

Molecular cloning and mRNA localization of tomato pollen profilin

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Abstract

The actin cytoskeleton plays an important role in the growth of pollen tube. The actin-binding protein profilin could play a role in regulating the organization of the actin filaments. Using the RT-PCR technique, we isolated a cDNA clone (designated *LePro1*) encoding profilin from pollen grains of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker). Sequence analysis of the insert shows 87% similarity to tobacco *ntPro2*, 78% to timothy grass profilin, 77% to *Arabidopsis* *AthPRF4*, 77% to maize *ZmPro3*, and 73% to birch profilin. Both quantitative PCR and RNA gel blot analyses demonstrated that *LePro1* is expressed in a tissue- or cell-type specific manner in the tomato plant. *In situ* hybridization of 2 μ m thick anther sections using a non-radioactive labeling method reveals that *LePro1* is expressed only in pollen grains, with undetectable transcription in other parts of the anther or other organs. Phylogenetic analysis of amino acid sequences of 18 plant profilins indicates that two distinct profilin gene classes are present in higher plants. One is pollen-specific, another is constitutive. *LePro1* belongs to the former class.

Introduction

Actin and its associated proteins are fundamental elements of the cytoskeleton which together play an important role in plant cell morphogenesis, mitogenesis, mobility and other cellular processes [1, 22, 26, 27]. The actin cytoskeleton is composed of a network of actin filaments whose precise organization is regulated by a number of actin-binding proteins. One of them is profilin, a small (12–15 kDa) monomeric actin-binding protein. The functions that have been associated with the action of profilin, and may be temporally the spatially correlated, include: (1) actin monomer and filament end binding [28, 29]; (2) positive or negative control of actin nucleation and polymerization [2, 5, 12, 30]; (3) promotion of the exchange of ATP or ADP bound to actin for ATP [11, 14, 21]; (4) participation in the phosphoinositide secondary messenger signaling pathway [1, 10, 16, 17, 37].

Profilin was first identified in plants as an ubiquitous allergen from birch pollen [32, 33]. Later, cDNA clones encoding profilin were isolated from other plant species such as maize [28], timothy grass [34], wheat [23], tobacco [20], common bean [36] and *Arabidopsis* [4, 13]. Other than the interaction of profilin with actin and phospholipids, little is known about the biological role of plant profilin.

There is evidence in plants to suggest that profilin transcription and translation levels dramatically increase during pollen maturation and its tube growth [20]. We are particularly interested in understanding the biological function of profilin in pollen development and tube growth at the molecular level. Here, we report the isolation and characterization of a pollen-specific cDNA encoding profilin from tomato using polymerase chain reaction (PCR) and *in situ* hybridization techniques.

The nucleotide sequence data reported will appear in GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number U50159.

Materials and methods

Plant materials

Tomato (*Lycopersicon esculentum* Mill cv. Money-maker) seeds were germinated and grown under greenhouse conditions. Tissue collection for RNA isolation was as follows: pollen grains were collected by buzzing open flowers at the 1.4 cm length stage (about 2 days before anthesis) with a Squiggle Wiggle Writer toy (Hart Enterprises). Other parts of the flower and other organs were collected at the same stage. All plant tissues were frozen in liquid nitrogen immediately after collection.

