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A Strong Inhibitor of Gene Expression in the 5′ Untranslated Region of the Pollen-Specific *LAT59* Gene of Tomato

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Promoter sequences that direct pollen-specific expression have been previously identified in the *LAT59* (for late anther tomato) gene. Here, we show that the *LAT59* sequences encoding the 5′ untranslated region inhibit expression of reporter genes by >20-fold in transient expression experiments and up to 300-fold after stable transformation. Inhibition occurred in somatic cells as well as in pollen. Our results indicate that the inhibitor still functions after pollen germination and therefore does not modulate the level of the LAT59 protein during pollen development. The presence of the leader sequence dramatically decreased mRNA accumulation but without affecting translation rate and mRNA stability. We believe that the leader inhibits transcription. We mapped the inhibitor to a region in the leader that coincides with a putative stem–loop and present evidence that this stem–loop participates in inhibition.

INTRODUCTION

The 5′ untranslated region (5′ UTR) of a gene often can affect gene expression and frequently does so via translational control (Geballe et al., 1986; Kaspar et al., 1992; Kempe et al., 1993; Stripecke et al., 1994). In many viral and eukaryotic genes, the mechanisms underlying this process have been well documented and involve the presence of either secondary structures or upstream open reading frames (reviewed in Kozak, 1991). However, in a few cases, leader regions have been shown to act transcriptionally; examples include the Drosophila *hsp22* heat shock gene, in which the first 20 nucleotides of the transcribed region contain overlapping transcriptional and translational control elements (Hultmark et al., 1986), and the human gastrin (Theill et al., 1987) and γ-globin (Amrolia et al., 1995) genes. In the last decade, transcriptional regulation by inhibiting elongation (often called attenuation) rather than initiation has also been documented in eukaryotes (reviewed in Wright, 1993). During attenuation, the RNA polymerase II complex pauses, usually early in the transcription process, in response to cellular or environmental conditions. Among the best characterized cases of transcriptional attenuation are the human c-*myc* gene, whose elongation is blocked upon cell differentiation (Bentley and Groudine, 1986), the murine adenosine deaminase gene that is regulated by transcription elongation in a tissue-dependent manner (Chinsky et al., 1989), and the human immunodeficiency virus HIV-1 gene in which premature termination of transcription is relieved by the *tat* gene product (Kao et al., 1987).

Little is known about regulation by sequences located in the transcribed region of plant genes. To date, the clearest evidence for the presence of transcriptional *cis*-acting elements in the transcribed region comes from analysis of genes that encode thylakoid proteins. Bolle et al. (1994) used run-on transcription assays to show that the leader sequences of the *PetE*, *PetH*, and *PsaF* genes quantitatively influence expression and that, at least in the *PsaF* gene, the leader contains *cis* elements that can interact with nuclear factors. Both the pea and Arabidopsis genes encoding ferredoxin contain light-regulatory elements in their transcribed region (Dickey et al., 1992, 1994; Caspar and Quail, 1993; Bovy et al., 1995); however, it is not yet clear which of the transcriptional or post-transcriptional events is affected. Recently, a translational enhancer was identified in the 5′ UTR of the maize alcohol dehydrogenase *Adh1* gene that can confer higher expression under low-oxygen conditions (Fennoy and Bailey-Serres, 1995; Bailey-Serres and Dawe, 1996).

The *LAT59* (for late anther tomato) gene is a member of the "late" genes (Mascarenhas, 1990) that are coordinately activated during the late phase of microsporogenesis, that is, after the first microspore mitosis (McCormick, 1991a). Studies to determine the mechanisms of pollen-specific gene expression have focused on promoter sequences. In all species in which regulation of late gene expression has been studied, pollen specificity is transcriptionally controlled by the proximal promoter region (Guerrero et al., 1990; Twell et al., 1991; Albani et al., 1992; Eyal et al., 1995; Weterings

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et al., 1995). However, there is also evidence that expression of late genes can be post-transcriptionally regulated. First, although the late genes are transcribed as early as microspore mitosis, the proteins they encode are thought to play their role only during pollen maturation and/or germination (Mascarenhas, 1990). Second, in several species, the transcriptional inhibitor actinomycin D does not affect early pollen tube growth in vitro, whereas the translational inhibitor cycloheximide does, indicating that initial pollen tube growth mainly relies on preexisting mRNAs in these species (reviewed in Mascarenhas, 1993). There is evidence of post-transcriptional regulation for the maize *Zm13* gene, whose mRNA is only translated at anthesis, 10 days after it first appears (Mascarenhas, 1993), and a tobacco mRNA encoding a 69-kD protein that is abundant in pollen tubes but whose translation is inhibited in mature pollen (Storchova et al., 1994).

These findings prompted interest in determining the mechanisms of post-transcriptional regulation of late genes. For example, Bate et al. (1996) recently showed that the *LAT52* gene contains a translational enhancer in its 5′ UTR that upregulates expression specifically in the late stages of pollen maturation. In contrast, we observed that the sequence encoding the *LAT59* 5′ UTR inhibits gene expression. In this study, we characterize the inhibitor activity of the *LAT59* leader. We show that inhibition involves a putative stem–loop structure but unexpectedly seems to be transcriptionally regulated. We use transient assays and stable transformation in somatic cells to show that *LAT59* 5′ UTR inhibitor activity is not restricted to pollen. We show that 5′ UTR inhibitor activity is not modulated upon pollen germination.

RESULTS

Characterization of an Inhibitor in the *LAT59* and *Nt59* Leaders

We previously characterized *cis* elements in the proximal promoter of the *LAT59* gene that are necessary and sufficient to drive pollen-specific gene expression (Eyal et al., 1995). The constructs used for those experiments did not contain the sequence encoding the 5′ UTR region of *LAT59* because we had found that it negatively affected gene expression. To further characterize this region of the *LAT59* gene, we created a translational fusion between the luciferase (*LUC*) open reading frame (Ow et al., 1986) and *LAT59* gene sequences by using the −115 minimal promoter (Eyal et al., 1995) and various portions of the sequence encoding the 5′ UTR (Figure 1): either the full-length 236-bp construct (59L), a 210-bp internal deletion (construct 59Δ210), or only the 10 most upstream base pairs (construct 59ΔL). We tested the activity of these constructs in transient expression assays by using particle bombardment of pollen, as previously described (Twell et al., 1989; Eyal et al., 1995). In these experiments, the LUC plasmids were cobombarded with a β-glucuronidase (GUS) plasmid (see Methods) used as an internal control for the bombardment. The relative LUC activity obtained after transient expression in pollen is shown in Figure 1. Deletion of the majority of the leader region in both 59Δ210 and 59ΔL resulted in a 20-fold increase in LUC activity relative to the 59L construct, demonstrating that the segment encoding the *LAT59* 5′ UTR negatively controls gene expression.

