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Biochemical characterization and baculovirus expression of the pectate lyase-like LAT56 and LAT59 pollen proteins of tomato

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Abstract

The LAT56 and LAT59 tomato (*Lycopersicon esculentum*) genes are preferentially expressed in pollen, and the proteins they encode are similar to pollen proteins from diverse plant species including monocots (lily, *Lilium longiflorum* and corn, *Zea mays*), dicots (tobacco, *Nicotiana tabacum* and short ragweed, *Ambrosia artemisiifolia*), and a gymnosperm (Japanese cedar, *Cryptomeria japonica*), as well as to a tomato pistil-expressed protein. The short ragweed and Japanese cedar proteins are major pollen allergens. All of these plant proteins show some sequence similarity to bacterial pectate lyases (EC 4.2.2.2). The LAT56 and LAT59 proteins have potential signal sequences. We have raised an antiserum to a TrpE-LAT56 fusion protein that recognizes both LAT56 and LAT59. We show that these proteins are present in mature pollen, and that LAT56 is glycosylated in pollen, consistent with a cell wall localization. We use a baculovirus expression system to show that both proteins enter the secretory pathway and are glycosylated in insect cells. Like the short ragweed allergens, LAT56 is rapidly degraded in pollen extracts, but in contrast to the heat-labile short ragweed and Japanese cedar allergens, LAT56 is a heat-stable protein. Although LAT56 and LAT59 share significant sequence similarity to bacterial pectate lyases, we could not demonstrate pectate lyase activity in pollen extracts or in culture medium or in extracts from insect cells expressing LAT56 or LAT59.

Key words

Allergen, heat stability, protein glycosylation, protein secretion, tissue prints.

Abbreviations

Anti-56, antiserum raised against the TrpE-LAT56 protein; endo F or H, endoglycosidase F or H; ER, endoplasmic reticulum; 56-Bac, baculovirus expressing LAT56; 59-Bac, baculovirus expressing LAT59; LAT, late anther tomato; pel, pectate lyase; PMSF, phenylmethanesulphonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sf9, fall army worm ovary cell line; TBS, Tris-buffered saline.

INTRODUCTION

Pollen is transcriptionally active during most of its development and many of its structural and enzymatic components are pollen-derived. Around 10-20% of the genes expressed in pollen are specific to pollen (reviewed in Mascarenhas, 1990a), and encode proteins believed to be required for pollen maturation, pollen-pistil interactions, or pollen tube elongation. Pollen-expressed genes have been grouped into two temporal classes, early and late (reviewed in Mascarenhas, 1990b). Early genes are expressed

after release of the microspores from the tetrad, while late genes are expressed after first microspore mitosis (reviewed in McCormick, 1991; 1993). The late mRNAs accumulate during maturation and reach their highest levels in mature pollen. Many pollen proteins that are required for germination and pollen tube growth are thought to be translated from pre-synthesized messages, since pollen tube growth does not require new RNA synthesis (for review, see Mascarenhas, 1993). The expression of two tomato (*Lycopersicon esculentum*) late-class genes, LAT56 and LAT59, was characterized by measuring steady-

state RNA levels or expression of a reporter gene driven from the *LAT56* or *LAT59* promoters (Ursin *et al.*, 1989; Wing *et al.*, 1989; Twell *et al.*, 1990; 1991). These experiments provided no information about translation of the native message or protein stability and thus it was unknown whether the *LAT56* and *LAT59* proteins were present prior to pollen germination or if their messages were pre-synthesized for later translation.

Database comparisons had indicated that *LAT56* and *LAT59* are similar to pectate lyases (EC 4.2.2.2) from *Erwinia chrysanthemi* and *E. carotovora*, and to pollen allergens from short ragweed, *Ambrosia artemisiifolia* (an angiosperm) and Japanese cedar, *Cryptomeria japonica* (a gymnosperm) (reviewed in McCormick, 1991). Genes encoding similar proteins have since been identified in tobacco, *Nicotiana tabacum* (Rogers *et al.*, 1992), corn, *Zea mays* (Turcich *et al.*, 1993), and lily, *Lilium longiflorum* (Kim *et al.*, 1994). The amino acid sequences predicted for the *LAT56* and *LAT59* proteins have potential endoplasmic reticulum (ER) signal sequences and N-linked glycosylation sites (Wing *et al.*, 1989). In addition, their similarity to bacterial pectate lyases, which are secreted (Keen and Tamaki, 1986; Tamaki *et al.*, 1988), and to pollen allergens, which are displayed on the pollen surface (Knox *et al.*, 1970), suggested that the *LAT56* and *LAT59* proteins might be secreted and glycosylated, and that they might function in the cell wall to, for example, mediate pollen tube growth. We therefore sought to determine the sub-cellular destination of the *LAT56* and *LAT59* proteins.

In this paper we use an anti-*LAT56* antiserum to show that the *LAT56* and *LAT59* proteins are present in mature pollen and in pollen tubes, and that *LAT56* is glycosylated in pollen. We also demonstrate that *LAT56* is heat-stable but is rapidly degraded in pollen extracts. We use a baculovirus expression system (reviewed in O'Reilly *et al.*, 1992) to show that the *LAT56* and *LAT59* proteins traverse the secretory

pathway and are glycosylated in insect cells and to test these proteins for pectate lyase activity.