RNA extraction and northern hybridization

Total RNA was extracted from pollen grains, anthers, ovaries, leaves, roots and stems using the RNeasy Plant Total RNA Kit (Qiagen) according to the manufacturer's protocol. Poly(A)⁺ RNA was isolated as follows. Frozen plant tissues were ground in liquid nitrogen with a mortar and a pestle till a fine powder was formed. The powder was then transferred to a beaker containing 0.1 M NaCl, 50 mM Tris-HCl pH 9.0, 10 mM EDTA, 2% sodium dodecyl sulfate (SDS) and 0.1 mg/ml proteinase K and homogenized in polytron for 2 min. The homogenate was shaken at 250 rpm for 15 min. After phenol extraction, the aqueous phase was transferred to a new tube, adjusted to 0.5 M NaCl, and then 0.5 g oligo-d(T) cellulose was added to 50 ml of the homogenate. The mixture was shaken at 250 rpm for at least 15 min to allow poly(A)⁺ RNA to bind to the cellulose. The oligo-d(T) cellulose was collected by centrifugation at 2000×g for 1 min, then loaded on column made in a 5 ml syringe with loading buffer containing 0.4 M NaCl, 10 mM Tris-HCl pH 7.5 and 1 mM EDTA. After washing with loading buffer, the poly(A)⁺ RNA was eluted with TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). RNA gel blots were performed according to Sambrook *et al.* [24]. Total RNA was blotted onto Hybond N membrane (Amersham) and cross-linked under UV light. It was then hybridized with the ³²P-labeled *LePro1* probe in a buffer containing 1% SDS, 1 M NaCl, 50% formamide and 10% dextran sulfate at 42 °C overnight. The membrane was then washed in 2× SSC (a combination of sodium chloride and sodium citrate) at room temperature for 2×5 min, then in 2× SSC and 1% SDS at 60 °C for 2× 30 min, and exposed on Fuji X-ray film at -70 °C overnight.

cDNA cloning by reverse transcriptase-polymerase chain reaction (RT-PCR)

For RT-PCR, 0.5 µg poly(A)⁺ RNA was used for synthesizing first-strand cDNA by AMV reverse transcriptase (Invitrogen). When the cDNA synthesis was complete, the RNA-DNA hybrids were denatured by heating at 95 °C for 2 min. The denatured cDNA was then directly used in the PCR amplification. Of the reverse transcription products 2 µl was added to 50 µl PCR solution containing 5 µl of 10× PCR buffer (Invitrogen), 1 µl of 100 mM dNTPs, and 2.5 U of *Taq* DNA polymerase (Life Technology). Two gene-specific primers, P1 (5'-ATGTCGTGGCAAACATATGTG) and P2 (5'-CTCAACCACCATGTTGCACTG) derived from *ntPro2* were synthesized and added to the PCR mixture at 1 µm each. The amplification was performed in a Thermal Cycler (Perkin Elmer Cetus) for 30 cycles with 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C. After the last cycle, the amplification was extended to 7 min at 72 °C. The amplified cDNA was then cloned into the pCRII plasmid vector using the TA Cloning Kit (Invitrogen).

The 3' rapid amplification of cDNA ends (RACE) was performed according to Frohman *et al.* [7]. A gene-specific primer, P3 (TCAAGGAGAACCTGGT-GCTG) derived from 231–251 of the *LePro1* sequence was synthesized for this purpose. An adapter primer, GGCCACGCGTCGAGTACT (17), and an amplification primer, GGCCACGCGTCGAGTAC, were commercially obtained from Pharmacia.

Nucleotide sequencing and data analysis

Nucleotide sequencing was carried out by a fluorescence cycle sequencing method using the new Ampli *Taq* polymerase FS enzyme (Life Technology). Both strands were sequenced and the sequence data were computerized using the Lasergene program (DNASTAR).

Plant tissue fixation, embedding, and sectioning

Plant materials were fixed in a fixative composed of 4% paraformaldehyde, 0.5% glutaraldehyde and 0.05 M PIPES (pH 6.8) for 2 h at room temperature. The fixed tissues were then dehydrated in a graded ethanol series containing dithiothreitol (DTT) (1 mM for 10–95% ethanol or 10 mM for 100% ethanol), then progressively infiltrated with butylmethac-

rylate/methylmethacrylate (4:1, v/v), 4–8 h each step, with three changes of 100% methacrylate, at least 24 h for the last change. Benzoin ethylether was added to the last change of methacrylate at 0.5% final concentration 4 h before embedding. The gaseous nitrogen was vigorously bubbled through the methacrylate mixture to displace oxygen. Embedding was done in gelatin capsules and the polymerization was carried out at 65 °C for at least 24 h. Polymerized blocks were stored at 4 °C until sectioning. 1–3 μ m thick sections were cut dry and then placed on Superfrost plus slides (Fisher Scientific) with a drop of diethyl pyrocarbonate (DEPC)-treated water. The slides were warmed 10–15 min on a slide warmer at 60 °C before processing *in situ* hybridization.