We tested whether the inhibitor function of the *LAT59* leader is conserved in *Nt59*, its tobacco homolog (Kulikauskas and McCormick, 1997). *Nt59* shares 86% nucleotide identity with *LAT59* in the coding region and 74% in the 5′ UTR sequence. The entire *Nt59* 5′ UTR, starting at position +3, was subcloned into 59ΔL (construct Nt59L) to produce a chimeric 5′ UTR that retains the same context of transcription initiation as the LAT59 fusions (Figure 1). Nt59L inhibited expression 50-fold relative to 59ΔL in transient expression assays, demonstrating that the inhibitor function has been conserved between the two species.

The *LAT59* Inhibitor Is Active in Somatic Cells

To determine whether inhibitor activity is pollen specific, we tested its activity in somatic cells. Because the *LAT59* promoter is not active in somatic tissue, transcription of the *LUC* fusions was driven by the cauliflower mosaic virus (CaMV) 35S promoter–enhancer. The two constructs, 35S/59L and 35S/59ΔL, shown in Figure 2, were designed so that the transcripts produced in somatic cells would be identical to the transcripts tested in pollen. The 35S/59L and 35S/59ΔL constructs were bombarded into tobacco BY-2 cells with a CaMV 35S promoter–*GUS* fusion as an internal control.

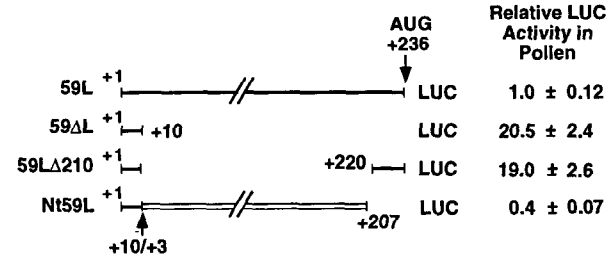

Figure 1. Identification of an Inhibitor in the *LAT59* and *Nt59* Leader Sequences.

Shown is a schematic representation of the mRNA transcripts produced under the control of the −115 *LAT59* promoter (Eyal et al., 1995). Borders of the constructs and the translational fusion site with the *LUC* reporter gene are indicated relative to the transcription initiation site. Constructs were expressed by transient expression assay in tobacco pollen. Relative LUC activity represents the ratio between the test (LUC) and the reference (GUS) plasmids and is the mean of from at least six up to 21 independent experiments.

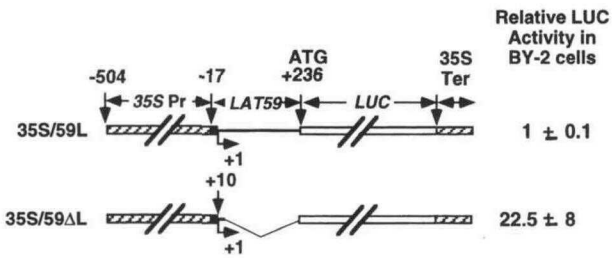

Figure 2. Activity of the Inhibitor in Transient Assays with Somatic Cells.

Shown are schematic representations of the 35S promoter–LAT59 leader–*LUC* fusions containing either the *LAT59* (positions −17 to +236) region (construct 35S/59L) or the *LAT59* (positions −17 to +10) region (construct 35S/59ΔL). Constructs were transiently expressed in tobacco BY-2 cells. Relative LUC activity represents the ratio between the test (LUC) and the reference (GUS) plasmids and is the mean of nine independent experiments. Pr, promoter; Ter, terminator.

Relative LUC activity is reported in Figure 2. Deletion of the leader sequence resulted in an increase in the level of expression similar to the one measured in transient expression in pollen. Therefore, we conclude that the inhibitor activity is not pollen specific.

The *LAT59* Inhibitor Is Active in Stable Transformants

To confirm the presence of an inhibitor in the segment encoding the 5′ UTR of the *LAT59* gene, we examined the effect of its deletion on reporter gene expression in stably transformed tomato plants. For these experiments, we replaced the *LUC* gene with the *Escherichia coli uidA* gene encoding GUS to allow histochemical localization (Jefferson et al., 1987). Pollen harvested from the flowers of the primary transformants was assayed for GUS enzymatic activity, and the results are reported in Figure 3. We observed a ninefold increase in mean GUS activity upon deletion of the *LAT59* leader, which is consistent with our transient expression results. However, there was a high variance, which is probably due to position effects and the relative weakness of the promoter (Eyal et al., 1995).

Because we had shown that the leader sequence could also inhibit expression in BY-2 cells, we investigated its effect on stable expression in transgenic BY-2 cell lines. Independent transformed BY-2 cell lines were generated that express either the 35S/59L or the 35S/59ΔL fusion. An internal control (CaMV 35S–*GUS* fusion) on the same T-DNA was included to eliminate variability due to position effects and thus reduce the number of independent transformants required for the analysis. Transformed cell lines were assayed for LUC and GUS activity at the exponential phase (3 or 4 days). Figure 4 shows the results obtained from five and four independent transformants, respectively, for the 59L and 59ΔL constructs. Cell lines with a deleted leader (35S/59ΔL) showed an average of 300 times higher relative LUC activity than those transformed with the full-length leader (35S/59L). This indicates that the region of the *LAT59* gene encoding the 5′ UTR strongly inhibits expression and confirms data obtained with the transient expression assay.

Inhibitor Activity Is Not Alleviated during Pollen Germination

The potential function of the LAT59 *protein as a pectate* lyase suggests that it may play an important role during pollen tube growth (Dircks et al., 1996). The LAT59 protein and its tobacco homolog, the Nt59 protein, show very high sequence homology (93.5% similarity at the amino acid level) (Kulikauskas and McCormick, 1997) as well as a similar pollen-specific pattern of expression; therefore, they are likely to play the same role in tomato and tobacco, respectively. In

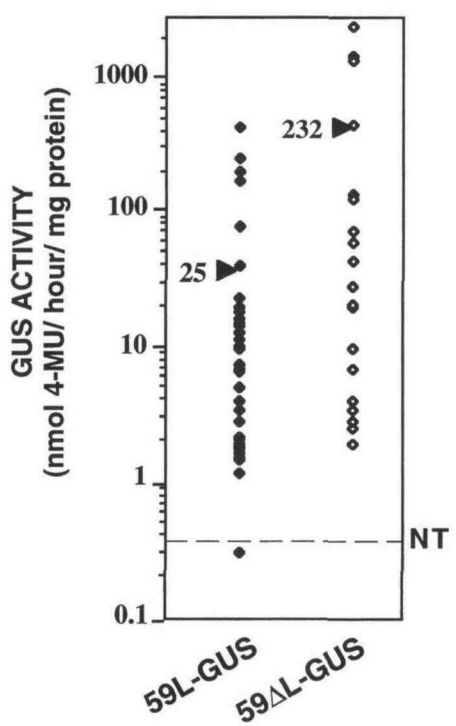

Figure 3. Effect of the *LAT59* Leader on Reporter Gene Expression in Pollen of Stably Transformed Tomato Plants.

