

Molecular cloning and characterization of profilin from tobacco (*Nicotiana tabacum*): increased profilin expression during pollen maturation

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Abstract

Profilin has recently been identified as an actin-binding protein in higher plants. A cDNA coding for tobacco profilin, which shared an average sequence identity of 75% with other plant profilins, was isolated from a tobacco pollen cDNA library by antibody screening. Tobacco profilin was expressed in *Escherichia coli* and purified by affinity to poly-(L-proline) Sepharose. A rabbit antiserum was raised against recombinant tobacco profilin and used to estimate the amount of profilin expressed in different tobacco tissues. Profilin can be detected in different somatic tissues, but the expression is 50–100 fold higher in mature pollen. Immunofluorescence and confocal laser scanning microscopy showed a homogeneous distribution of profilin in the cytoplasm of *in vitro* cultured pollen grains and pollen tubes of tobacco whereas some growing pollen tubes were stained more intensively at their tip. A possible role of pollen profilin as a developmentally upregulated microfilament precursor in mature pollen is discussed.

Introduction

Profilins represent actin-binding proteins which are linked to the signal transduction cascade via the phosphoinositide pathways in eukaryotes [27]. Recently profilins have been discovered as plant allergens [44, 45, 46, 49] and shown to function as actin-binding proteins also in higher plants [38, 47]. In addition to their role as actin-

binding proteins, plant profilins bind to PIP₂ (phosphatidylinositol 4,5-bisphosphate) and via this mechanism inhibit phospholipase C [11]. As yet, plant profilins were characterized from birch (*Betula verrucosa*) [45], timothy grass (*Phleum pratense*) [48] and maize (*Zea mays*) [38]. Profilins from mono- and dicotyledonic plants show an average sequence identity of 75% compared to an average low sequence identity of 35% with

other eukaryotic profilins. Despite this low degree of sequence identity, plant profilins efficiently bind to plant and mammalian actins *in vitro* [47] and *in vivo* [34, 39]. The ability to sequester monomeric actin seems therefore to represent one of the important functions of profilins in plants. To gain more information about the biological functions of plant profilins, we isolated cDNAs coding for tobacco profilin and investigated the expression of profilin in *Nicotiana tabacum*, which represents a well established plant system for the study of pollen maturation and development as well as pollen germination [4, 5]. Small amounts of profilin could be detected in leaves and seeds and early in pollen development whereas most profilin was expressed in mature and germinated tobacco pollen.

By immunofluorescence profilin could be detected homogeneously distributed in the cytoplasm of resting pollen compared to a more intense staining observed in the tips of germinated tobacco pollen tubes. Possible functions of plant profilins during pollen germination are discussed in view of the above findings.

Materials and methods

Materials, plants, antibodies

Tobacco plants (*Nicotiana tabacum* cv. Petit Havanna) were raised in climate chambers under controlled conditions. Pollen was collected at various stages of development, i.e. uninucleate microspores, mid-binucleate pollen prior to starch formation, embryogenic pollen produced by a 7-day starvation treatment of mid-binucleate pollen [5], mature, dry pollen and germinated pollen that had been incubated in Brewbaker-Kwack medium [8]. Tobacco suspension cultures were grown according to Murashige and Skoog [30].

An oligonucleotide complementary to plant 18S ribosomal RNAs (5'-CTAGAGCTAATACGTGCAACAAACCCCGAC-3') was used for northern blot control hybridizations. All antibodies which were used are described [47]. The antibodies used for immunohistochemistry were purified by protein G affinity using Mab traps

(Pharmacia, Uppsala, Sweden). The contents of purified immunoglobulins in all antibody preparations was comparable according to photometry (280 nm). Pollen and plant tissues were harvested into liquid nitrogen freshly from tobacco plants (cv. Petit Havanna) grown in a glasshouse.

Construction of the tobacco pollen cDNA library, immunoscreening and characterization of a cDNA clone coding for tobacco profilin

Total RNA was prepared from mature tobacco pollen harvested from cultivated tobacco plants (cultivar Petit Havanna) according to [2], poly(A)⁺ RNA was enriched by oligo-dT cellulose chromatography and reversely transcribed into cDNA. An expression cDNA library was prepared in phage λ ZAP (Stratagene, La Jolla, CA). 2000000 phage were screened with serum IgE from a profilin allergic patient as described [45]. More than 150 immunopositive clones were obtained which were further tested with profilin-specific antibodies [47]. After *in vivo* excision plasmid pBSk⁺ containing the cDNA of clone 4 was subjected to DNA sequencing [35] using ³⁵SdCTP (NEN, Stevenage, UK) and a T7 sequencing kit (Pharmacia, Uppsala, Sweden).