In situ hybridization

Sense and antisense RNA probes were synthesized by *in vitro* transcription of LePro1 cDNA cloned in pCRII (Invitrogen) using a digoxigenin (DIG) RNA Labeling Kit (Boehringer Mannheim). Methacrylate was removed from the sections by incubating the slides in acetone for 5–30 min, then gradually rehydrated. The sections were treated with 0.2 M HCl for 20 min, rinsed twice in 2 $\times$ SSC, each for 5 min, then treated with proteinase K for 30 min at 37 °C, rinsed with DEPC-water, followed by a treatment in 0.1 M triethanolamine (pH 8.0) for 5 min. The slides were transferred to a fresh 0.1 M triethanolamine containing 0.25% (v/v) acetic anhydride with gentle stirring for 10 min, and then rinsed twice in 2 $\times$ SSC, each time for 5 min. Prehybridization was carried out by applying 200 μ l prehybridization solution containing 5 $\times$ SSC, 2% block solution (Boehringer), 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 50% (v/v) formamide, 5% (w/v) dextran sulfate, denatured 0.5 mg/ml salmon sperm DNA and 0.25 mg/ml yeast tRNA on each slide, covered with coverslips and incubated for 2 h at 42 °C. After prehybridization, the solution was replaced by 100 μ l hybridization solution containing denatured DIG-labeled probes and the prehybridization solution. Hybridization was done at 42 °C overnight. Slides were then washed twice in 2 $\times$ SSC at room temperature, each time for 15 min, then in 2 $\times$ SSC and 0.1% SDS at 60 °C for 15 min. Immunological detection was performed according to Boehringer's protocol included with the kits. Briefly, DIG-labeled RNA was detected using an anti-DIG alkaline phosphatase conjugate. A subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phos-

phate and nitroblue tetrazolium salt produced an insoluble blue precipitate which visualized hybridization signals.

Results and discussion

Isolation of profilin cDNA from tomato pollen by RT-PCR

Out of many recombinant clones obtained, ten were randomly chosen for restriction mapping. Analysis of the digested plasmids showed that each clone contained an insert of the predicted size, and they all hybridized to the *ntPro2* probe, suggesting that all ten clones contained the same cDNA fragment. Sequence analysis of one of them, clone 1 showed that it contained a profilin cDNA fragment (designated LePro1-1) from the translation initiation codon to the amino acid position 121. Compared to *ntPro2*, it lacks only the last 10 amino acids at the C-terminus. This is to be expected since the selection of PCR primers was based on *ntPro2*. Nucleotide sequence homology search in GenBank showed that this cDNA fragment has a similarity of 87% to tobacco *ntPro2*, 78% to timothy grass profilin, 77% to *Arabidopsis* *AthPRF4*, 77% to maize *ZmPro3*, and 73% to birch profilin.

The missing part of the C-terminus and the 3'-untranslated region of LePro1 was amplified using 3'-RACE and cloned into the pCRII vector. One clone, designated LePro1-2, was sequenced and found to contain the 3'-untranslated region and an upstream 130 bp coding sequence including the missing part at the C-terminus. The 5' end of LePro1-2 overlapped with the 3' end of LePro1-1. A unique *SacII* site was located in the overlapping region of two clones, which permitted the identical digestion and subsequent religation of two fragments into one single clone, LePro1. The entire cDNA length is 625 bp containing a complete 399 bp open reading frame that encodes a polypeptide of 133 amino acids with a predicted molecular weight of 14.435 kDa and an isoelectric point of 4.978, and a 3'-untranslated region in which two putative polyadenylation signals are present at 89 and 180 bp upstream from the poly(A) tail, respectively. The alignment of the deduced amino acid sequence of profilins from pollen shows a widespread similarity among them (Figure 1). High levels of similarity between LePro1 and *ntPro2* (87%) indicate a close genetic relation between them as it is also the case between *MzPro3* and timothy grass profilin (82%). Most divergence

was found between *MzPro3* and *LePro1*. *Arabidopsis* pollen-specific profilin, *Ath PRF4* shows 76% similarity to *LePro1*. A putative G-actin-binding domain [25, 35] and a phosphatidylinositol 4,5-bisphosphate (PIP₂) binding domain [15] was located at 6–13 amino acid from the C-terminus and at 84–96 amino acid from N-terminus, respectively.