RESULTS

Pollen-expressed pectate lyase-like gene families

The similarity between the pollen-expressed proteins *LAT56* and *LAT59* and pectate lyases from plant pathogenic bacteria was detected by database searches (Wing *et al.*, 1989). The sequence conservation is particularly pronounced in the so-called (Keen and Tamaki, 1986) region I-interregion-region II portion of the proteins (fig. 1), which is conserved among known *Erwinia* pectate lyases. The similarity of *LAT56* and *LAT59* to the short ragweed pollen allergen *Amb a* I was noticed later (McCormick, 1991). Many plant representatives fall into this superfamily of pectate lyase-like genes, from dicots (Budelier *et al.*, 1990; Rogers *et al.*, 1992; Kulikauskas and McCormick, unpublished observations), from monocots (Turcich *et al.*, 1993; Kim *et al.*, 1994), and from a gymnosperm (Sone *et al.*, 1994). Figure 1 shows an alignment of some of the predicted plant pectate lyase-like proteins. Although it is difficult to strictly define a protein in one species as the ortholog of another in another species, these predicted proteins can be grouped by pattern of tissue expression (pollen vs. pistil), by intron positions in the coding sequence, and by deduced amino acid sequence similarity. Most of these genes are expressed in pollen, although one, 9612 (Budelier *et al.*, 1990) is expressed in pistils. *LAT56* and *LAT59* each have two introns in non-homologous positions, while *LMP131*, *Zm58*, and 9612 have no introns (Kulikauskas and McCormick, unpublished observation; Turcich *et al.*, 1993; and Budelier *et al.*, 1990, respectively). *G10* has introns in positions homologous to those in *LAT56* (Rogers *et al.*, 1992) and the deduced amino acid sequences of *LAT56* and *G10* are more similar to each other (83%) than to the other proteins. The predicted *LAT59*, *LMP131*, and

Figure 1. Alignment of the predicted amino acid sequences of the pollen-expressed proteins and the pistil-expressed protein 9612. *LMP131*, *Zm58-2*, *G10*, *Amb a* I.1, and *Cry j* I are from lily, corn, tobacco, short ragweed, and cedar, respectively. The sequences similar to regions I and II of *Erwinia* pectate lyase genes are underlined and overlined. The region of *LAT56* and *LAT59* used to raise antisera are indicated with dotted underlines. Amino acids conserved between all sequences are shaded. Conserved amino acids are grouped as follows: (K,R), (E,D), (Q,N), (S,T), (F,Y,W,H), (L,I,V,A), (P,G), and (M,C). The published *LAT56* sequence (Wing *et al.*, 1989) had a frame shift (Turcich *et al.*, 1993) which, when corrected, extends the predicted sequence so that *LAT56* aligns with the other plant proteins at the carboxyl-terminus. Changes in the *LAT56* protein from the sequence published previously (Wing *et al.*, 1989) are at codon 98 (F to G), codon 124 (K to D), codons 136-139 (RCGQ to DRQK), codon 151 (L to AD), codon 191 (R to T), codon 216 (E to G), and codons 423 and 429 (introduction of an A at each); all corrections have been entered in the databases.

Zm58 proteins are more similar to each other than to the other predicted proteins in that each has an amino-terminal extension not present in the others.

Considering that pectate lyase-like genes are expressed in pollen of diverse plant species, including angiosperms and a gymnosperm, and that the sequence conservation within the group is high, it seems likely that these proteins play an important role in some pollen function. To determine what that function might be, we attempted to phenocopy a LAT56 or LAT59 null mutation by expressing antisense constructs in tomato, but we were unable to detect a pollen phenotype in any of the antisense transgenic plants (data not shown). A possible explanation for this result is that because several pectate lyase-like genes are expressed in pollen in one species (*i.e.*, short ragweed; Rafner *et al.*, 1991), the reduction in expression of only one gene has no effect, because other family members can substitute for the missing protein.

LAT56 and LAT59 proteins are present before pollen germination

We required antisera recognizing LAT56 and LAT59 in order to investigate when LAT56 and LAT59 proteins are present in pollen. We had difficulty in obtaining high-level expression from full-length versions of LAT56 and LAT59 in *Escherichia coli* (data not shown), so we attempted to raise mouse antisera to TrpE-LAT56 and TrpE-LAT59 fusion proteins, as described in Methods. The fusion constructs were made using the regions predicted to be the most antigenic portions of the pollen proteins (Hopp and Woods, 1981; indicated in figure 1 by dotted underlines). We were only successful in raising an anti-serum against the TrpE-LAT56 fusion protein. Figure 2 shows that the anti-56 antiserum recognizes two protein bands in total pollen extracts, and that there is no signal detected in leaf or petal. In pistil extracts the antibody detects no discrete proteins of the sizes predicted for LAT56, LAT59 or 9612, but only a diffuse smear. In order to verify that the two proteins detected in pollen extracts correspond to LAT56 and LAT59, we tested the anti-56 antiserum for recognition of LAT56 and LAT59 by expressing LAT56 and LAT59 in insect cells using the baculovirus expression system. The anti-56 antiserum recognizes a protein in 56-Bac-infected cell extracts that co-migrates with the smaller protein from pollen, and that is absent in both the 59-Bac-infected and the no insert control virus-infected extracts. This pollen protein is

