the phenotype of a mutant allele reverts to that of its progenitor. Here we present the characterization of five dominant *Mu*-suppressible alleles of the *knox* (*knotted1*-like h**o**meobo**x**) genes *liguleless3* and *rough sheath1*, which exhibit neomorphic phenotypes in the leaves. RNA blot analysis suggests that *Mu* suppression affects only the neomorphic aspect of the allele, not the wild-type aspect. Additionally, *Mu* suppression appears to be exerting its effects at the level of transcription or transcript accumulation. We show that truncated transcripts are produced by three alleles, implying a mechanism for *Mu* suppression of 5′ untranslated region insertion alleles distinct from that which has been described previously. Additionally, it is found that *Mu* suppression can be caused by at least three different types of *Mutator* elements. Evidence presented here suggests that whether an allele is suppressible or not may depend upon the site of insertion. We cite previous work on the *knox* gene *kn1*, and discuss our results in the context of interactions between *Mu*-encoded products and the inherently negative regulation of neomorphic *liguleless3* and *rough sheath1* transcription.

THE insertion of transposable elements into genes can have diverse consequences for gene regulation. Transposon-induced alleles, while often thought of as primarily resulting in loss-of-function "knock-outs," actually exhibit a fascinating array of regulatory alterations. These alterations include overexpression or misexpression of the gene, alterations in the start of transcription initiation, as well as commandeering the gene's expression completely through the interaction of *trans*-acting factors with the inserted element.

Insertions of the retrotransposons *gypsy* or *copia* in Drosophila can cause the overexpression of the gene into which they have inserted. Examples of this include the Dominant *Hairy-Wing* (*Hw*) alleles at the *achaete-scute* locus (Campuzano *et al.* 1986). Misexpression is exemplified by *tom* retrotransposon insertions in Drosophila. *tom* appears to contain sequences which can function as an eye enhancer, resulting in dominant eye phenotypes (Tanda and Corces 1991). An example of a transposable element whose insertions cause mutant phenotypes dependent upon endogenous *trans*-acting factors is the Drosophila retrotransposon *gypsy.* The 5′ untranslated region (5′UTR) of the *gypsy* element contains binding sites for the Suppressor of Hairy-wing protein. Experiments have shown that the inserted *gypsy* element, bound by Su(Hw), functions as an insulator, preventing the interaction of distal enhancers with inappropriate promoters and resulting in tissue-specific mutations (Dorsett 1990; Holdridge and Dorsett 1991; Jack *et al.* 1991; Geyer and Corces 1992).

A transposon can also usurp entirely the promoter function of the gene into which it has inserted. *high chlorophyll flourescence106* (*hcf106*), is a gene involved in the maize chloroplast electron transport pathway. *hcf106::Mu1* is a recessive loss-of-function mutation caused by the insertion of a member of the *Mutator* (*Mu*) family of transposable elements (*Mu1*; Barkan and Martienssen 1991). Under some circumstances, the *Mu* element can act as a cryptic promoter, initiating transcripts extending outward from its terminal inverted repeat (TIR) restoring gene function.

The *Mutator* system of transposons in maize is made up of at least five nonautonomous elements, all under the control of the system's autonomous regulator *MuDR* (Chomet *et al.* 1991; Hershberger *et al.* 1991; Qin *et al.* 1991; reviewed by Chandler and Hardeman 1992; James *et al.* 1993). All of these elements share sequence homology only in their ∼220-bp TIRs; their internal sequences are unique. The 4.9-kb *MuDR* element encodes two transcripts, *mudrA* and *mudrB* (Hershberger *et al.* 1991). *mudrA* has significant homology with the *IS10* family of bacterial transposases (Eisen *et al.* 1994). Analysis of *MuDR* deletion derivatives has shown that, while a functional *mudrA* gene is required for excision, transposition, and suppression, the *mudrB* gene is necessary only for transposition and suppression (Lisch *et*

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al. 1999). *Mutator* activity is correlated with *mudrA* and *mudrB* transcript accumulation, hypomethylation of the TIRs, excisions, new transpositions, and the appearance of extrachromosomal, supercoiled, circular forms of *Mu* (reviewed in Chandler and Hardeman 1992). The *Mutator* system is extremely active. Estimates have placed the transposition frequency between 17.5 and 51.5% of new positions of total insertion sites, depending upon the chromosomal position of a single *MuDR-1* element (Lisch *et al.* 1995). This variability in transposition frequency dependent upon the chromosomal position of the autonomous element is in contrast to the maize transposon system *Suppressor-mutator* (*Spm/dSpm*; Raina *et al.* 1993). Unlike another maize transposon system, *Activator/Dissociation* (*Ac/Ds*), *Mu* generally inserts into unlinked sites (Lisch *et al.* 1995). This feature, along with its high rate of transposition, makes it an excellent tool for mutagenesis. *Mutator* is thought to transpose using a "cut-and-paste" mechanism with subsequent gap repair off the sister strand at the donor site, much like *P* elements in Drosophila (Engels *et al.* 1990; Lisch *et al.* 1995). *Mutator*-induced mutations have been instrumental in identifying and characterizing numerous genes in maize including the *knotted1*-like h**o**meobo**x** (*knox)* genes *liguleless3* and *rough sheath1*, the subjects of this study.

The *knox* genes *knotted1*, *liguleless3*, *rough sheath1*, *gnarley1*, and *liguleless4* were first defined by a series of dominant mutations exhibiting similar, yet distinguishable, phenotypes in the leaf (Figure 1; Freeling 1992). These mutations all show perturbations at the blade-sheath boundary, the junction between the distal, dark-green, highly photosynthetic blade and the more proximal yellow sheath. In these mutants, the more distal organ regions, such as blade, acquire more proximal identities, such as sheath (Freeling 1992). *knox* genes are normally expressed in shoot apical cells. The dominant phenotypes are caused by their ectopic expression in the leaf (Smith *et al.* 1992; Schneeberger *et al.* 1995; Muehlbauer *et al.* 1999).