A phylogenetic analysis was done among the eighteen plant profilins sequences available in GenBank, using the Clustal method (DNASTAR). These plant profilins could be grouped into two clusters (Figure 2). Cluster 1 contains eight profilins including three *Arabidopsis* profilins (*At1–3*), one common bean profilin (*Pv*), three wheat profilins (*Ta1–3*) and one barley profilin (*Hv*). Cluster 2 contains ten profilins including three from maize (*Zm1–3*), one from timothy grass (*Pp*), one from birch (*Bv*), three from tobacco (*Nt1–3*), one from tomato (*Le*) and one from *Arabidopsis* (*At4*). Genes from dicotyledons were distinguished from monocotyledons and formed two subclusters, dicotyledons subcluster and monocotyledons subcluster in each cluster. Interestingly, all genes in cluster 1 were isolated from vegetative organs, and those in cluster 2 were from pollen or strongly expressed in pollen. Huang *et al.* [13] analyzed some plant profilin genes and have suggested two profilin gene classes (constitutive and pollen-specific) in plants. Our results with all plant profilin genes strongly supports this assumption.

Specificity of *LePro1* expression

The analysis of PCR products (Figure 3a) shows a different expression pattern among various organs and tissues. A strong band was found in both pollen grains and anthers, but no band or a very weak one was detected in other organs or tissues. When they were probed with *LePro1*, each PCR product showed an hybridization signal (Figure 3b), and the pattern was very similar to that in Figure 3a. This result suggests that the *LePro1* is expressed in an organ or cell-type-specific manner. A comparison of gene expression of *LePro1* at the mRNA level was also conducted between pollen and pistil using 3'-RACE. Pollen RNA showed a strong band with the expected size corresponding to the *LePro1*, while no band was found in pistil RNA, confirming that the *LePro1* was not transcribed in the gynoecium at anthesis (data not shown). Identical results were also obtained in RNA gel blot analysis. An abundance of *LePro1* transcripts was only detected in pollen grains and anthers, but not in other organs (Figure 4). We noticed that in both RT-PCR and RNA gel

blot analyses, the signal of the *LePro1* transcripts in anthers was less intense than that in pollen grains. To address the question of whether the signal that appeared in the anther RNA is derived from the entire anther (pollen grains and other parts) or only from pollen grains, we attempted to remove all pollen grains from the anther before RNA extraction without success. We therefore decided to resolve this problem by employing *in situ* hybridization. Our initial attempt to this end using paraffin-embedded anthers was also inconclusive since the positive signal in pollen was inconsistent although the rest of the anther exhibited no hybridization. This inconsistency of the signal in pollen was probably due to the 15–20 μ m thickness of the paraffin sections being too thick to cut through most of the pollen grains which are 15–20 μ m in diameter. To ensure that most pollen grains would be sectioned, we subsequently used the methacrylate embedding medium to obtain 2 μ m sections of anthers. A very strong hybridization signal was found in pollen grains but not in other parts of the anther when the antisense probe was used for *in situ* hybridization (Figure 5a–c). The control hybridization with the sense probe yielded no signal (Figure 5d). The *in situ* hybridization results clearly indicate that the *LePro1* is expressed in a pollen-specific manner in tomato.