most likely LAT56. The antiserum also recognizes a larger pollen protein and a protein in the 59-Bac-infected extract that is absent in both the 56-Bac- and no insert control-infected extracts (*fig. 2*). Despite the slight difference in migration of the insect cell-derived LAT59 protein and the putative pollen LAT59 protein, the larger pollen protein is most likely LAT59; a recently obtained LAT59-specific antibody recognizes both the baculovirus expressed LAT59 and the larger pollen protein (Curie and McCormick, unpublished observation). The difference in migration of the insect cell-derived and pollen LAT59 protein might be due to differences in post-translational modifications. Thus, the anti-56 antiserum can recognize both LAT56 and LAT59 proteins. In the immunoblot shown in figure 2, extracts from equal numbers of insect cells were loaded, but LAT56 detection is stronger than LAT59 detection. We cannot accurately determine the relative amounts of LAT56 and LAT59 because the anti-56 antiserum may have a higher affinity for LAT56.

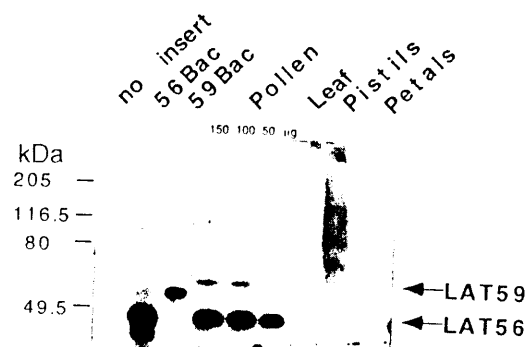


Figure 2. LAT56 and LAT59 proteins are preferentially expressed in pollen. Protein extracts were prepared from insect cells and tomato tissues as described in Methods. Extracts from approximately 1.8×10^5 insect cells, 150 μ g, 100 μ g, 50 μ g pollen, and 50 μ g of leaf, petal or pistil were loaded as indicated. Proteins were separated by SDS-PAGE and immunoblotted with the anti-56 antiserum.

LAT56 and LAT59 proteins enter the secretory pathway

Since LAT56 has two and LAT59 has three potential N-linked glycosylation consensus sequences, we assayed for the presence of glycan residues on LAT56 and LAT59. The presence of glycan residues directly demonstrates that a protein traverses the secretory pathway, because N-linked glycosylation occurs only in the ER and Golgi (Faye *et al.*, 1989). We treated pollen extracts with endo H, which cleaves the high mannose glycans that are

attached to proteins in the ER, or with endo F, which cleaves both high mannose glycans and the complex glycans that result from further modification of high mannose glycans in the Golgi (Thotakura and Bahl, 1987). LAT56 from pollen treated with endo H and endo F co-migrates with untreated LAT56 (fig. 3A), even on high resolution gels that could have detected a 2 kDa size difference. Thus pollen LAT56 is resistant to both endo H and endo F. Because another tomato pollen protein, LAT52, was sensitive to endo F deglycosylation in the same pollen extracts (Muschietti *et al.*, 1994), we believe that the resistance of pollen LAT56 to endo F is not due to an inhibitor of the enzyme in pollen extracts.

Because endo H and endo F do not cleave some types of glycans from proteins (Thotakura and Bahl, 1987), we tried to detect glycans on LAT56 by covalently modifying glycoproteins from pollen extracts with digoxigenin-succinyl- ϵ -amidocaproic acid hydrazide using a glycan detection kit, as described in Methods. When treated pollen proteins were immunoblotted using the anti-56 antiserum, the LAT56 protein from the treated extract migrated more slowly than LAT56 from an untreated extract (fig. 3B). This observation is consistent with a mobility shift of LAT56 due to covalently attached digoxigenin-succinyl- ϵ -amidocaproic acid hydrazide molecules on the glycan residues and therefore confirms that LAT56 is glycosylated in pollen. Glycosylation of LAT59 in pollen could not be assessed because the protein concentration after the glycan assay was too low for antibody detection.

Several plant proteins (Bustos *et al.*, 1988; Vernet *et al.*, 1990; MacDonald *et al.*, 1994; Muschietti *et al.*, 1994) have been expressed in insect cells using the baculovirus expression system and were shown to enter the secretory pathway, as demonstrated by the acquisition of glycans. We used the baculovirus expression system to explore the glycosylation of LAT56 and LAT59 in more detail (fig. 3C, fig. 4). In the experiment shown in figure 4, Sf9 cells infected with 56-Bac, 59-Bac, or the no insert control were labeled with [^{35} S] methionine and incubated in cold medium as described in Methods. After 2 and 4 h incubations in cold medium unique bands (indicated by black dots, fig. 4) appeared in the medium. Duplicate samples run on the same gel were immunoblotted (data not shown) to confirm that the bands marked by black dots (fig. 4) co-migrate with LAT56 and LAT59 proteins. Additional smaller bands (indicated by white dots, fig. 4) are most likely either