GUS expression levels were determined in transgenic tomato plants harboring *LAT59* fused to the *uidA* reporter gene with (59L–GUS) or without (59ΔL–GUS) the *LAT59* leader sequence. GUS levels in the pollen extract are represented in nanomoles of 4-methylumbelliferone (4-MU) generated per hour per milligram of protein. NT, untransformed control line.

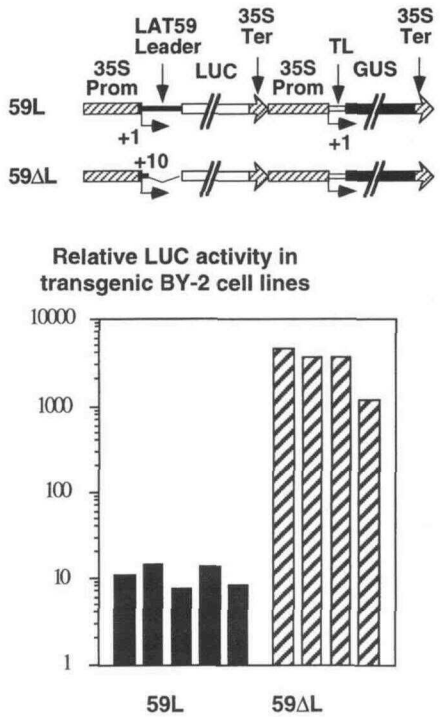

Figure 4. The *LAT59* Leader Sequence Has a Strong Inhibitor Activity in Transgenic BY-2 Cells.

A schematic representation of the *LUC* (test) and *GUS* (reference) gene fusions present on the same T-DNA used to stably transform tobacco BY-2 cells is shown above. Relative LUC activity represents the ratio between LUC and GUS activities and is shown for independent transgenic cell lines below. Angled arrows indicate the transcription initiation sites. Prom, promoter; Ter, terminator; TL, tobacco mosaic virus leader sequence.

addition, we have shown that the inhibitor activity present in the *LAT59* 5′ UTR is functionally replaced by the *Nt59* 5′ UTR sequence. We wondered whether a change in inhibitor activity could control a differential expression between mature pollen and germinated pollen. First, we needed to establish the pattern of accumulation of LAT59 and of Nt59 in mature pollen and during germination. Protein extracts from mature and in vitro–germinated pollen were immunoblotted and probed with the anti-59 antibody. Figure 5A shows that the antibody detected an ~50-kD doublet in mature pollen extracts. After 15 hr of pollen germination, the amount of the LAT59 protein increased, whereas the Nt59 protein level did not change.

To test for a regulatory role for the 5′ UTR, we measured the transcript level only in tobacco, because of the limited amount of germinated tomato pollen available. Tobacco total RNA from mature pollen and germinated pollen was probed with the *Nt59* gene. Figure 5B shows an ~2.5-fold decrease in the steady state level of *Nt59* mRNA upon germination, after correction of the hybridization intensities with the loading control (ethidium bromide staining of rRNA). Extrapolation of this result to the *LAT59* gene would indicate that the increase in LAT59 protein upon germination does not result from a decrease in the 5′ UTR inhibitor activity.

To verify the absence of a regulatory role for the *LAT59* 5′ UTR inhibitor, we measured the effect of its deletion on transient gene expression in germinating pollen. Tobacco pollen was bombarded with either 59L–LUC or 59ΔL–LUC plasmids and then split into two batches. One batch was directly extracted, and the other batch was germinated overnight. Bombardment had no discernable effect on germination frequency. Relative LUC activity, obtained after correction for bombardment variations with the GUS internal control, was measured for the 59L and 59ΔL constructs in mature and germinated pollen. Results of this experiment are shown in Table 1. The role played by the leader in gene expression at each stage can be estimated by the ratio between the 59ΔL and 59L LUC activities. For both mature and germinated pollen, the 59ΔL/59L ratios were similar, although the LUC activity was higher in germinated pollen than in mature pollen. We also assayed pollen of three independent 59L–GUS transgenic tomato plants before and after in vitro germination. We found that the effect of the leader on *GUS* expression did not change after germination (data not shown). Because the leader inhibits reporter gene expression at a similar level in mature and germinated pollen, it is unlikely to be responsible for the increase in LAT59 protein levels after germination.

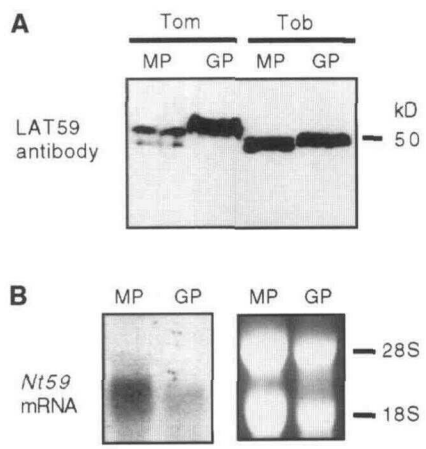

Figure 5. LAT59 and Nt59 Protein and *Nt59* mRNA Steady State Levels during Pollen Development.

(A) Shown is an immunoblot of a gel containing 50 μg of total protein from mature pollen (MP) and germinated pollen (GP) of tomato (Tom) and tobacco (Tob), probed with the LAT59 antiserum.

(B) Shown at right is ethidium bromide staining of a formaldehyde–agarose gel containing 20 μg of total RNA from mature pollen (MP) and germinated pollen (GP) of tobacco, showing 18S and 28S rRNAs. A blot of the same gel probed with the *Nt59* cDNA is shown at left.

Table 1. Comparison of Inhibitor Effect in Mature (MP) and Germinated Pollen (GP)

Construct	LUC Activity (Light Units/μg Protein)[a]	
	MP	GP
59L	280 ± 90	9,000 ± 60
59ΔL	6,000 ± 900	120,000 ± 4,000
Ratio $\frac{59\Delta L}{59L}$	21	13

[a] Results are from six independent bombardments.