Prospect and significance

Using *in situ* hybridization, we have demonstrated the specificity in expression of one profilin gene, *LePro1*, in tomato pollen grains. Although a few investigators have reported that several isoforms of profilin were specifically expressed in pollen grains of maize [28], tobacco [20] and *Arabidopsis* [4, 13], no mRNA localization has been done before. Our *in situ* hybridization results have clearly demonstrated that *LePro1* is strongly expressed only in pollen grains. A number of pollen-specific genes have been identified during the past few years [19]. Some of them are expressed in the early stage of microsporogenesis and the expression is then reduced during pollen maturation ('early pollen genes'). Others do not start their expression until mitosis and the expression increases dramatically during pollen maturation ('late pollen genes') [18]. Our unpublished data indicate that *LePro1* transcripts can be detected only from the stage when the flowers are about 1.2 cm long. This stage is about four days before anthesis in cv. MoneyMaker. The transcripts accumulation became more abundant as the pollen matured. Thus the *LePro1* gene seems to belong to 'late pollen

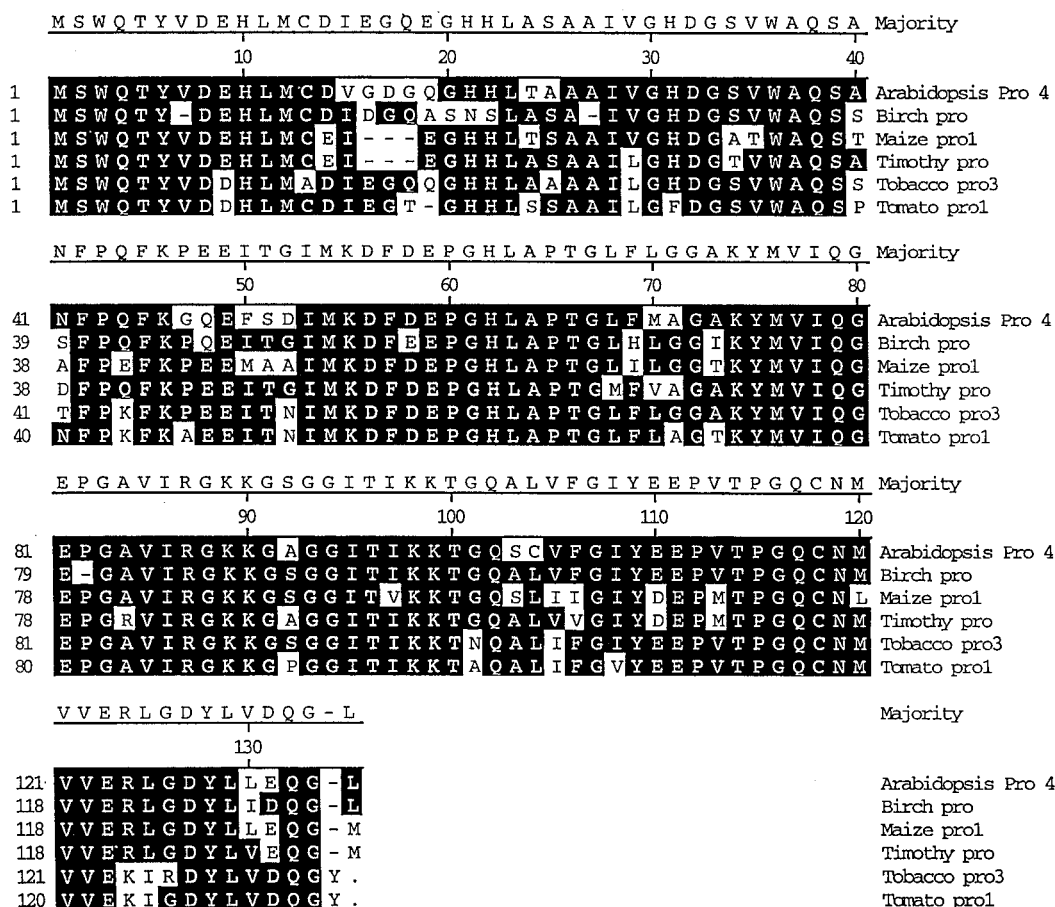


Figure 1. Alignment of deduced amino acid sequences of profilins isolated from the pollen of different plant species, using the Clustal method of the Lasergene program. The consensus residues are shaded with solid black. The protein sequences are: Arabidopsis pro4, deduced from the pollen-specific profilin, Ath PRF4 [13]. Birch pro, deduced from birch pollen profilin cDNA clone 4 [33]. Maize pro1, deduced from maize pollen profilin cDNA ZmPro1 [28]. Timothy pro, deduced from timothy grass pollen cDNA clone 3 [34]. Tobacco pro3, deduced from tobacco pollen profilin cDNA ntpro3 [20]. Tomato pro1, deduced from the tomato pollen-specific profilin cDNA, LePro1.