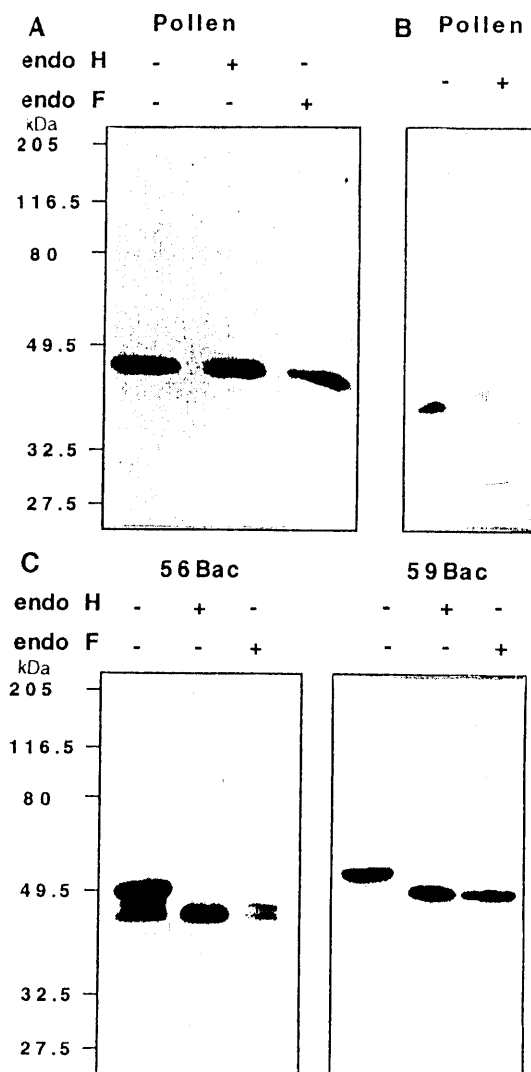


Figure 3. LAT56 and LAT59 are glycosylated. **A**, pollen extracts were mock-treated or treated with endo H or endo F as indicated; **B**, glycosylated pollen proteins were mock-treated (-) or modified with digoxigenin-succinyl- ϵ -amidocaproic acid hydrazide (+) as described in Methods; **C**, Sf9 cells were infected with either 56-Bac or 59-Bac. 72 h post infection cells were harvested and mock-treated or treated with endo H or endo F, as indicated. Samples were separated by SDS-PAGE and immunoblotted with the anti-56 antiserum.

degradation products of LAT56 and LAT59 or less extensively glycosylated versions of the proteins. We believe that the proteins in the medium are secreted rather than simply released into the medium by cell lysis because several major proteins detectable in cell extracts do not appear in the medium fraction (fig. 4). In the experiment shown in figure 3C, cells

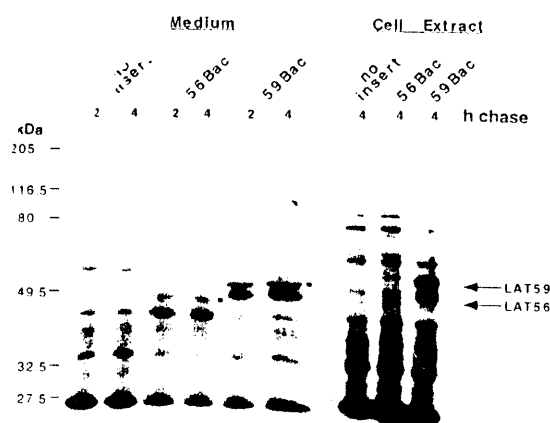


Figure 4. LAT56 and LAT59 are secreted in insect cells. Sf9 cells were infected with either the control virus, 56-Bac, or 59-Bac. 72 h post infection the cells were labeled for 2 h with [^{35} S]methionine and chased for 2 or 4 h. Supernatants were taken at the indicated times and centrifuged to pellet cellular debris. After a 4 h chase, cells were lysed in SDS sample buffer. Samples were separated by SDS-PAGE and autoradiographed.

infected with either 56-Bac or 59-Bac were lysed and the extracts were treated with endo H or endo F. The proteins were immunoblotted with the anti-56 antiserum. LAT56 and LAT59 treated with endo H and endo F migrate faster than the untreated proteins. These results thus indicate that both LAT56 and LAT59 are translocated across the insect cell ER membranes where they are modified by glycosylation.

We attempted to immunolocalize LAT56 on fixed and embedded sections of mature pollen, however we were unsuccessful. A possible explanation could lie in the fact that pollen allergens are extremely water soluble and rapidly lost when pollen is hydrated (Grote *et al.*, 1993). We therefore used a modified form of tissue printing to indirectly show that LAT56 (and/or LAT59) are localized to pollen tubes. Pollen was germinated in liquid for either 45 min or 3 h and fixed to nitrocellulose by mild vacuum filtration. The membranes were baked briefly at 80°C and further fixed to the membranes before immunoblotting. This procedure conserves most of the pollen morphology and the affixed pollen could be stained and examined with UV microscopy to determine the location of the pollen nuclei (data not shown). The anti-56 antiserum reacts strongly with the pollen tubes, at both the 45 min and 3 h time points (*fig. 5*). The rounded surface of the pollen grain (30 μ m diameter) appears intact and does not react with the antibody, but the heat treatment and fixation is probably permeabilizing the pollen tube cell wall and plasma membrane. No



Figure 5. Immunolocalization of LAT56 on pollen tissue prints. Pollen was germinated in liquid for either 45 min (A) or 3 h (B) and the pollen tubes were affixed to nitrocellulose membranes and immunoblotted with anti-56 antiserum.

signal was observed when equivalent tissue prints were incubated with pre-immune serum or with an anti-LAT52 antibody (Muschietti *et al.*, 1994) that can only recognize antigen under denaturing conditions (data not shown). While we cannot definitely conclude that the antigen is cell wall localized, our data from the 3 h time point is consistent with such a location. The antigen signal is seen throughout the length of the tube, although the pollen cytoplasm has moved towards the distal half of the tubes.