Sequences Encoding the *LAT59* 5′ UTR Are Likely to Affect Transcription

We initially tried to compare *GUS* mRNA and protein steady state levels in pollen of individual transgenic tomato plants expressing 59L–GUS or 59ΔL–GUS. However, the high variability in the level of *GUS* mRNA did not correlate with the enzymatic activity measured, and we could not correct for this variability because the plants had not been transformed with an internal control. Because comparable levels of inhibition were obtained by transient assays in pollen and BY-2 cells, we opted to use BY-2 cells to study the mechanism of inhibition. Therefore, we measured the mRNA level and enzyme activity for the *LUC* and *GUS* genes in transgenic cell lines expressing 35S/59L or 35S/59ΔL. We extracted mRNA from and conducted enzymatic assays with the same aliquot to avoid stage-dependent variation in expression. Figure 6 shows the results with a representative transformant of each fusion; similar results were obtained with four independent transformants. Figure 6A shows that the LUC activity is reduced in the presence of the leader, and Figure 6B shows that there is less *LUC* mRNA when the leader is present. A comparison of the signals shows that 50 μg of total RNA from the 59L transformant contains less *LUC* mRNA than 1 μg of total RNA from the 59ΔL transformant. Therefore, we estimate that the difference in *LUC* mRNA between these two lines is >50. Calculations from the GUS enzymatic and mRNA levels indicate that the 59L cell line has five times more expression than does the 59ΔL cell line; thus, the steady state level of *LUC* mRNA is at least 250 times less when the sequence for the *LAT59* 5′ UTR is present. We conclude that the *LAT59* 5′ UTR inhibitor affects the cellular mRNA level by either decreasing transcription or increasing mRNA degradation.

The *LAT59* and *Nt59* 5′ UTRs contain predicted stem–loop structures (PC Gene, RNA folding program). In *LAT59*, the stem–loop spans the region from +62 to +180 and has a calculated stability of $\Delta G = -47$ kcal mol^{-1}. Such stem–loops in the 5′ transcribed region were found to inhibit translation initiation in several genes (Kozak, 1991). The predicted stem–loops in *LAT59* and *Nt59* prompted us to test whether the *LAT59* 5′ UTR might affect translation as well as transcript accumulation.

Synthetic RNAs, with the LUC open reading frame fused either to the full-length *LAT59* 5′ UTR or to its deleted 59ΔL version, were produced and translated in a cell-free translation system. Figure 7 shows that both synthetic mRNAs are translated at a similar rate over 2 hr, reaching a plateau in <1 hr. Results were similar whether the mRNAs were capped or uncapped and whether translation was performed in wheat germ extracts (Figure 7) or in rabbit reticulocyte lysates (data not shown).

We also examined the role of the *LAT59* 5′ UTR on translation in protoplasts, because it was possible that the conditions of the in vitro assay did not allow the proper folding of the putative stem–loop. For these experiments, mRNA was introduced into protoplasts via electroporation (Callis et al., 1987; Gallie et al., 1989), and transient expression was monitored, allowing us to directly address post-transcriptional control mechanisms. The importance of a cap and a poly(A) tail for efficient translation in plant protoplasts has been demonstrated previously (Gallie, 1991). Capped and polyadenylated synthetic mRNAs were produced that contained a *LUC* fusion with *LAT59* leader sequence, with or without the inhibitor element, using the vector pT7-LUC-A_{50} (Gallie et al., 1991). Synthetic mRNAs were electroporated into BY-2 protoplasts, and LUC enzymatic activity was assayed after 4

A

Cell line	59L	59ΔL
LUC Activity (light Units/mg prot)	1190	33360
GUS Activity (nM MU/min/mg prot)	148	22
LUC/GUS	8	1516

B

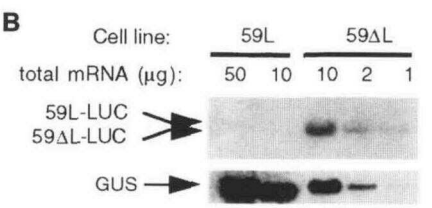

Figure 6. Effect of the *LAT59* Leader Sequence on mRNA Level and GUS and LUC Activity in Transgenic BY-2 Cells.

mRNA and protein extracts were prepared from the same culture at exponential phase. Results from one transformant for each construct are shown.

(A) Enzymatic activities of 4-day-old BY-2 cell cultures expressing the 35S–*GUS* transgene and either 59L–LUC or 59ΔL–LUC. prot, protein; MU, 4-methylumbelliferone.

(B) RNA gel blot. The blot was first hybridized with *LUC* and then stripped before hybridization with *GUS*. The blot was exposed with a PhosphorImager for 15 hr.

The smaller size of the 59ΔL *LUC* mRNA is due to the deletion of 226 bp of the sequence encoding the 5′ UTR.

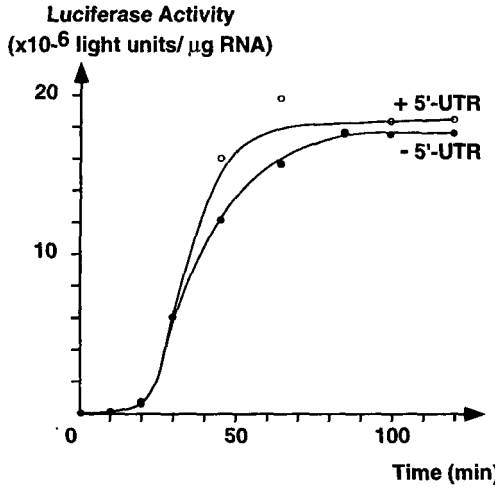

Figure 7. Effect of the 5′ UTR on the Rate of Translation in Vitro.

Capped synthetic *LUC* transcripts fused either to the full-length *LAT59* 5′ UTR (+ 5′-UTR) or to 59ΔL (− 5′-UTR) were translated in a wheat germ extract. Aliquots of the reaction were taken at various time intervals and assayed for LUC activity.

hr of transient expression. Both mRNAs yielded the same amount of LUC activity (~25 light units mg of protein^{-1} hr $^{-1}$). The *LAT59* 5′ UTR clearly does not affect translation, because it had no effect on expression after in vitro translation or when introduced into protoplasts as mRNA, but it inhibited expression 20-fold when introduced as DNA.

We estimated the stability of the LUC transcript by determining the functional half-life in the presence or absence of the *LAT59* 5′ UTR. The functional half-life of an mRNA is defined as the time required to reach 50% of the maximum LUC activity (Gallie, 1991). Synthetic mRNAs were electroporated into BY-2 protoplasts, and aliquots were harvested at various time intervals and assayed for LUC activity, as shown in Figure 8. LUC activity dropped after 7 hr, although the viability of the protoplasts remained constant over 24 hr (Figure 8, open circles). If the leader decreases the stability of the mRNA, the accumulation of LUC should reach an earlier plateau when the 5′ UTR is present on the message. Figure 8 shows that both transcripts led to approximately the same level of LUC activity and showed a similar functional half-life: 130 min in the presence of the 5′ UTR and 150 min in the absence of the 5′ UTR. Therefore, a difference in mRNA stability cannot explain the 20-fold difference in expression observed in transient assays with tobacco BY-2 protoplasts. We conclude that the *LAT59* 5′ UTR inhibitor does not act via alteration of mRNA stability. Having demonstrated that neither the efficiency of translation nor the stability of the transcript is affected by the 5′ UTR, we indirectly show that inhibition occurs through a transcriptional mechanism.