Expression and purification of recombinant tobacco pollen profilin, production of a rabbit anti-recombinant tobacco profilin serum

Plasmid pBSk⁺ containing the clone 4 cDNA was used to express recombinant tobacco profilin. Transformed *E. coli* XL-1 Blue were grown in LB containing 100 μ g/ml ampicillin to an optical density (600 nm) of 0.3 until IPTG was added to a final concentration of 3 mM. *E. coli* were grown further for 4 h, harvested by centrifugation and washed twice in 0.9% sodium chloride. Pellets were then homogenized and high-speed supernatants were incubated with poly(L-proline) Sepharose as described [25, 46]. Bound profilin was eluted as 2 M and 6 M urea fractions as described [46]. More than 95% pure tobacco

profilin was used to immunize a rabbit with complete Freund's adjuvant. The rabbit was boosted twice with antigen and incomplete Freund's adjuvant. The antiserum which was tested for reactivity with protein extracts from tobacco pollen and pollen from different monocotyledonous and dicotyledonous plants strongly reacted with bands at around 14 kDa molecular mass.

Northern blot analysis and immunoblotting

RNA was prepared from pollen and tobacco tissues according to Ausubel *et al.* [2]. 20 µg of total RNA were separated by denaturing agarose gel electrophoresis and transferred onto nylon membranes [2]. RNA was UV-cross-linked to the membrane which was hybridized with the random hexamer ³²P-dCTP (NEN, Stevenage, UK) primed [12] complete cDNA of clone 4. After autoradiography the same membrane was stripped in 0.1% SDS at 95 °C, checked by autoradiography and subsequently hybridized with an oligonucleotide complementary to 18S plant ribosomal RNAs. The oligonucleotide was 5' end-labelled with ³²γ-ATP (NEN, Stevenage, UK). Plant protein extracts were prepared by grinding the tissues under liquid nitrogen to a fine powder which was extracted as described [18a]. According to protein determinations [7], comparable amounts of proteins were loaded onto 12.5% denaturing SDS-PAGE [19] and proteins were then electroblotted to nitrocellulose (Schleicher & Schuell, Dassel, Germany) according to [43]. Membranes were incubated with rabbit or mouse monoclonal antibodies and ¹²⁵I-labelled secondary antibodies as described [47]. Autoradiographies were also subjected to densitometrical analysis with a densitometer (Hirschmann, Unterhaching, Germany) to estimate the intensity of the bands.

Immunofluorescence and confocal laser scanning

Pollen was collected from *Nicotiana tabacum* plants. About 5 mg of mature pollen was hydrated

for 1 h in a moist chamber and subsequently allowed to germinate in 25 ml Erlenmeyerflasks containing 2 ml medium [8] prepared with 10% sucrose. The Erlenmeyers were rotated for 1 h or 3 h at 25 °C. Cultured pollen was fixed for 30 min in 2% paraformaldehyde (pH 7.0) freshly dissolved in phosphate-buffered saline (PBS) supplemented with 5% sucrose-Brewbaker-Kwack medium, under continuous agitation. Pollen was concentrated by low-speed centrifugation and spontaneous sedimentation and fixative was discarded. After 3 rinsings in PBS, cell walls were partly digested in 1% cellulysin for 3 min in the dark and rinsed again three times in PBS. In some experiments, cells underwent an additional permeabilization with 1% Triton X-100 for 3 min and another 3 rinsings in PBS. Pollen was then incubated overnight in a 1:10 diluted affinity-purified RP1 or RP2. After 3 rinsings in PBS the cells were incubated with a 1:40 FITC-labelled sheep anti-rabbit for 1 h in the dark and washed again with PBS. Cells were mounted on glass slides in inclusion medium containing citifluor (Agar, Stansted, Essex, UK). The results are based on 6 separate experiments, in which the pollen tubes were exposed to both the primary and the secondary antibody, and additional series of control experiments, in which the first or secondary antibody was omitted or a 1:10 diluted affinity-purified normal rabbit serum was used which contained similar amounts of immunoglobulin as the specific antibodies. Parallel experiments (10 in total) have been carried out with pollen of *Lilium longiflorum*. Altogether several hundreds of tobacco pollen grains and tubes have been screened first by conventional epifluorescence microscopy. Preparations were analysed in epifluorescence mode on an Optiphot Nikon microscope using a 420–490 nm excitation filter, a 510 nm dichroic mirror and a 520 nm bypass filter. In addition, optical sections and three-dimensional reconstructions (maximal projections of x-y sections) of the fluorescence labelling pattern of pollen grains and tubes were made with a MRC-500 BioRad (Hemel, Hampstead, UK) Confocal Laser Scanning Microscope (CLSM) used with the following settings: the 488 nm

10 mW line of an argon laser dimmed with 1 or 3% neutral-density filters, a BHS fluorescence filter (dichroic reflector 510 nm LB, barrier filter CG 515 nm) in channel 1, maximum black level 6.2, maximum gain 4.8, moderately closed pin-hole, stepper motor 1 μ m. Non-confocal transmission microscopy images of the labelled cells were obtained using fibre optics connected to channels 2 of the CLSM. Photographs were taken from the monitor on Kodak TMX-100 films (Kodak, Heidelberg, Germany).