Several of the *Mutator*-induced *liguleless3* (*lg3*) and *rough sheath1* (*rs1*) alleles identified are *Mu* suppressible. *Mu*-suppressible alleles are those alleles whose phenotypes are dependent upon whether they are in a *Mu*-active or -inactive background. More formally stated, *Mu* suppression occurs when the phenotype of the *Mu*-induced allele returns to that of its progenitor in the absence of *Mu* activity. *hcf106::Mu1*, discussed earlier, is an example of a *Mu*-suppressible allele. When *Mu* is active, *hcf106::Mu1* transcripts fail to accumulate, and the plant appears mutant. When *Mu* is inactive, transcription once again resumes, restoring the phenotype to that of the progenitor (Barkan and Martienssen 1991). The *Lg3-O* and *Rs1-O* alleles are both dominant, gain-of-function mutations, and the lesions that cause these mutations are not known. The *liguleless3*- and *rough sheath1*-suppressible alleles (*Lg3-Or331*, *Lg3-Or422*, *Lg3-Or1021*, *Lg3-Or211*, and *Rs1-Or11*) were identified in screens for revertants of the reference alleles *Rs1-O* and *Lg3-O* (Muehlbauer *et al.* 1999; R. Schneeberger, unpublished results). They have been designated *Lg3*- or *Rs1-Or#*, with *O* for original, *r* for revertant, and then an assigned number. For each of these suppressible alleles, when *Mu* is active, the plant appears wild type; when *Mu* is inactive, the phenotype reverts to that of the progenitor, in this case the reference allele, and the plant appears mutant (Figure 1).

The mechanism by which *Mu* activity is able to act as a switch, turning on and off the mutant phenotypes of suppressible alleles, is not well understood. Suppression of *hcf106::Mu1* is postulated to be the result of a transcriptional block (Barkan and Martienssen 1991). It was proposed that when *Mu* is active, proteins, presumably transposases, are bound to the ends of the inserted element blocking transcription, but when *Mu* is inactive, these proteins are no longer bound and transcription can occur. It has been suggested that dominant suppressible alleles of *Knotted1*, caused by insertions into the third intron, are due to *Mu* activity interfering with the binding of a silencer element (Greene *et al.* 1994).

Suppression is found in other systems besides *Mutator.* Insertions of *Suppressor-mutator* (*Spm)*, another maize transposable element system, can also result in suppressible alleles. When *Spm* is active, alleles containing the transposon display the null phenotypes. When *Spm* is inactive, if the element is inserted with its transcription unit opposite to that of the gene, it can be spliced out, and the mutant phenotype is suppressed (Gierl *et al.* 1985; Kim *et al.* 1987). Mutant phenotypes caused by insertions of the retrotransposons *gypsy* and *copia* in Drosophila can be suppressed by the effects of several unlinked modifier genes including *suppressor of Hairy-wing* [*su(Hw)*], *enhancer of white eosin* [$e(w^e)$], and *suppressor of forked* [*su(f)*], each named after the first allele they were found to modify (Rutledge *et al.* 1988). The mechanism by which each of these modifiers exerts their influence is not well understood.

To better understand the mechanism of *Mu* suppression, we have characterized five suppressible alleles of *lg3* and *rs1.* These alleles represent insertions into both introns and the 5′UTR of the genes. We have discovered that at least two mechanisms exist for *Mu* suppression caused by insertions into the 5′UTR, and these are likely to be distinct from how *Mu* suppression functions in intron insertions. Understanding the molecular basis of *Mutator* suppression is likely to contribute to our understanding not only of transposon biology, but also to our understanding of the spatial regulation of *Lg3* and *Rs1.* There is evidence that one or more of the *knox* genes, including *rs1*, are subject to negative regulation by the MYB transcription factor RS2 (Schneeberger *et al.* 1998; Timmermans *et al.* 1999; Tsiantis *et al.* 1999), and suppressible alleles may highlight participating sites