Mapping of the Inhibitor Element(s)—Implication of the Stem–Loop Structure

To define sequences that are involved in the inhibition, we derived a series of 3′ to 5′ and internal deletions in the leader region of the 59L construct. The most informative of these constructs and their effect on LUC expression in pollen are shown in Figure 9. Removal of the sequence upstream of the secondary structure from positions +10 to +56 (construct 59L1) had no effect on LUC expression. On the contrary, deletion of the downstream region from +189 to the translational fusion site (construct 59L2) resulted in a threefold increase of LUC expression. We observed a similar derepression level with 59L3, whose translational context had been restored by addition of the original *LAT59* sequence flanking the AUG. These results indicate that the (+189 to +220) region participates in the inhibition. Deleting the internal region from +10 to +156 (construct 59L4) entirely eliminates the inhibition. Thus, essential elements reside upstream of position +156, and the downstream regulatory region alone (+189 to +220) cannot inhibit expression. In turn, further 3′ to 5′ deletions up to positions +136 and +91 (constructs 59L5 and 59L6) largely derepress expression.

We conclude from these experiments that the central region of the leader is essential for the inhibitor activity although not sufficient, because a small region located in the 3′ part of the leader participates in achieving full-level inhibition. The fact that a large region of the leader is involved in the inhibitor activity and that this region contains a putative stem–loop (from positions +62 to +180) raised the possibility that this stem–loop mediates the inhibition.

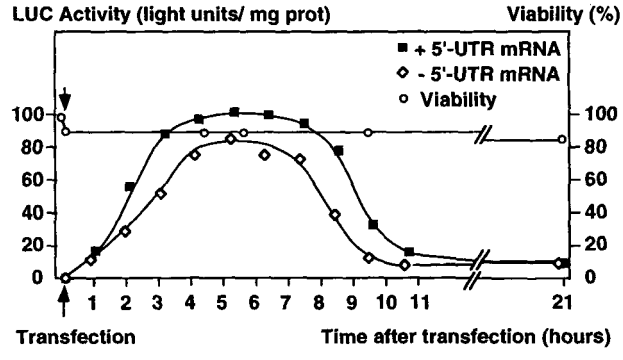

Figure 8. Effect of the 5′ UTR on the Stability of *LUC* mRNA in Electroporated BY-2 Protoplasts.

Capped and polyadenylated synthetic *LUC* transcripts, containing the full-length *LAT59* 5′ UTR (+ 5′-UTR) or a deleted version retaining the last 50 nucleotides (− 5′-UTR), were electroporated into BY-2 protoplasts. The arrows indicate the time of electroporation. Aliquots were taken at various time intervals, and LUC activity was assayed (left scale). The viability of the protoplasts was measured in the same aliquots (right scale). prot, protein.

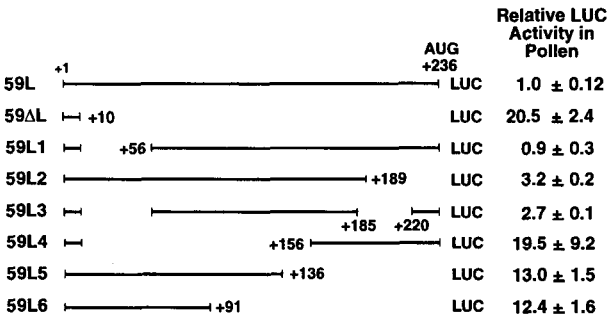

Figure 9. Deletion Analysis of the *LAT59* Leader Region.

A schematic representation of the mRNA transcripts produced under the control of the −115 *LAT59* promoter is shown. Borders of the constructs and the translational fusion site with the *LUC* reporter gene are indicated relative to the transcription initiation site. Constructs were introduced into tobacco pollen via particle bombardment. Relative LUC activity represents the ratio between the test (LUC) and the reference (GUS) plasmids and is the mean of from at least six up to 21 independent experiments.

We used site-directed mutagenesis to test whether the putative stem–loop or the sequence itself is important for the inhibitor activity. Alteration of a secondary structure by point mutations can be reversed by a second mutation that is complementary to the first one, resulting in an identical structure with an altered sequence. In this way, the effect on expression of a secondary structure can be dissociated from the effect of the sequence that constitutes it. Mutations resulting in destabilization of the *LAT59* putative stem–loop were introduced, singly or in combination with a complementary mutation, and their effect on expression was tested in pollen by using transient expression assays. To simplify the analysis, the mutations were made in the context of the 59L3 construct, which lacks the 5′ and 3′ dispensable parts of the leader but still shows considerable inhibition (6.2 times relative to 59ΔL). The calculated stability of the 59L3 leader is −49 kcal mol^{-1}, and the stability of each mutated leader sequence is indicated in Table 2. We could not identify pairs of mutations in which both members would significantly affect the calculated stability of the structure, probably because of the length of the stem–loop and its capacity to fold in alternative structures. Therefore, we tested three sets of mutations (M1 through M3, represented in Figure 10A) and were careful to keep the ratio between the AT and GC pairs constant between the wild-type and the double mutant sequences (see Methods).

The effects of the mutations on LUC activity are shown in Figure 10B. M1 and M1C mutations each derepressed expression more than twofold, whereas when both mutations were present, the inhibition level increased to four times higher than did the 59L3 sequence. This increase is consistent with the higher stability of the double mutant. Mutation M2 derepressed expression more than did M2C, but the double mutant M2M2C partially restored the inhibition. Similarly, the double mutant M3M3C derepressed expression to a lesser extent than did M3C alone. Therefore, in all three regions of the stem–loop, inhibitor activity of the double mutant is higher than one or both of the single mutations alone. This strongly supports the existence of a secondary structure in the leader and suggests that this structure mediates the inhibition. How the role of this stem–loop can be reconciled with a transcriptional control is discussed later in this article.

DISCUSSION

We identified an inhibitor element in the tomato *LAT59* gene leader and showed that the inhibition is functionally conserved in the leader of its tobacco homolog *Nt59*. Using transient expression in pollen, we defined two functionally different domains in the leader that account for the inhibitor activity. One large domain, located in the center of the leader, mediates a greater than sixfold inhibition and is essential for the inhibitor function. This domain encompasses a putative stem–loop structure spanning sequences from positions +62 to +180. That mutations affecting the sequence but conserving the secondary structure show inhibition strongly supports the formation of this stem–loop in vivo and suggests that it mediates the inhibition, as does the fact that a very similar stem–loop can be predicted in the leader of *Nt59*.