LAT56 is heat-stable but subject to proteolytic degradation

In the course of testing whether the protease inhibitor-like LAT 52 protein was heat stable (Muschiatti *et al.*, 1994), we used LAT56 as a control. Unexpectedly, the LAT56 protein is also heat stable (fig. 6A), although the vast majority of pollen proteins precipitate after heating in the absence of SDS, as can be assessed by the Coomassie-stained gel (data not shown; see Muschiatti *et al.*, 1994). To determine if this heat stability was a general feature of the pollen-expressed pectate lyase-like proteins, we tested the short ragweed pollen allergen *Amb a I* for heat stability, but more than 90% of the allergen precipitated upon heating in the absence of SDS (data not shown).

In initial experiments with the anti-56 antiserum and pollen extracts, the pattern of detected protein bands was variable from different preparations. To determine if this variability was due to the variable detection of degradation products of LAT56, we investigated the stability of LAT56 protein by incubating crude pollen extracts at 0°C, room temperature (25°C), or 37°C in the presence or absence of protease inhibitors. In freshly prepared pollen extracts and in the absence of protease inhibitor, the 44 kDa protein completely disappears within 30 min after incubation at 25°C or 37°C. Examination of the Coomassie-stained gel (fig. 6B) shows that most proteins in the extract are not affected. Degradation of LAT56 protein is prevented by incubation at 0°C or by addition of PMSF (at 25°C or 37°C; fig. 6B) or Pefabloc (at 20°C), (data not shown), which are serine protease inhibitors. Pollen extracts that are prepared in the absence of PMSF and then frozen and thawed before addition of PMSF also show no 44 kDa protein (fig. 6B). Other protease inhibitors, such as antipain, leupeptin, aprotinin, and pepstatin, were not effective in preventing LAT56 degradation (data not shown), although we included them in our standard protease inhibitor cocktail so that the insect cell extracts and the pollen extracts would be prepared in the same buffer. Furthermore, boiling of pollen extracts prior to incubation in the absence of PMSF stabilizes the LAT56 protein (data not shown), indicating that this degradation is dependent upon the presence of a heat sensitive factor.

Are LAT56 and LAT59 pectate lyases?

We tested pollen of several species that express pollen-specific pectate lyase-like proteins (tomato,

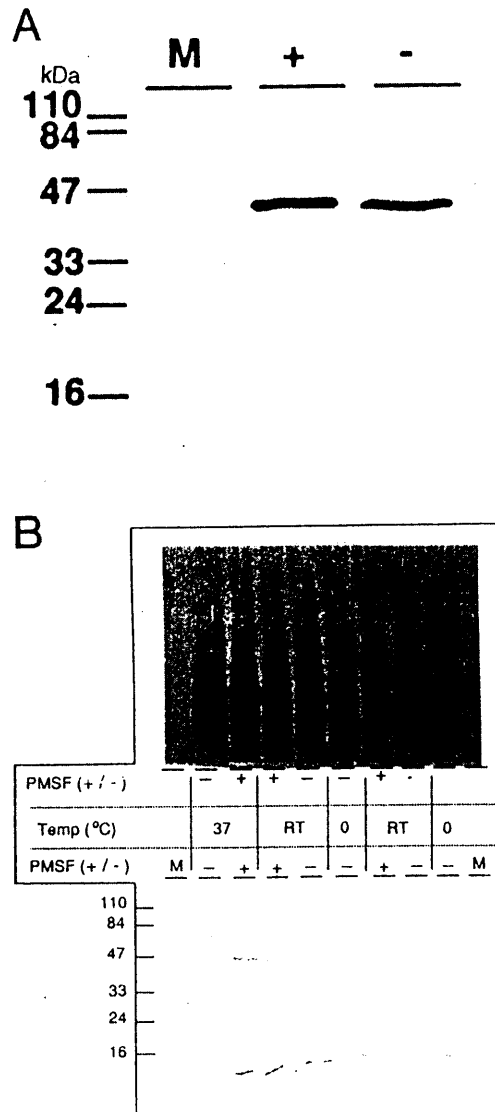


Figure 6. LAT56 is heat-stable and sensitive to a pollen protease. **A**, heat stability of LAT56 protein. Sample applied in + the lane represents 70 μ g protein of a pollen extract that was heated for 2 min at 90°C in the absence of SDS. Sample applied in the - lane represents 70 μ g protein of a pollen extract that was not heated. Lane M contains molecular mass markers. The samples were separated by SDS-PAGE and immunoblotted with the anti-56 antiserum; **B**, protease sensitivity of LAT56 in pollen extracts. Extracts were incubated at the indicated temperature for 30 min, in the absence or presence of PMSF, then separated by SDS-PAGE and immunoblotted with the anti-56 antiserum. Lanes 2-6 were from freshly extracted pollen; lanes 7-9 were from pollen extracts that were frozen and then thawed before addition of PMSF and incubation; lanes 1 and 10 are molecular mass markers. Duplicate samples were separated by SDS-PAGE and stained with Coomassie. The stained gel is oriented upside down, so that the lanes directly correspond to the immunoblot below.

tobacco and corn) for pectate lyase activity using a spectrophotometric assay (Collmer *et al.*, 1988). As a positive control, we used the medium of a bacterial culture of *E. coli* expressing *E. chrysanthemi* *pelB*. We were unable to demonstrate pectate lyase activity in pollen of any of these species.