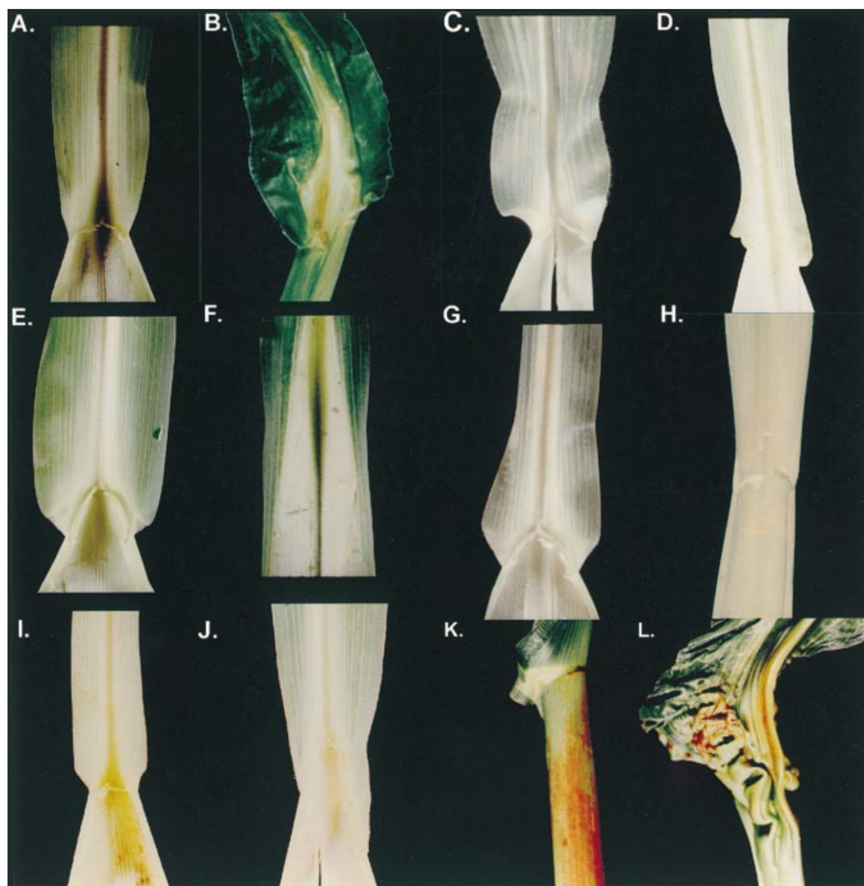

Figure 1.—*Lg3-O-* and *Rs1-O*-suppressible alleles display a range of leaf phenotypes. (A) +/+; (B) *Lg3-O*; (C) *Lg3-Or422/+ Mu*-on; (D) *Lg3-Or422/+ Mu*-off; (E) *Lg3-Or211/+ Mu*-on; (F) *Lg3-Or211/+ Mu*-off; (G) *Lg3-Or1021/+ Mu*-on; (H) *Lg3-Or1021/+ Mu*-off; (I) *Lg3-Or331/+ Mu*-on; (J) *Lg3-Or331/+ Mu*-off; (K) *Rs1-Or11/+ Mu*-on; (L) *Rs1-Or11/+ Mu*-off.

in *rs1* and functionally equivalent sites in the other *knox* genes.

MATERIALS AND METHODS

Genetic stocks: *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* were isolated by J. Fowler in a *Lg3-O* revertant screen as described in Muehlbauer *et al.* (1999). Each of these alleles has been introgressed three times into a lab inbred line that carries the *sh1-bz1-m4* deletion. We used the mutable *bronze* allele *bz1-mum9* to monitor *Mutator* activity. The *bz1-mum9* allele in the homozygous condition or with *sh1-bz1-m4* results in clonal purple spots on a bronze background in the aluerone layer of kernels when the *Mutator* system is active. The *Rs1-Or11* allele was isolated in a similar screen for revertants of *Rs1-O* (R. Schneeberger, unpublished results). This allele has been introgressed three and four times into the inbred line B73, and *Mu* activity was monitored using the *a1-mum2* allele. When *Mutator* is active, homozygous *a1-mum2* or *a1mum2/a1sh2* kernels exhibit red clonal sectors on a yellow background.

PCR: For the determination of *Mu* insertion sites, the *Mu* D09242 (5′ AGA GAA GCC AAC GCC AWC GCC TCY ATT TCGTC 3′) primer was used coupled with either *Lg3* R5′-1 (5′ CTG GTA TTC TAG TAC GCC 3′) for the *Lg3* 5′UTR insertions, *Rs1*-U6 (5′ TGG AGT TCC TCA AGC GGG TG 3′) for *Rs1-Or11*, or *Lg3* cDNA F1 (5′ CCC AAC CTC TCT CTC TCC CCC CTAG 3′) for *Lg3-Or211.* Amplification conditions were 94° for 2 min, 35× [94° for 1 min, 60° for 30 sec, 72° for 1 min]. PCR products were electrophoresed, and then purified using the QIAquick gel extraction kit (QIAGEN, Chatsworth, CA). Purified PCR products were then direct sequenced at the University of California, Berkeley DNA sequencing facility using the double-stranded dye termination technique on an ABI sequencer (Applied Biosystems, Foster City, CA). To determine which *Mu* element was inserted into *Rs1-Or11*, PCR primers PBO9 (5′ CGA TCC CAT CCA GCT TGT CACC 3′) and *Rs1*-U6 were used with the Extend Long Template PCR kit (Roche) according to the manufacturer's instructions, and products were sequenced as described above. Alterations in the regions 3′ of the *Lg3-Or1021* allele were investigated using the *Mu*DO9242 primer and and *Lg3*cDNA B1 (5′ CGC CTG AAT GCT GCT CAG GAA CGAC 3′) primer. Amplification conditions were the same as above, except extension time was increased to 1.5 min.

RT-PCR: RT-PCR was performed according to Bauer *et al.* (1994), except all samples were amplified for 35 cycles. PCR primers used were the *Lg3* cDNA B1 and *Lg3* cDNA F1 primers described above. *ubiquitin* primers used as controls were Ubi3 (5′ TAA GCT GCC GAT GTG CCT GCG TCG 3′) and Ubi4 (5′ TAA GCT GCC GAT GTG CCT GCG TCG 3′).

Rapid amplification of cDNA ends (RACE): RACE reactions were performed according to manufacturer's instructions using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) with 1 μg poly(A)$^+$ RNA from immature ear tissue and PCR primers *Lg3*-3′ (5′ CGC GGG ATC CAG TGG TGT ATG ATT CAG GGT CC 3′) and *Lg3*-D3 (5′ GAA GTA GAG TGT CGT CCC AGA AGA CCC ACC 3′) as a nested amplification primer.

RNA blot analysis: Total RNA was isolated from sheath or shoot of ∼5-wk-old plants using TRIZOL reagent (Gibco BRL, Gaithersburg, MD) according to manufacturer's instructions. For analysis of *rough sheath1* transcript, poly(A)$^+$ RNA was isolated on oligo(dT) cellulose columns according to Schnee-

berger *et al.* (1995). Approximately 20 μg of total RNA or 2 μg of poly(A)$^+$ was electrophoresed on 1.2% formaldehyde gels, transferred in 20× SSC overnight onto Duralon membrane (Stratagene, La Jolla, CA). Blots were then UV crosslinked in a Stratalinker (Stratagene), and prehybridized in 6× SSC, 2 mm EDTA, 10 mm Tris-Cl, pH 7.5, 5× Denhardt's, 0.2 mg/ml salmon sperm DNA, 20 mm sodium phosphate buffer, pH 7, and 1% *N*-lauryl-sarkosyl at 65°. Probes were radiolabeled using Stratagene's Prime-It II kit according to manufacturer's instructions. *lg3* probes used are shown in Figure 5. The *lg3* 3′ probe was shown to be unique by sequence comparison with other *knox* genes (P. Bauer, unpublished results). *Rough sheath1* RNA expression was analyzed using the pVM4.1 *rs1* cDNA as a probe. After hybridization, blots were washed in 0.2× SSC, 0.5× SDS at 65°, and exposed on Kodak X-OMAT AR film for 1–3 days.