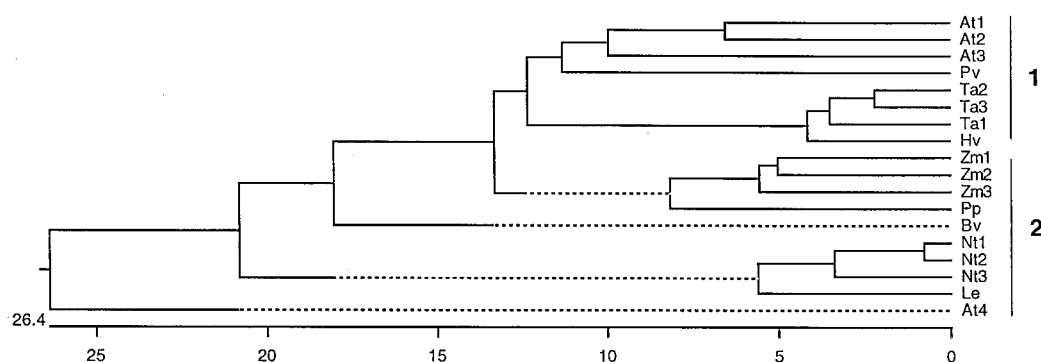


Figure 2. Phylogenetic tree of plant profilins, constructed by comparing the amino acid sequences of 18 plant profilins using the Cluster method (DNASTAR): At1-4, *Arabidopsis thaliana*; Pv, *Phaseolus vulgaris*; Ta1-3, *Triticum aestivum*; Hv, *Hordeum vulgare*; Zm1-3, *Zea mays*; Pp, *Phleum pratense*; Bv, *Betula verrucosa*; Nt1-3, *Nicotiana tabacum*; Le, *Lycopersicon esculentum*. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences. Units indicate the number of substitution events. Dotted lines indicate a negative branch length (introduced by averaging the tree).