Because of the possibility that the amount of pectate lyase activity in pollen might be below the limit of detection, we assayed for pectate lyase activity in both cell extracts and the medium of 56-Bac and 59-Bac-infected cells, but detected no activity. We mixed 56-Bac- and 59-Bac-infected extracts in case an interaction between the two proteins was necessary for activity, to no effect. We considered the possibility that the tomato enzymes might have co-factor requirements different from the bacterial enzymes. These co-factors should be present in a pollen or pistil extract. To test this possibility we spiked the insect cell extracts with extract from pollen and/or pistil to 3% (v/v) of the total substrate solution. We were still unable to detect pectate lyase activity. As a final control, we also added the *pelB* positive control to pollen extracts and insect cell extracts to exclude the possibility that an inhibitor of pectate lyase activity was present in the extracts. The pectate lyase activity in the pollen or the insect cell extracts with added *pelB* was similar to that seen with an equal volume of *pelB* control alone. Thus, we conclude that the lack of pectate lyase activity in pollen or insect cells is not due to an inhibitor of pectate lyase activity.

The LAT56 and LAT59 proteins are secreted from insect cells (fig. 3 and 4), but do not accumulate to as high a level in the medium as in the cells. In order to determine the quantity of LAT56 protein present in the insect cell culture medium and cell extracts relative to that in pollen, we immunoblotted extracts containing 8, 40, 50, 80, and 100 μ g total protein from pollen and compared it to fractions of culture medium and extracts from cells expressing LAT56 (data not shown). We estimated that the amount of the LAT56 protein in the assayed culture medium was equivalent to that in 0.04 mg (dry weight) pollen or 2.4×10^4 pollen grains, and that the LAT56 protein in the assayed insect cell extracts was equivalent to that in 2.7 mg (dry weight) tomato pollen or 1.6×10^6 pollen grains.

DISCUSSION

The antiserum we raised against the TrpE-LAT56 fusion protein recognized both proteins and allowed us

to explore the expression patterns and characteristics of LAT56 and LAT59. The antiserum does not recognize leaf or petal proteins. However, it does strongly but rather non-specifically cross-react with pistil extracts, perhaps with highly glycosylated versions of pistil-expressed family members, such as 9612 (Budelier *et al.*, 1990). The LAT56 protein is detected in pollen extracts at about a 20-fold higher level than LAT59, which most probably reflects differences in the epitope specificity of the antiserum. It is interesting to note, however, that the anti-56 antiserum does not cross-react with any tobacco pollen protein (data not shown), and that the LAT56 and tobacco G10 proteins are more similar than LAT56 and LAT59 (84% similar vs. 74% and 76% identical vs. 61%, respectively) in the region used for antiserum preparation (fig. 1).

Our experiments indicate that the LAT56 and LAT59 proteins enter the secretory pathway. The proteins are secreted from insect cells into the medium (fig. 4) and the proteins made in insect cells are sensitive to endo H and endo F (fig. 3), which indicates that they are glycosylated. The LAT56 protein from pollen extracts treated with digoxigenin-succinyl- ϵ -amidocaproic acid hydrazide migrated with a slower mobility than LAT56 from untreated extracts, indicating the presence of glycans. It is surprising that LAT56 from pollen was not sensitive to endo H or endo F. The most likely explanation for this result is that endo H and endo F cannot cleave the particular structure of the glycans on pollen LAT56. Although LAT56 and LAT59 clearly enter the secretory pathway (fig. 3 and 4), we cannot exclude the possibility that LAT56 and/or LAT59 are secreted in insect cells by default. It is possible that one or both is mislocalized outside the insect cells, because secretion appears to be the default pathway for overexpressed proteins that normally reside in the vacuole or other compartments. For example, phaseolin, a vacuolar protein, is largely secreted in insect cells (Bustos *et al.*, 1988).

LAT56 is heat-stable and remains soluble when pollen extracts are boiled (fig. 6 A). In contrast, *Amb a* I.1 (data not shown) and *Cry j* I (Yasueda *et al.*, 1983) are heat-sensitive. LAT56 in pollen extracts is sensitive to proteolytic degradation by a serine protease (fig. 6 B). The *Amb a* I.1 protein is specifically cleaved by an endogenous protease in pollen extracts (Smith *et al.*, 1988) to yield a C-terminus derived- 26 kDa peptide and a N-terminus-derived 12 kDa peptide. These results are analogous to ours, in that we sometimes (fig. 6 B) detect a

degradation product of about 12 kDa in tomato pollen extracts. Since our antiserum was raised to the amino-terminal portion of LAT56, we can only detect the smaller of the degradation products of LAT56. Thus both LAT56 and *Amb a* 1.1 may be cleaved by a pollen endo-protease. The significance of the heat-stability and protease-susceptibility of LAT56 remain unclear. The protease-susceptibility might reflect spurious degradation of LAT56 by a protease that it would not normally contact in intact pollen. Alternatively, the protease-susceptibility may be part of the mechanism regulating LAT56 protein levels during pollen development.