DNA gel blot analysis: Genomic DNA isolation and DNA gel blot analysis were performed according to Lisch *et al.* (1995).

RESULTS

Suppressible dominant alleles delineate two functions in the mutant; suppression affects only one: The dominant *Rs1* and *Lg3* mutations are made up of two components, a wild-type component and a gain-of-function component. It is possible that *Mu* suppression acts as a general repressor of these genes. This would not be immediately distinguishable, as single mutant loss-of-function phenotypes for either of these genes probably do not exist (R. Tyres and M. Freeling in collaboration with Pioneer Hi-Bred Seed, Inc., unpublished results). Another possibility is that *Mu* suppression may act exclusively on the dominant ectopic function. To distinguish between these possibilities, we used RNA blot analysis to examine *lg3* or *rs1* expression in *Mu*-active, homozygous shoot tissue. In this experiment, because only the dominant allele was present, expression detected in this tissue would indicate that wild-type expression is not affected by suppression. This is precisely what we found (Figure 2). Total RNA from wild-type shoot tissue and shoot tissue from *Mu*-active, homozygous *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* plants was analyzed by RNA gel blot analysis, using the *lg3* 3′UTR region as a probe (see Figure 5). This region has been found to be unique by sequence comparisons with other *knox* genes (P. Bauer, unpublished results). While *Mu* activity is able to suppress the neomorphic leaf phenotype, wild-type expression is unaffected, as assayed by expression in the shoot (Figure 2).

***Mu* suppression is independent of element type and can result from insertions at multiple sites:** To determine the context within which *Mu* suppression was functioning in *Lg3* and *Rs1*, we determined where in each of the suppressible alleles the *Mutator* elements were inserted (Figure 3). We used gene-specific PCR primers coupled with a primer that amplifies from the end of all *Mu* elements to determine the sites of *Mu* insertions. We found that the *Rs1-Or11* allele was caused by the insertion of a *Mu* element 154 bp into the third intron.

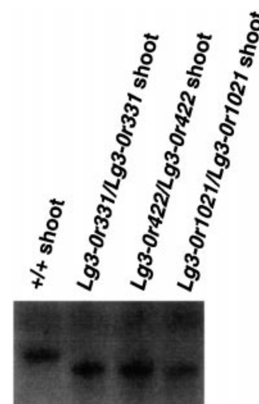

Figure 2.—*Mu* suppression affects only the gain-of-function aspect, leaving the wild-type aspect unaltered. RNA gel blot analysis of *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* homozygous shoot tissue probed with the *lg3* 3′UTR. Note the distinctive size of the transcripts produced by the mutant alleles. This is addressed later. The gel was stained with ethidium bromide to ensure approximately equal loading (not shown).

The *Lg3-Or211* allele was found to be inserted at the 3′ intron/exon junction of the first intron. The *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* alleles were found to be caused by *Mu* insertions into the same site, +29, in the 5′UTR. These results show that *Mu* suppression can be caused by insertions into a variety of sites.

We found that *Mu* suppression of *Lg3* and *Rs1* could be caused by three types of *Mu* elements. PCR amplification followed by direct sequencing of the *Mu* element inserted into the *Rs1-Or11* allele revealed it to be a deletion derivative of *MuDR.* The *MuDR* element undergoes frequent automutagenesis, likely as a result of interrupted double-stranded gap repair (Lisch *et al.* 1995). The *Mu* element in *Lg3-Or211* had been shown previously to be a *Mu1* element (Fowler *et al.* 1996).

To determine which *Mu* elements were inserted into *Lg3-Or331*, *Lg3-Or422*, and *Lg3–Or1021*, plants from families segregating for these alleles were first genotyped by digesting genomic DNA from individual leaf tissue with *Xba*I and hybridizing with a *lg3* 5′ (see Figure 5) probe, which reveals an ~6.9-kb band that segregates with the mutant phenotype (data not shown). These same blots were then stripped and reprobed with a *Mu3*-specific probe. This probe hybridized with the same segregating band as the *lg3* 5′ probe did (Figure 4), suggesting that the polymorphism is due to the insertion of a *Mu3* element. The same samples were also digested with *Eco*RI, which cuts once in *Mu3* and once in *lg3* (Figure 5), and were hybridized with the *lg3* 5′ probe. This resulted in a 1.1-kb band consistent with the insertion of a *Mu3* element into the site in the 5′UTR previously determined (data not shown). The element in *Lg3-Or1021* is likely a *Mu3*-like element (Figure 4). This element cross-hybridizes with a *Mu3* probe, but is polymorphic within the *Xba*I fragment containing it. This

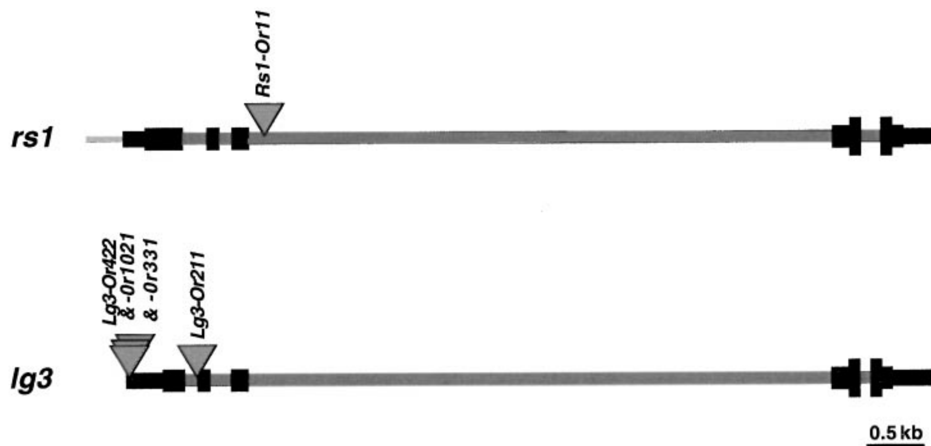

Figure 3.—Locations of suppressible insertions. The *Rs1-Or11* allele contains a *Mu* insertion into the beginning of the third intron. The *Lg3-Or211* allele is caused by a *Mu* insertion into the end of the first intron. The *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* alleles are caused by insertions into the same site in the 5′UTR.

polymorphism is not due to any gross alterations within the *Xba*I fragment, *Eco*RI fragment, or in the region at least 1 kb 3′ of the insertion as determined by PCR (data not shown), and is therefore likely to be due to alterations either 5′ of the insertion or within the element itself. Each of these elements is in the same orientation, as indicated in Figure 5.