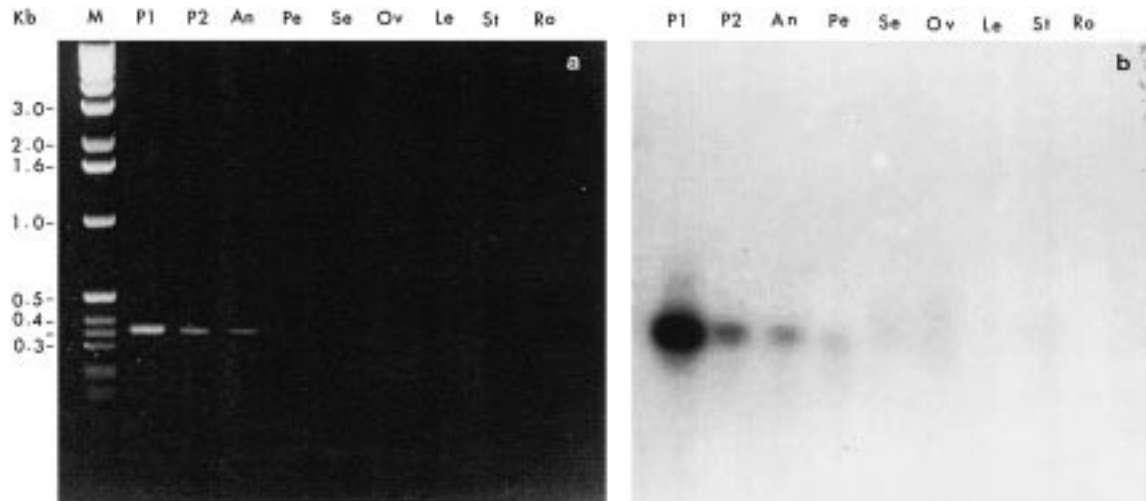


Figure 3. RT-PCR products amplified from tomato RNA using profilin-specific primers. The lanes are: 1 kb DNA ladder (M), pollen grains (P1 and P2), anthers (An), petals (Pe), sepals (Se), ovaries (Ov), leaves (Le), stems (St) and roots (Ro) using profilin-specific primers—0.5 μ g of total RNA was used for reverse transcription except for lane P1 where 0.2 μ g poly(A)⁺ RNA was used. a, 15 μ l of each PCR product was loaded on 1.4% agarose gel and stained with ethidium bromide; b, the same gel presented in panel a was blotted onto Hybond N membrane and probed with ³²P-labeled *LePro1*, showing a strong hybridization with the PCR product for pollen grains and anthers.

genes'. In tobacco, no profilin was detected up to the early binucleate stage, but very strong accumulation of profilin transcripts were present in mature and germinated pollen [20]. Similar results were also obtained in maize, in which three pollen- or anther-specific profilin genes (*ZmPro1*, *ZmPro2* and *ZmPro3*) could only be detected in mature and germinated pollen grains [28].

The reason for the presence of profilin in relatively large quantities in mature pollen can only be speculated. The presence of profilin is well correlated with actin-dependent tip growth of fibroblast cells [9] and the acrosomal process of sperm [30]. Similarly, profilin may be required for the proper modulation of the actin cytoskeleton involved in the pollen germination and the tube growth. Animal profilin is known to form a 1:1 complex with the monomeric G-actin [3] and hence can act as a G-actin-sequestering protein. In ungerminated pollen grains, profilin could sequester G-actin, and under proper stimulation such as pollen hydration and/or germination, rapid polymerization could be made possible by an unknown desequestering mechanism. For example, pollen grains of pear lack filamentous actin in their dehydrated state but rapid polymerization of actin occurs within minutes of hydration [31].

Recombinant plant profilin has been shown to bind *in vitro* to the PIP₂ as well and inhibit phospholipase C γ [6], the first enzyme in the phosphoinositide-based-

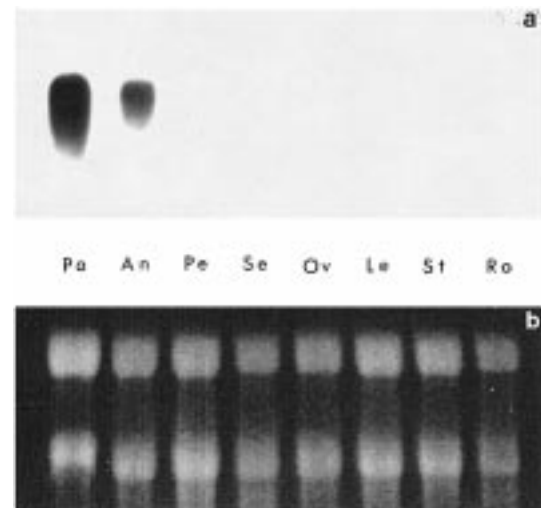


Figure 4. Gel blot analysis of RNA from tomato pollen grains (Po), anthers (An), petals (Pe), sepals (Se), ovaries (Ov), leaves (Le), stems (St) and roots (Ro). a. The RNA was blotted onto Hybond N membrane (Amersham) and probed with ³²P-labeled *LePro1*. A strong hybridization signal was detected in both pollen grains and anthers, but not in other organs. b. The total RNA was purified by a RNeasy Plant total RNA kit (Qiagen), and the quality of RNA was examined by loading the same amount of RNA from the respective organs/parts in each lane, and staining the gel with ethidium bromide.

cell signaling cascade. Thus pollen profilin could participate in actin cytoskeleton modulation as well as in