The crystal structures of pectate lyase C and E from *E. chrysanthemi* have been used for a structure-based alignment of the proteins in the pectate lyase superfamily (Henrissat *et al.*, 1995). In addition to the already noted evolutionary-based sequence similarity to pectate lyases (Wing *et al.*, 1989; McCormick, 1991) the structure-based alignment suggests that the pollen proteins also share the parallel β -helix topology of *bona fide* pectate lyases, adding more support to the supposition that the pollen proteins are pectate lyases. We have been unable to detect pectate lyase activity in extracts of tomato pollen or from insect cells expressing LAT56 or LAT59. Taniguchi *et al.* (1995), however, recently detected pectate lyase activity associated with a purified preparation of the cedar allergen *Cry j* 1, although they did not report whether pectate lyase activity could be measured in crude pollen extracts of Japanese cedar pollen. The specific activity of the purified cedar allergen was similar to that of the bacterial pectate lyases. This observation shows that at least some of the pollen pectate lyase-like proteins are, in fact, pectate lyases. One possible explanation why we could not detect pectate lyase activity is that neither the pollen extracts, the insect cell culture medium, nor the insect cell extracts contained enough LAT56 or LAT59 protein. Taniguchi *et al.* (1995) performed pectate lyase assays on 0.04 mg of *Cry j* 1 protein, which had 14 units of activity. We typically used 0.2–0.25 mg total pollen extract protein in a pectate lyase assay. In order to assay the same amount of LAT56 or LAT59 as Taniguchi *et al.* (1995) used, the LAT56 and LAT59 proteins would have to comprise 16–20% of the total pollen proteins, which is clearly not the case. Nonetheless, we calculate that we could have detected 0.062 units of pectate lyase activity. Thus, if the LAT56 and LAT59 had a specific activity similar to the cedar allergen and comprised at least

0.07% of the total cellular protein, we could have detected pectate lyase activity. It is also possible that the natural substrates for LAT56 and LAT59 cannot be mimicked in the *in vitro* assay. In this light, it is noteworthy that *E. chrysanthemi* EC16 produces several isozymes of pectate lyase that differ in their abilities to cleave polygalacturonate *in vitro* and to macerate plant tissue (Payne *et al.*, 1987). Thus, perhaps LAT56 and LAT59 are indeed *bona fide* pectate lyases that are weakly active, or that require a different substrate or assay conditions for detection of activity. Pectin-degrading enzymes are thought to mediate pollen tube growth by increasing the rate of hydrolysis of tip wall components, based partly on the observation that pectinase enhanced pollen tube elongation rates *in vitro* (Roggen and Stanley, 1969). An alternative function for less active pectin-degrading proteins might be to cleave the intine pectate at a low level in order to produce elicitors of the pathway for germination and pollen tube growth.

METHODS

Production of anti-56 antibody. A TrpE-LAT56 fusion protein comprising amino acids 49 to 165 of LAT56 was constructed by ligating the 360 bp *EcoRI* fragment of pLAT56 (Wing *et al.*, 1989) into pATH 3, which differs from pATH 2 (Dieckman and Tzagoloff, 1985) in the multiple cloning site. A TrpE-LAT59 fusion protein comprising amino acids 98 to 139 of LAT59 was constructed by ligating the 120 bp *NsiI-EcoRI* fragment of pLAT59 into pATH 2. The constructs were expressed in XL Blue cells (Stratagene, La Jolla). The induction and partial purification of the fusion proteins were performed according to Spindler *et al.* (1984). The proteins were further purified by SDS-PAGE. The proteins were eluted from an excised gel fragment and used to immunize mice. Approximately 20 μ g protein in incomplete Freund's adjuvant was used per immunization for four boosts. The immune response was monitored by ELISA on tail bleeds ten days after immunization. Ascites fluids were induced by the sarcoma line T180 (ATTC #TIB 66), and were collected approximately 7, 10, and 13 days after immunization. Ascites fluids were used directly for immunodetection at a dilution of 1:1000 (or 1:500, fig. 5 and 6).

Preparation of plant tissue extracts. All tissues were obtained from *Lycopersicon esculentum* cv. VF36. Pollen was harvested from mature flowers (stage 4 flowers, Ursin *et al.*, 1989) by vibrating the flower pedicel with a Bio-Vortexer (model 1083-MC). Pollen was ground with sea sand in 100 mM Tris (pH 7.4), 1 mM EDTA, 1 mM Pefabloc (Boehringer Mannheim Biochemicals) and a proteinase

inhibitor cocktail composed of 0.5 $\mu\text{g ml}^{-1}$ each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin A (Sigma) in a marble mortar chilled to dry ice temperature. After thawing, the extract was centrifuged at $14,000 \times g$ for 2 min at 4°C and the aqueous phase was reserved. Leaves, petals, and pistils were harvested from growing plants, frozen in liquid nitrogen, and ground in a marble mortar in the same buffer used for pollen extracts, and processed as above. Total protein of extracts was determined by the bicinchoninic acid method according to manufacturer's instructions (Pierce).