The *Lg3-Or331* and *Lg3-Or422* alleles are caused by insertions of the same *Mu* element into the same site in the 5′UTR and are monomorphic at the level of a Southern blot. Thus, it begs the question of whether these alleles represent independent reversion events or repeat isolations of the same event. *Mutator* mutagenesis for the screen that produced these revertant alleles occurred in the male parent, so a reversion event that takes place prior to meiosis I in the pollen cell lineage

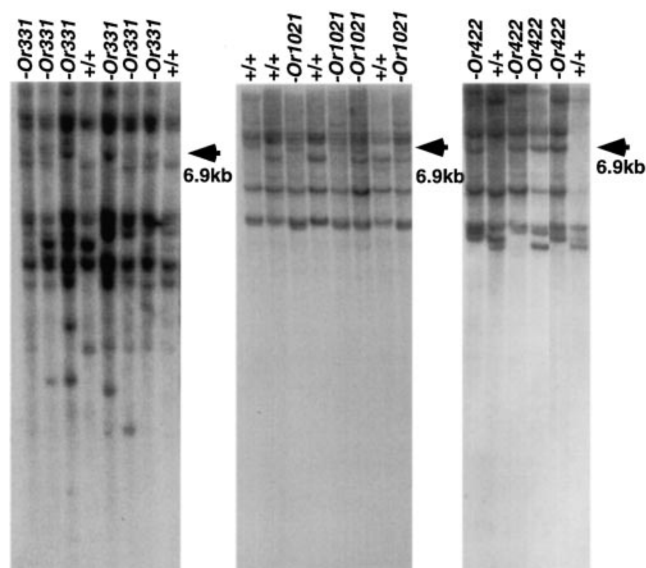

Figure 4.—The *Lg3-Or331*, *Lg3-0r422*, and *Lg3-Or1021* alleles are caused by insertions of a *Mu3* and a *Mu3*-like element. Genomic DNA was isolated from wild-type and mutant segregants, digested with *Xba*I, and probed with a *Mu3*. This revealed an ~6.9-kb band present only in the mutants (see arrow).

could result in a number of plants heterozygous for the same reversion event (Fowler 1994). However, we have evidence supporting the two alleles being independent reversion events. *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* were each found, upon sequencing, to have small genomic deletions in similar regions of the 5′UTR (data not shown), which are not found in their progenitor, *Lg3-O*, or in wild-type siblings. *Lg3-Or331* is missing the base pairs $+140 \rightarrow +90$, while *Lg3-Or422* has a slightly different deletion spanning $+145 \rightarrow +96$. Additionally, *Lg3-Or211*, which contains an insertion into the end of the first intron, is missing $+140 \rightarrow +90$. The deletions are not linked with suppressibility, however, because the nonsuppressible *Lg3-Or81* allele also has a deletion from base pairs $+140 \rightarrow +90$. One possible mechanism for these strange incidences would be if each allele underwent a *Mutator* excision followed by exonuclease cleavage, followed by a subsequent reinsertion into either the intron or 5′UTR. Alternatively, the region in the 5′UTR where these deletions occurred is highly G + C rich and contains numerous direct repeats; therefore, it is possible that these deletions arose from strand slippage during replication.

***Mu* activity abolishes ectopic transcript accumulation resulting in a mutant plant appearing indistinguishable from wild type:** Each of the suppressible *lg3* and *rs1* alleles discussed here appears identical to wild type when they are in a *Mutator*-active background. The mutant phenotype manifests itself only when *Mu* is inactive. We wanted to know whether this was due to an absence of ectopic transcripts or whether *Mu* activity was acting post-transcriptionally. To investigate this, we used RNA blot analysis to examine RNA accumulation in sheath tissue of *Mu*-active heterozygous plants, using the *lg3* 3′UTR as a probe (Figure 5). RNA blot analysis indicated that for each of the alleles, when *Mu* was active and the plants appeared wild type, transcripts failed to accumulate (data not shown) as shown for the *Lg3-Or211* allele (Figure 6). These results suggest that suppression is operating at the level of transcription. Because we did not perform transcription run-on assays,

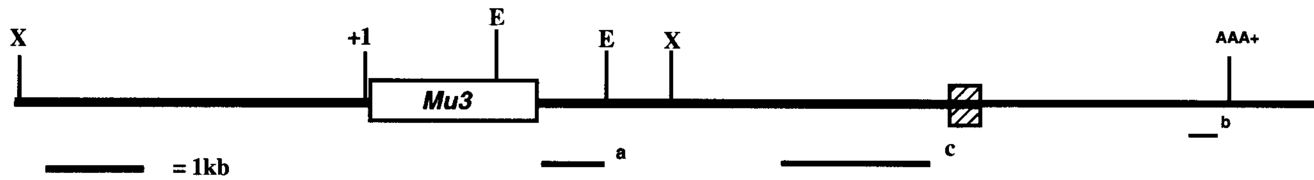

Figure 5.—Schematic diagram of *Lg3* 5′UTR suppressible alleles. The homeobox region is illustrated as a hatched box. The *lg3* 5′ probe spans the first 607 bp of the cDNA. The *lg3* 3′ probe consists of the ~300-bp 3′UTR. The open box denotes the site and orientation of the *Mu3* element. (▨) Homeobox region, (a) *lg3* 5′ probe, (b) *lg3* 3′ probe, (c) *lg3* cDNA probe. The cDNA probe spans the entire 1.5-kb transcript. E, *Eco*RI, X, *Xba*I.

it remains possible that transcripts are initiated but are unstable.