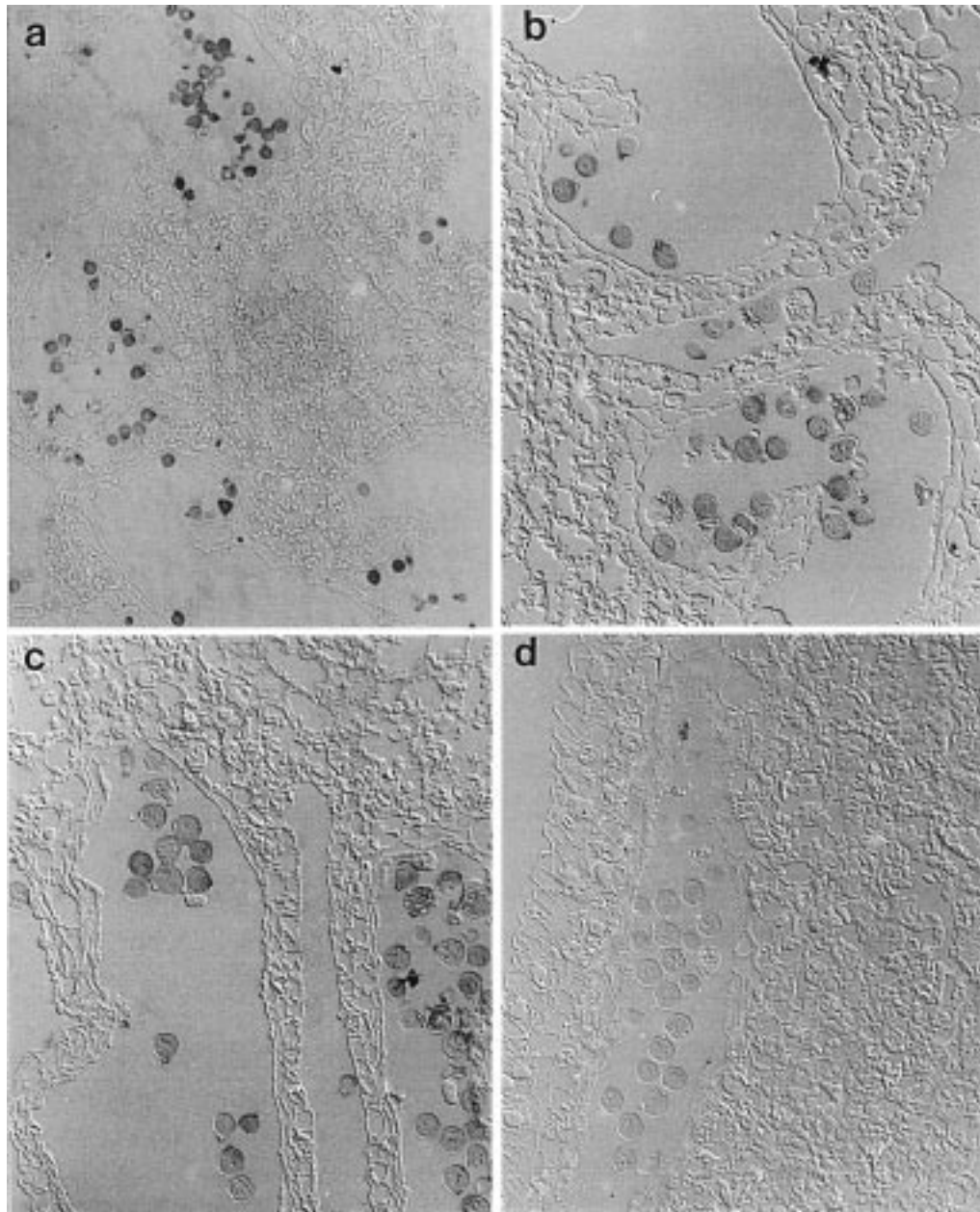


Figure 5. *In situ* hybridization of non-radioactive DIG-labeled antisense (a–c) or sense (d) *LePro1* to anther sections from 1.4 cm long flowers (two days before anthesis). a. Bright field image showing transverse section of an anther, and specific hybridization signal in the pollen grains ($\times 38$). b. Higher magnification of the same anther section from a (Nomarski DIC optics) ($\times 98$). c. Longitudinal section of an anther from a different flower showing pollen-specific hybridization signal (Nomarski DIC optics) ($\times 98$). d. Longitudinal section of an anther showing the absence of signal in the sense control (Nomarski DIC optics) ($\times 98$).

signaling system during pollen germination and tube growth.

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