However, in BY-2 cells, three lines of evidence demonstrate indirectly that the inhibitor exerts its control at the transcriptional level. (1) The steady state mRNA level is lowered by the *LAT59* leader, as shown by RNA gel blot experiments with transgenic BY-2 cell lines, and thus parallels the decrease in gene expression caused by the leader sequence.

Table 2. Predicted Stability of Stem–Loop Mutants

Construct	ΔG (kcal.mol^{-1})[a]
59L	−60.6
59ΔL	~0
59L3	−49.7
- M1[b]	−46.6
- M1C	−52.2
- M1M1C	−62.1
- M2	−39.2
- M2C	−39.3
- M2M2C	−48.9
- M3	−40.6
- M3C	−37.7
- M3M3C	−51.2

[a] Predicted stability calculated using the PC Gene RNA folding program, according to the Zucker algorithm.
[b] Dashes indicate that the mutations were introduced into 59L3.

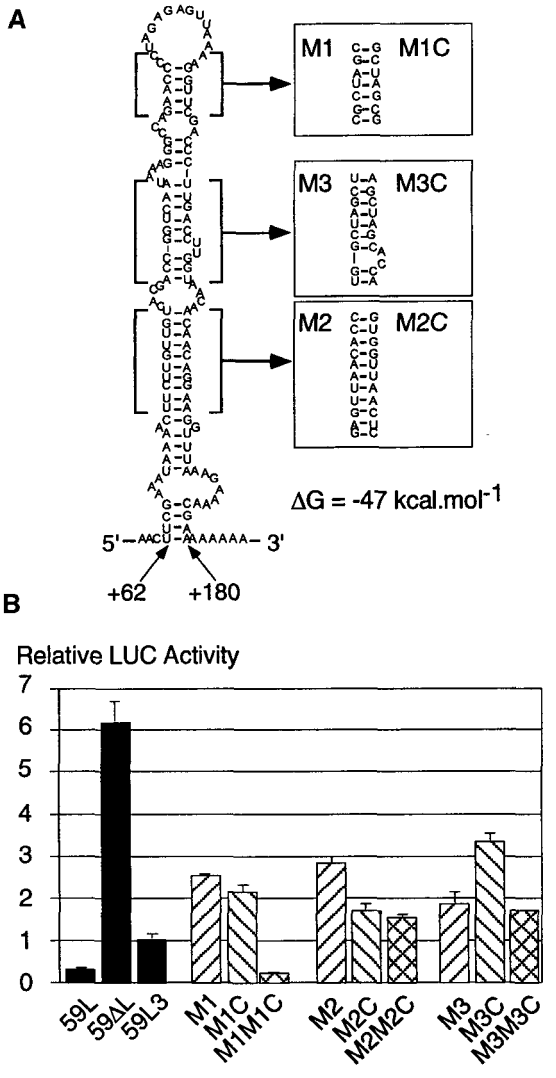

Figure 10. Site-Directed Mutagenesis of the *LAT59* Putative Stem–Loop Structure.

(A) The putative stem–loop structure of the *LAT59* leader. Mutations M1, M2, and M3, and M1C, M2C, and M3C (C for complementary mutation) were introduced in the context of the 59L3 construct, and their sequences are indicated to the right of the stem–loop structure. Numbers indicate the nucleotide position relative to the transcription start site.

(B) Relative LUC activity after transient expression in tobacco pollen. Results are the mean ± SD of six independent experiments. Relative LUC activity obtained with the 59L3 construct was set to 1. M1M1C, M2M2C, and M3M3C correspond to double mutants.

(2) Both in vitro and in vivo translation assays failed to show an effect of the 5′ UTR on translation efficiency. Points (1) and (2) together clearly exclude an effect of the 5′ UTR on translation. (3) The *LAT59* 5′ UTR does not significantly alter the mRNA functional half-life, as determined by time-course experiments in protoplasts electroporated with mRNA. Hence, mRNA instability cannot account for the inhibition. Future work will include run-on transcription assays to test directly for transcriptional control mediated by the leader sequence in these cells. But more importantly, we need to investigate whether the results obtained in BY-2 cells are relevant to pollen. Because the *LAT59* gene is specifically expressed in pollen, it is conceivable that pollen factors might differentially control the inhibitor activity.

Secondary structures that are located in the 5′ region of mRNA have been shown to inhibit translation in eukaryotes (Kozak, 1991). However, it is most unusual that regions of dyad symmetry in the corresponding DNA would play a role in controlling gene expression. One plausible explanation for our results could be that the *LAT59* leader affects transcription elongation rather than transcription initiation. It is then easy to imagine how such a secondary structure could be an obstacle for the RNA polymerase and prevent its progression. Regions of dyad symmetry have been shown to occur directly upstream of an RNA polymerase II pausing site in c-*myc* (Bentley and Groudine, 1988), although their role in the attenuation process has not been shown.

The c-*myc* attenuation site has been mapped to a T-rich sequence, as have other characterized attenuation sites. This is reminiscent of the bacterial *rho*-independent terminators that are composed of a stem–loop followed by a sequence rich in T residues (d'Aubenton Carafa et al., 1990). The second domain in the *LAT59* leader that is involved in the inhibitor activity is located downstream of the stem–loop, between positions +185 and +220. This domain is not able to inhibit expression on its own but is necessary to achieve full-level inhibition. Interestingly, these 35 bp are extremely AT rich in the *LAT59* and *Nt59* genes (94 and 91% AT, respectively). Whether the two defined domains in the *LAT59* leader can act as a premature termination site for the RNA polymerase requires further investigation. Position dependence of the inhibitor element relative to the promoter will be tested, because it has been shown to affect the c-*myc* attenuator function in Xenopus oocytes (Roberts and Bentley, 1992). The fact that the inhibitor functions independently of the linked promoter (*LAT59* or CaMV 35S) suggests that it does not interfere with elements of the basic transcriptional machinery but rather supports a role in controlling elongation.

Why does the *LAT59* gene have this inhibitor? Inhibition is certainly not a general feature of the leaders of late pollen-expressed genes. We found at most a twofold level of inhibition when the leader region of the related *LAT56* gene was tested in transient expression assays (data not shown). The presence of the *LAT52* leader enhances expression (Bate et al., 1996).