Construction of recombinant baculovirus strains. 56-Bac (baculovirus expressing LAT56) was constructed by fusing the entire coding region of LAT56 (Wing *et al.*, 1989) to the baculovirus polyhedrin promoter in pVL1392 (O'Reilly *et al.*, 1992). This vector, designated pVL56c, was co-infected into *Spodoptera frugiperda* (Sf9) cells with linearized baculogold baculovirus genome (PharMingen) according to supplier's instructions. Recombinant virus was identified and amplified according to O'Reilly *et al.*, 1992. 59-Bac was constructed by fusing the entire coding region of LAT59 (Wing *et al.*, 1989) to the baculovirus polyhedrin promoter in pVL941. This vector, designated pVL59, was co-infected and identified and amplified as described above. The "no insert" control was constructed by co-infecting with the transfer plasmid pVL941 (lacking any exogenous DNA sequence) and identified and amplified as described above.

Expression and localization of LAT56 and LAT59 in insect cells. Sf9 cells grown in Grace's medium (Gibco/BRL), supplemented with fetal calf serum to 10%, were infected at a multiplicity of infection (MOI) of 200. At 72 h post infection, cells were labeled with [^{35}S]methionine for 2 h, incubated in the absence of [^{35}S] methionine for 2 or 4 h, and separated from the medium. Each fraction was separated by SDS-PAGE and protein signals were amplified by fluorography. In parallel, cells of 4 h time point samples were lysed in SDS sample buffer, separated by SDS-PAGE on the same gel as above, and immunoblotted with the anti-56 antiserum.

Detection of glycans. Glycan cleavage: Sf9 cells grown in Sf900II were infected with 56-Bac or 59-Bac. At 72 h post-infection, cells were lysed in elution buffer (50 mM Tris (pH 6.8), 0.5% SDS, 0.1% β -mercaptoethanol). Pollen was ground in a mortar in elution buffer. Sf9 and pollen extracts were boiled for 5 min and digested with endo H or endo F according to supplier's instructions (Boehringer-Mannheim). The extracts were separated by SDS-PAGE and immunoblotted with the anti-56 antiserum. Glycan modification: Mature tomato pollen was ground in a marble mortar cooled to dry ice temperature in 100 mM sodium acetate (pH 5.5) containing 1 mM Pefabloc (Boehringer-Mannheim). Pollen glycoproteins were modified with digoxigenin-succinyl- ϵ -amidocaproic acid hydrazide according to supplier's instructions (Boehringer-Mannheim),

separated by SDS-PAGE and immunoblotted with the anti-56 antiserum.

Tissue prints. Tomato (cv. VF36) pollen was germinated in liquid pollen germination medium (Jahnen *et al.*, 1989) for 45 min or 3 h. The pollen suspension was slowly pipetted, using a 1 ml pipetteman, onto a square of 0.2 μm S&S nitrocellulose membrane (pre-wetted in TBS) that had been placed on the platform of a Nalgene vacuum filtration device. A slow vacuum was pulled for 1 min, using water aspiration. The membranes were baked at 80°C for 3 min and then treated for 10 min in 0.25% glutaraldehyde, 2.5% *p*-formaldehyde in phosphate buffer. The membranes were washed in water, blocked with 2.5% dry milk in TBS and incubated with the primary antibody (1:500) overnight and in the secondary antibody for 2 h.

Pectate lyase assays. Sf9 cells grown in Sf900II medium were infected with 56-Bac, 59-Bac, or the no-insert control virus. At 72 h post-infection, culture medium was separated from cells by centrifugation and used directly in assays. Cells were harvested by washing twice in ice-cold 25 mM Tris-HCl (pH 8.8) containing proteinase inhibitor cocktail, and scraped into the same buffer. The cells were disrupted by three cycles of 5 s of sonication followed by 20 s on ice using a Tekmar sonic disruptor set to 60. Pollen extracts were prepared as described above, except that the extraction buffer did not contain EDTA. The supernatants from these insect cell and pollen extracts were used in the pectate lyase assays. One part extract was added to ten parts substrate mix (0.24% polygalacturonic acid (Sigma), 25 mM Tris-HCl (pH 8.8), 0.67 mM CaCl_2) and the increase in absorbance at 232 nm was measured over 2 to 5 min at room temperature (Collmer *et al.*, 1988), or at 65°C , the reported optimal temperature for the *Cry j 1* enzyme (Taniguchi *et al.*, 1995). The positive control for the pectate lyase assays was the cell-free medium from a culture of *E. coli* strain DH5 α containing pPEL344 (Keen and Tamaki, 1986), which encodes *E. chrysanthemi pelB*. Cells were grown for one to two days at 37°C in LB supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin, then shifted overnight to 28°C . The culture was centrifuged for one minute at $14,000 \times g$ to pellet the cells, and the supernatant was reserved. Enzyme activity was comparable to that reported by Keen and Tamaki (1986).

Miscellaneous methods. SDS-PAGE separations (Laemmli, 1970) were done on 8.5% gels. Immunoblots were developed by the ECL (Amersham) method (fig. 2, 3 and 4) or with the alkaline-phosphatase-linked (Promega) method (fig. 5 and 6). DNA sequencing, plasmid DNA isolation, and transformation of *E. coli* strains were done according to standard procedures (Sambrook *et al.*, 1989). Amino acid sequence comparisons were done using the Genetics Computer Group sequence analysis software package Version 7. Data base searches were done using the GenBank Genetic Sequence Data Bank, Release 81.0, 1994.

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