Suppressible intronic insertions mediate normal transcription from a distance: Previous work on *Mu* suppression of *hcf106* had shown that *Mu* is able to function as an outward-reading promoter (Barkan and Martienssen 1991). We wanted to determine if similar mechanisms were operating at the suppressible *lg3* and *rs1* alleles we were studying. Such mechanisms would be especially interesting given the intron insertions we were studying, as this would result in proteins missing exons that could still affect a phenotype. Instead, we found that there must be more than one mechanism to explain *Mutator* suppression. We examined RNA expression in sheath tissue from *Mu*-inactive *Lg3-Or211*/+ or *Rs1-Or11*/+ plants, using the entire cDNA as a probe, to compare the size of the transcript produced to that of wild type, assayed in immature ear tissue (Figure 7). We are confident that it is ectopic *rough sheath1* transcript that we are detecting in the leaf and not ectopic expression of the highly homologous *gnarley1* (Foster *et al.* 1999), based on experiments that find no *gnarley1* expression in *Rs1* mutant leaves (R. Schneeberger, unpublished results). We found that for both alleles the size of the transcript ectopically produced by the dominant allele was identical in size to wild type. If suppression of these alleles were occurring in a manner similar to *hcf106*, then we would expect significantly smaller transcripts containing only the region distal to the insertion. When *Mutator* is active, no ectopic transcripts accumulate in these alleles (Figure 6; and our unpublished results). These results suggest that the suppressible-*Mu* intron insertions may prevent ectopic transcription in a *Mu*-activity-dependent manner.

***Lg3-Or331*, *Lg3-Or422*, and *Lg3–Or1021* produce altered transcripts:** *Lg3-Or331*, *Lg3-Or422*, and *Lg3–Or1021* are caused by insertions into the 5′UTR, so we thought these alleles might be likely candidates for using *Mu* as an outward-reading promoter. Instead, we found that when *Mu* is inactive, these alleles produce a transcript that is significantly shorter than that of wild type (Figure 8A). These transcripts appear too short to be explained by transcripts initiating from the end of the inserted element. These truncated transcripts are seen

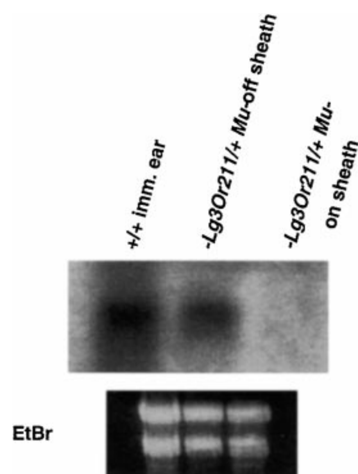

Figure 6.—*Mu* activity abolishes ectopic *Lg3-Or211* accumulation. Wild-type immature ear RNA and sheath RNA from *Lg3-Or211*/+ *Mu*-off and *Lg3-Or211*/+ *Mu*-on plants were hybridized to the *lg3* cDNA probe. The ethidium bromide-stained gel is shown below for a loading control.

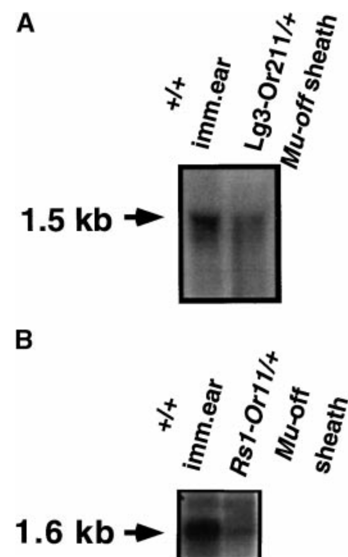

Figure 7.—Suppressible intron insertions mediate ectopic transcription from a distance. RNA blot analysis of (A) wild-type immature ear and *Lg3-Or211*/+ *Mu*-off sheath tissue and (B) *Rs1-Or11 Mu*-off sheath tissue, hybridized with the *lg3* cDNA or *rs1* cDNA, respectively. The asterisk indicates incompletely spliced *Rs1-Or11* message (Schneeberger *et al.* 1995).