Two hypotheses of regulation via the inhibitor element were considered. (1) Inhibition that occurs in mature pollen could be alleviated during germination to allow for the accumulation of the LAT59 protein. However, we found that the steady state mRNA level of the *Nt59* gene, its tobacco ho-

molog, decreases upon germination, indicating that there is not transcriptional upregulation of these genes during pollen germination and suggesting that the increase in LAT59 protein occurs via a post-transcriptional mechanism. Furthermore, we found no significant modulation of the activity of the 5′ UTR inhibitor upon germination in transient expression experiments. (2) Pectate lyases in pollen are thought to be involved in pollen tube growth either by destabilizing the pectic linkages to help construct the wall or by facilitating the penetration of the tube through the pectin-rich matrix of the style. Although there are many pectate lyase–like genes expressed in pollen (McCormick, 1991a; Dircks et al., 1996), it is not known whether there are differences in substrate specificity and whether the precise functions of the proteins differ. Perhaps the expression of some pectate lyase–like proteins must be tightly regulated in pollen. Similarly, if the product of *LAT59* could compromise cell wall integrity, perhaps its expression must be prevented in somatic cells. Thus, inhibition by the leader might prevent expression in somatic tissues but allow expression in pollen. Consistent with this idea is the much more dramatic inhibition obtained in the transgenic BY-2 cells compared with transgenic tomato pollen.

An alternative interpretation is that the inhibitory sequence lacks a regulatory role and simply keeps *LAT59* expression low at all times, while other mechanisms allow expression in pollen. Further study is needed to determine whether the inhibitor provides additional control over gene expression.

METHODS

Plasmid Constructs

The 59L construct contains 115 bp of the promoter and the entire 236-bp leader of the late anther tomato *LAT59* gene and was created by translational fusion with the luciferase (*LUC*) open reading frame (Ow et al., 1986) fused to the cauliflower mosaic virus (CaMV) 35S termination sequence. Construct 59ΔL was described previously under the name p59ΔILUC (Eyal et al., 1995). The 59ΔL210 plasmid was obtained by cloning a double-stranded oligonucleotide corresponding to the *LAT59* (positions +220 to +236) sequence into the NcoI site of 59ΔL. 59L2, 59L5, and 59L6 are 3′ deletions generated after the introduction of an NcoI site by polymerase chain reaction (PCR) at positions +189, +136, and +91, respectively, and subsequent deletion of the thus generated downstream NcoI fragment. To obtain the internal deletions 59L1, 59L3, and 59L4, a BclI restriction site was introduced at position +10 in 59L–LUC. The BclI-NcoI fragment of the resulting plasmid was replaced by a PCR-made BclI-NcoI fragment corresponding to various lengths of the *LAT59* 5′ untranslated region (5′ UTR). In the case of 59L3, the 3′ oligonucleotide used in the PCR reaction contained a 16-nucleotide tail sequence spanning a region (positions +220 to +236) of the 5′ UTR. M1 to M3, M1C to M3C, and M1M1C to M3M3C mutations were created by site-directed mutagenesis (Kunkel et al., 1987) followed by replacing the wild-type 5′ UTR of 59L by the mutated sequences. Nucleotide substitutions introduced are as follows (the dash corresponds to an unchanged nucleotide): M1, +109 (CGCTAG) +114; M1C, +127 (GCTAGCG) +133; M2, +75 (GAGT–AACACC) +85; M2C, +154 (GTGGTTAACTC) +164; M3, +90 (TGGCTAGCT) +98; and M3C, +139 (AGCTAGCACCA) +149.

The (+3 to +207) fragment of the *Nt59* gene encompassing most of the leader sequence was obtained by PCR, using genomic DNA as a template. The AflIII-NcoI fragment was thus generated and then cloned into the NcoI site of 59ΔL.

The 35S/59L and 35S/59ΔL constructs, which were bombarded in tobacco BY-2 cells, contain the CaMV 35S gene promoter–enhancer region (−504 to −19) and include the TATA box, fused to either the (−17 to +236) L region or the (−17 to +10) ΔL region of *LAT59* obtained by PCR.

Plasmids used in the BY-2 protoplast transient experiments were generated from the pT7-LUC-A_{50} plasmid kindly provided by D. Gallie (University of California, Riverside; Gallie et al., 1991). Because the DraI site used for linearization before in vitro transcription is present twice in the *LAT59* leader, the pT7-LUC-A_{50} plasmid was modified by the addition of a BglII site between the DraI and EcoRI sites downstream of the $(A)_{50}$ sequence. The (+21 to +236) and (+185 to +236) *LAT59* PCR fragments were inserted between the HindIII and NcoI sites of this modified pT7-LUC-A_{50}.

β-Glucuronidase (*GUS*) fusions used in tomato (*Lycopersicon esculentum* cv VF36) stable transformation were as described previously (Eyal et al., 1995). 59L and 59ΔL constructs that were stably introduced into BY-2 cells were constructed in the pBin19 vector (Bevan, 1984). They contained a *LUC* and a *GUS* reporter gene fusion in direct orientation in the T-DNA (Figure 4). The 35S–*GUS* fusion, used as internal control for the transformation experiment, corresponded to the excised PstI fragment from the pRTL2–GUS vector (Carrington and Freed, 1990), whereas the 35S/59–*LUC* cassettes, containing either the 59L or the 59ΔL region, were identical to those used in transient expression assays in tobacco BY-2 cells.

Transient Expression

Particle bombardment experiments with tobacco pollen and tobacco BY-2 cell culture, as well as processing of the tissue for analysis of LUC and GUS activities, were as previously described (Twell et al., 1991) and later modified (Eyal et al., 1995). In each experiment, the test plasmid was cobombarded with a reference plasmid: pBI223 (Clontech, Palo Alto, CA) was used for assays with BY-2 cells, and pLAT56-12 (Twell et al., 1991) was used for assays of *LAT59* constructs in pollen. Between six and 21 independent bombardments were performed with each construct.

Tobacco BY-2 cells (*Nicotiana tabacum* cv Bright Yellow 2) were maintained in culture, as described previously (An, 1985). Protoplasts were isolated essentially as described for the isolation of carrot protoplasts (Gallie et al., 1995), except that the isolation buffer contained 1% cellulase Onozuka R10 (Yakult Honsha CD, Ltd., Tokyo, Japan) and 0.1% pectolyase Y23 (Seishin Pharmaceutical Co., Tokyo, Japan), and digestion was for 3 hr at 28°C with 50 rpm agitation. In vitro–synthesized *LUC* mRNAs (2 μg) were mixed with 10^6 protoplasts (in 1 mL) immediately before electroporation (pulse 5 msec at 150 V, using a homemade unit of unknown capacitance). Electroporated protoplasts were then transferred to BY-2 medium (An, 1985) containing 200 mM mannitol and assayed at the time intervals indicated. Processing of the protoplasts before the LUC assay was as described by Gallie et al. (1995). Protoplast viability was determined by staining with 1% fluorescein diacetate (Sigma).