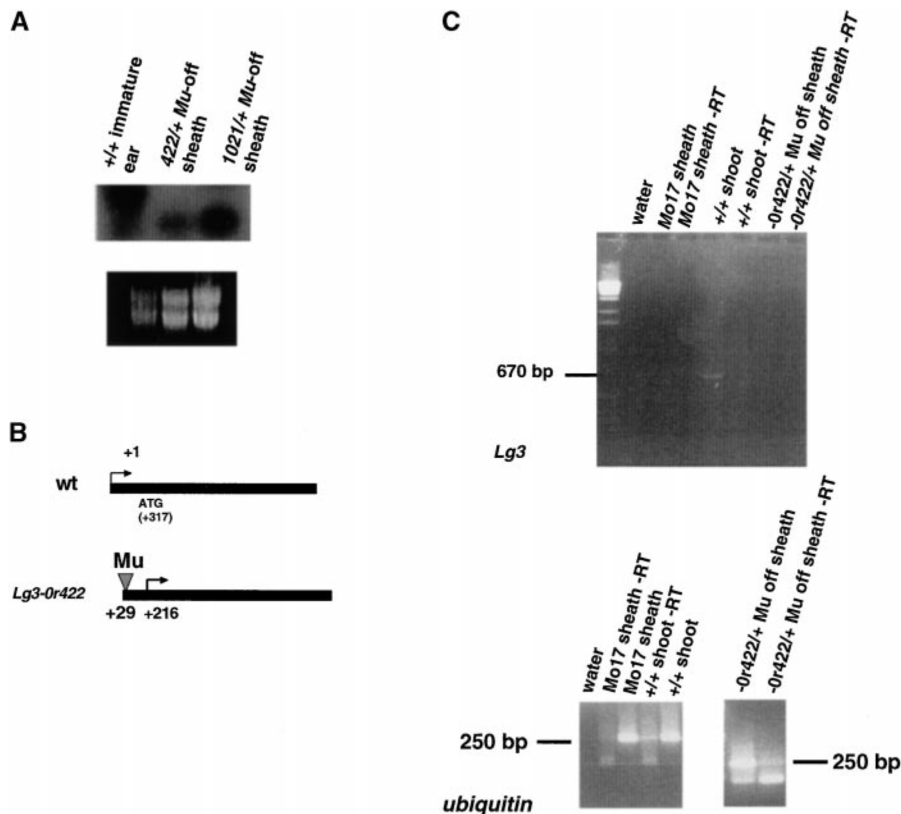

Figure 8.—The *Lg3-O*-suppressible alleles caused by 5′UTR insertions produce an altered transcript. (A) RNA blot analysis of *Lg3-Or422/+* and *Lg3-Or1021 Mu*-off sheath, probed with the *lg3* cDNA, produces a transcript smaller than wild type (*Lg3-Or331* produces a transcript identical in appearance to −*Or422/+* and −*Or1021/+*, data not shown). (B) RACE characterization of the *Lg3-Or422/+* cDNA (see materials and methods) placed the start of transcription at +216 in the 5′UTR. (C) RT-PCR of *Lg3-Or422/+ Mu*-off sheath tissue. cDNA from sheath tissue of the inbred Mo17 was used as a negative control, and cDNA from shoot tissue of a segregating wild-type was used as a positive control. *ubiquitin* primers were used to ensure template quality, producing a 250-bp product. Gene-specific *lg3* primers, the 5′ primer contained in the region believed to be deleted in *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021*, amplify the expected 670-bp cDNA product from shoot but not from Mo17 sheath *Lg3-Or422/+ Mu*-off sheath tissue. As an additional negative control, PCR was done on samples that were treated identically, except no reverse transcriptase was added (−RT).

ectopically expressed in the leaf, as well as where *lg3* is normally expressed in shoot meristematic tissue. Transcripts identical in size were also detected associated with the *Lg3-Or331* allele (data not shown). To characterize these transcripts further, we made a RACE library using tissue from *Mu*-inactive, homozygous *Lg3-Or422* immature ears. From this library, we cloned the cDNA corresponding to *Lg3-Or422* transcript, and found it to start 187 bp downstream of the inserted element (Figure 8B), a truncation of the transcript that is in the range predicted by the size discrepancy seen on the RNA blot.

To further support our finding that the 5′ region of these transcripts was altered, we performed RT-PCR using gene-specific primers, one of which primed off of sequences contained within the region we believe to be absent in transcripts from these altered alleles. We prepared cDNA from wild-type sheath, which is not expected to ectopically express *lg3*, as a negative control, and wild-type shoot as a positive control. We used *ubiquitin* primers to ensure the integrity of the template as well as *lg3* gene-specific primers that span an intron to verify template source. The *lg3* primers were determined to be gene specific for this RT-PCR assay by sequence comparison with the other *knox* genes. Using this assay, we found that while we successfully amplified a band of the expected size from wild-type shoot, no product is seen using cDNA from *Lg3-Or422/+ Mu*-inactive sheath tissue, consistent with the deletion of the region that includes the binding site for the 5′ PCR primer (Figure 8C). In summary, three *lg3* alleles caused by *Mu* insertions into the 5′UTR repress ectopic transcription in the presence of *Mu* activity. When *Mu* is inactive, they exhibit a normal expression pattern, although they are associated with truncated transcripts.

DISCUSSION

We have presented the characterization of the *Mutator*-suppressible *Liguleless3-0* and *Rough Sheath1-0* alleles: *Lg3-Or331*, *Lg3-Or422*, *Lg3-Or1021*, *Lg3-Or211*, and *Rs1-Or11.* These alleles represent *Mutator* insertions into introns as well as into the 5′UTR. Analysis of the 5′UTR insertions suggests an additional mechanism for *Mu* suppression of 5′ insertions distinct from that described for *hcf106* (Barkan and Martienssen 1991).

We have found that suppressible alleles can be caused by three types of *Mutator* elements: *Mu1*, *Mu3*, and *MuDR.* Based upon work by Greene and co-workers that describes suppressible *knotted1* alleles caused by *Mu1* insertions in both orientations (Greene *et al.* 1994), suppression appears to be orientation independent. Interestingly, *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021*, each of which is due to an insertion into the same site by the same element in the same orientation, have subtly different phenotypes (Figure 1), and the severity of the phenotype has been found to positively correlate with levels of ectopic expression (Muehlbauer *et al.* 1999).

The suppressible intron insertions we describe (*Lg3-*

Or211 and *Rs1-Or11*) both produce a wild-type size transcript when *Mu* is inactive. These results support the idea that transcription through *Mutator* transposons is not the limiting factor affecting insertion alleles. It has been found that some *Mutator* insertions into introns can be spliced out along with surrounding sequences (Ortiz and Strommer 1990), although some are processed using the *Mu* ends as splice donor or acceptor sites. Numerous alleles containing the maize transposons *Spm/dSpm* (Kim *et al.* 1987; Raboy *et al.* 1989) and *Ac/Ds* (Doring *et al.* 1984; Peacock *et al.* 1984; Weil and Wessler 1990) have been isolated in which intron insertions as well as exon insertions are transcribed through and then subsequently spliced, although this is dependent upon the orientation of the element (Gierl *et al.* 1985; Weil and Wessler 1990).

When *Mu* is inactive, the *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* 5′UTR insertion alleles produce a transcript that initiates 216 bp downstream of the normal site and 187 bp downstream of the inserted *Mu* element. Our results show that when *Mu* is off, the *Mu* element appears to redirect the start of transcription, although we cannot formally exclude the possibility that the effect is post-transcriptional. A similar example of transposon-induced alteration of transcription initiation has been found in *Antirrhinum majus* (snapdragons) at the *Tam1*-induced allele *niv-5311* (Sommer *et al.* 1988). The *nivea* gene encodes an enzyme in the anthocyanin biosynthetic pathway. *niv-5311* is a revertant of the *niv-rec53*::*Tam1* allele, which has a *Tam1* element inserted at −47. In the revertant, the *Tam1* element has excised, additionally deleting 66 bp that removes the TATA box. Despite the original transcription initiation site being present, this allele now initiates transcription 20 bp downstream of it. This may have to do primarily with the absence of the TATA box because other revertants that had deletions upstream of the TATA box retained their correct site of initiation. It is possible that the *Mu* insertions into *Lg3-Or331*, *Lg3-Or422*, and *Lg3-Or1021* could favor the utilization of a cryptic initiator sequence in the 5′UTR. The preference for more downstream initiation could result if the inserted *Mu* element were able to induce secondary structure into the region reaching up to +1, making transcription initiation at the normal site sterically hindered.

So how is it that some *Mu* insertions result in suppressible alleles while others do not? Any model that attempts to explain *Mu* suppression of these alleles must take into account that the *lg3*- and *rs1*-suppressible alleles were identified as revertants of their reference alleles, *Lg3-O* and *Rs1-O*, which are both gain-of-function mutations with as yet undescribed lesions.

Our results, taken with evidence from work on *knotted1* suppressible alleles (Greene *et al.* 1994), imply that whether a *Mu*-induced allele is suppressible or not depends on the site of insertion. As first suggested by Greene and co-workers, *Mutator* may be capable of functioning as an insulator. Insulators, also sometimes called boundary elements, are thought to functionally isolate genes by preventing interactions between distal enhancers and inappropriate promoters (reviewed by Corces and Gerasimova 1997). Suppressible *Mu* insertions may be those that have inserted into a site in which they can function as an insulator by preventing, in a *Mu*-activity-dependent manner, neomorphic activation of the gene in the leaves. Alternatively, the *Lg3-O* and *Rs1-O* mutations could be caused by the removal of an insulator sequence that is normally present in the wild-type gene, allowing interactions between an existing leaf enhancer and the promoter that are normally prevented by the insulator element. If this were the case, *Mu* activity could rescue the lesion by substituting for the loss of the insulator element in an activity-dependent manner. Reversion events similar to both of these possibilities have been suggested for revertants of the *Nasobemia* (*Antp*[ns]) alleles of the Drosophila homeotic gene *Antennapedia* (Talbert and Garber 1994).

Another possibility is that *Mu* insertions into critical sites of *Lg3-O* or *Rs1-O* could function, in an activity-dependent manner, to recruit novel silencing complexes "seeded" by the transposon-encoded proteins, to quench the dominant phenotype. It has been shown in Drosophila that *Dorsal*-mediated repression at the ventral silencer requires the formation of a multiprotein complex (Valentine *et al.* 1998). Additionally, *Dorsal* repression activity is dependent upon binding site context. If *Mutator*-activity-dependent seeding of silencer complexes were context dependent, then this could explain why only a small subset of *Mu* insertions is suppressible.

We have considerable evidence that at least three class I *knox* genes, including *rs1* and *lg3*, are under negative regulation. *rough sheath2*, which encodes a member of the MYB family of transcription factors, has been found to negatively regulate *lg3*, *rs1*, and *kn1* (Schneeberger *et al.* 1998). Furthermore, there is evidence that suggests, but does not prove, that negative regulatory information is located in the introns of these genes (Hake *et al.* 1989).

Other plant gene introns have been found also to contain regulatory sites. Insertion of a *Tam3* element into the intron of *plena* in Antirrhinum, the homolog of *AGAMOUS* in Arabadopsis, results in a gain-of-function ovulata phenotype in which sterile floral organs are replaced by sex organs due to ectopic expression of *plena* (Bradley *et al.* 1993). Bradley and co-workers propose that the gain-of-function phenotype is due to the interference of the action of a negative regulator caused by the insertion. Interestingly, the introns of *AGAMOUS* were found to be required for correct spatial and temporal expression of the gene (Sieburth and Meyerowitz 1997). Sequence analysis of *Lg3* and *Rs1* introns, as well as *Mu* elements, have revealed numerous potential regulatory sites, including MYB binding sites

and matrix attachment regions (Gasser *et al.* 1989); however, none of these sites have yet been tested functionally. Thus, given the evidence for negative regulation of these genes, models that take this into account are favored.

While our work does not implicate directly a molecular mechanism that describes *Mu* suppression of *Lg3-O* and *Rs1-O* ectopic transcription, it does set constraints upon the contributing factors. The variety of effects that transposons can have on the genes they insert into is still not completely understood. The absence of a clearly understood mechanism for *Mu* suppression has not, however, prevented it from being utilized as a genetic tool. *Mu* suppression has been used successfully to turn genes on and off in marked sectors at various developmental times (Martienssen and Baron 1994; Muehlbauer *et al.* 1997). A better mechanistic understanding of *Mu* suppression would engender better *Mutator*-based genetic tools. In a broader sense, *Mutator* suppression provides an excellent system in which to exploit the intricate relationship between transposon and host chromosome.

We thank David Braun and Albert Erives for critically reading the manuscript and Sarah Hake for helping us interpret some of the data. We also thank Richard Schneeberger for the *Rs1-Or11* pictures in Figure 1. The authors also thank Gary Muehlbauer, Richard Schneeberger, and Petra Bauer for contributory discussions and stocks, Randall Tyers for PCR technical advice, and Barbara Kloeckner-Gruissem for her invaluable consultations. This work was supported by National Science Foundation grant no. MCB 9603119 to M.F.

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Communicating editor: J. A. Birchler