In Vitro Pollen Germination

Pollen from tomato or tobacco was added at a ratio of 1 to 2 mg/mL to germination medium described previously (Jahnen et al., 1989), except that 24% polyethylene glycol 3350 (Sigma) was used; it was swirled at 50 rpm at 28°C for 15 hr. Germination was consistently near 100%. Germinated pollen was collected on a nitrocellulose membrane by vacuum, washed several times with MSO medium (McCormick, 1991b), and processed for protein or RNA extraction, or for LUC and GUS enzymatic assays.

Stable Transformation

Constructs cloned into pBIN19 were introduced into tomato by using *Agrobacterium tumefaciens* LBA4404, as previously described (McCormick, 1991b). We generated 34 and 23 independent transformants, respectively, for the 59L and 59ΔL constructs. The pBin19 constructs were introduced into BY-2 cells via Agrobacterium LBA4404, essentially as described by An (1985), except that 300 mg/L of carbenicillin and kanamycin were used. Four-day-old subcultures of BY-2 cells were cocultivated with a saturated culture of Agrobacterium for 3 days. After selection for 3 to 4 weeks, kanamycin-resistant calli were transferred to liquid medium and cultured at 28°C. Aliquots of the cell cultures were sedimented at 700*g* for 3 min; the pellets were frozen in liquid nitrogen and lyophilyzed overnight. Cells were disrupted by vortexing in the presence of glass beads, as described previously (Van Hoof and Green, 1996), before analysis of LUC and GUS enzymatic activity or protein and RNA concentration.

In Vitro Transcription/Translation

mRNAs used in in vitro translation experiments and BY-2 protoplast transfections were synthesized in vitro by using the Ambion mMessage mMachine in vitro transcription kit (Ambion Inc., Austin, TX). RNA integrity was determined by agarose gel electrophoresis. In vitro translation reactions were performed with the Promega wheat germ extract, using optimum concentrations of 50 mM potassium and 2 mM magnesium. Reactions were incubated at 25°C. ^{35}S-methionine was incorporated in the translation products to allow their detection after separation by SDS-PAGE. The reaction (1 μL) was assayed for LUC activity and protein concentration.

Analysis of GUS and LUC Activities

GUS enzymatic activity was assayed fluorometrically by using 2 mM 4-methylumbelliferyl β-D-glucuronide as a substrate (Jefferson et al., 1987). GUS histochemical assays were performed with leaves, stems, roots, and flowers from the primary transformants of tomato, as previously described (Jefferson et al., 1987; Eyal et al., 1995). LUC activity was analyzed as previously described (Twell et al., 1991), except for the RNA electroporation into BY-2 protoplast experiments, when the more sensitive Promega luciferase assay system was used. Results are reported either as relative LUC activity, which represents the ratio between the test (LUC) and the reference (GUS) plasmids, or as LUC activity and GUS activity when corrected for variation in total protein content by using a bicinchoninic acid protein assay (Pierce, Rockford, IL).

Production of the LAT59 Peptide Fragment in *Escherichia coli* and Polyclonal Antibody Preparation

The LAT56 protein is 54% identical to the LAT59 protein, and the LAT56 antibody weakly cross-reacts with the LAT59 protein (Dircks et al., 1996). To generate an antibody that would specifically recognize LAT59, we used a short polypeptide from the LAT59 N-terminal extension that is not present in LAT56. The histidine-tagged LAT59 construct was generated in the pRSET vector (Invitrogen, San Diego, CA). The nucleotide sequence corresponding to amino acid positions 29 to 91 of LAT59 polypeptide was synthesized by PCR with a NheI site at the 5′ end and with a TAG stop codon and a HindIII site at the 3′ end. Because no overexpression of this 62–amino acid polypeptide could be obtained in *E. coli*, the so-called *E. coli* rare codons AGG and AGA (Chen and Inouye, 1994), encoding arginine and present at positions 8, 9, 10, 56, and 57 in the cloned sequence, were mutated by PCR to CGT and CGC, respectively, also encoding arginine.

The pRSET-derived construct was introduced into the *E. coli* BL21 (Novagen Inc., Madison, WI). Cells were induced with 0.3 mM isopropyl β-D-thiogalactopyranoside for 2 hr at 30°C. The histidine-tagged LAT59 polypeptide was purified using the guanidine–HCl–based protocol of the QIAexpress System (Qiagen Inc., Chatsworth, CA), followed by affinity chromatography on Ni-NTA resin (Qiagen). The protein sample in urea was desalted in PBS. Mice were injected with 80 μg of protein in MPL plus TDM emulsion adjuvant (RIBI Immunochem Research, Hamilton, MT). Ascites fluids were induced by the sarcoma line T180 (ATCC accession number TIB 66) and directly used for immunodetection at a 1:5000 dilution. We verified that the anti-LAT59 antibody did not cross-react with the LAT56 protein in extracts prepared from insect cells infected with baculovirus expressing either the LAT56 or LAT59 cDNAs (Dircks et al., 1996; data not shown).

Protein Extraction and Immunochemistry

Mature pollen and in vitro–germinated pollen from tomato were disrupted in a glass–glass homogenizer on ice using 3 volumes of protein extraction buffer (10 mM Tris, pH 7.4, 1 mM DTT, 1 mM EDTA, and 1 mM Pefabloc [Boehringer Mannheim]). Cell debris was eliminated by centrifugation. Proteins were separated by SDS-PAGE and blotted to Hybond-ECL nitrocellulose membrane (Amersham) by electrotransfer. The membrane was blocked in TBS containing 2% glycine and 5% milk, incubated with a 1:5000 dilution of LAT59 antibody, and detected using enhanced chemiluminescence developer following the manufacturer's instructions (Amersham).

RNA Isolation and Hybridizations

Isolation of total RNA from BY-2 cell lines was performed essentially as described by Newman et al. (1993). The lyophilyzed pellet was powdered by vortexing 1 min in the presence of 25 glass beads of 3 mm in diameter. After phenol extraction, RNA was precipitated with 0.2 volumes of 1 M acetic acid and 0.7 volumes of ethanol. LiCl washes were replaced by 3 M sodium acetate, pH 5.2. RNA was electrophoresed and blotted according to standard methods (Sambrook et al., 1989). Total RNA from mature and germinated pollen of tobacco was isolated using a CsCl method, according to Chirgwin et al. (1979). The entire open reading frames of the *uidA*, *LUC*, and *Nt59* genes were labeled with phosphorus-32 by random priming (Feinberg

and Vogelstein, 1984) and used as probes. Signals were detected using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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