Results

Isolation and characterization of a cDNA clone coding for tobacco profilin

A cDNA (clone 4) coding for an isoform of tobacco profilin was isolated from a tobacco pollen expression cDNA library by antibody screening. The cDNA of clone 4 has a length of 653 nucleotides and the open reading frame of 405 nucleotides codes for a protein of 14577 Da deduced molecular weight. No N-glycosylation sites could be found in the deduced amino acid sequences of the clone. When compared with known profilin amino acid sequences an average sequence identity of 75% with profilins from birch (*Betula verrucosa*), timothy grass (*Phleum pratense*) and maize (*Zea mays*) (Fig. 1) and a comparable lower average sequence identity of 35% with other profilin sequences could be found. When the cDNA sequence of tobacco profilin was compared with the cDNA sequences in GenBank a 72% sequence identity spanning almost the complete coding region with an unpublished *Arabidopsis thaliana* cDNA clone (accession number: T04185 submitted by T. Newman) was found.

Expression and purification of recombinant tobacco pollen profilin; production of a rabbit anti-tobacco profilin antiserum

The cDNA of clone 4 was expressed in pBSk +, *E. coli* XL-1 Blue using 3 mM IPTG [37]. *E. coli*

	1		50
tobacco	MSWQTYVDDHLMADIEGQQGHHAAAAAILGHDGSVWAQSSTFFPK		
birch	-----E---C---D---GEELA-S- -V-----S--Q		
timothy	-----E---CE--- -S-----T---AD--Q		
ZmPro1	-----E---CE--- -TS---V---AT---TA--E		
ZmPro2	MSDRAK---A---E---CE--- -V-----AA---TA--E		
ZmPro3	-----E---CE--- -SS---V---A---TA--Q		
	51		100
tobacco	FKPEETINIMKDFDEPGHLAPTGLFLGGAKYMWIQGEPGAVIRGKKGSGG		
birch	---Q---G---E-----H---I-----A-----		
timothy	-----G-----M-VA-----R-----A--		
ZmPro1	---MAA-----I---T-----		
ZmPro2	--T-DMA-----PT-----		
ZmPro3	---M---I-----F---I---PT-----		
	101		140
tobacco	ITIKKTNQALIFGIYEFPVTPGQCNMVVEKIGDYLVDDQGY		
birch	-----G---V-----I---L		
timothy	-----G---VV---D---M-----RL---E---M		
ZmPro1	--V---G---S---I---D---M-----L---RL---LE---M		
ZmPro2	--V---G---VV---D---M-----RL---LE---M		
ZmPro3	--V---G---VI---D---M-----RL---E---L		

Fig. 1. Comparison of the tobacco pollen profilin amino acid sequences with other plant profilin sequences. The deduced amino acid sequence of tobacco profilin was compared with profilin sequences from birch (*Betula verrucosa*) [45], timothy grass (*Phleum pratense*) [48] and maize profilin isoforms ZmPro1, ZmPro2, ZmPro3 [38]. Amino acids are displayed in single letter code, identical amino acids are indicated by lines and gaps were added to ensure maximal fit of the sequences. The nucleotide sequence and deduced amino acid sequence of tobacco profilin has been submitted to the EMBL/SWISS-Prot library under the accession number X82120.

were harvested and recombinant tobacco pollen profilin was purified by affinity to poly-(L-proline) Sepharose as described [26]. Figure 2 shows that pure profilin (99%) was obtained which migrates slightly above 14 kDa in SDS-PAGE. The protein was tested with antibodies raised against different plant profilins. All polyclonal antibodies showed extensive cross-reactivities with recombinant tobacco profilin [47]. A sample of 200 μ g purified recombinant tobacco profilin was used to immunize a rabbit using Freund's complete adjuvant to generate a rabbit anti-tobacco profilin antiserum (RP 5). The obtained RP 5 antiserum showed extensive cross-reactivity with profilins from different tissues (leaves, roots and pollen) of monocotyledonous and dicotyledonous plants (data not shown).

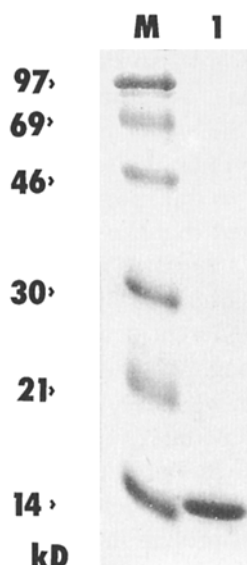


Fig. 2. Purification of recombinant tobacco profilin by poly-(*L*-proline) affinity chromatography. Tobacco profilin was expressed in plasmid pBSK + /*E. coli* XL-1 Blue 1. Total *E. coli* proteins incubated with poly-(*L*-proline) Sepharose and eluted in subsequent steps using 2 M and 6 M urea. Lane 1 shows ca. 5 μ g of recombinant tobacco pollen profilin derived from the 6 M urea elution which is more than 95% pure. Lane M is the molecular weight marker. Recombinant tobacco pollen profilin migrates slightly above the 14 kDa lysozyme band.

Different expression of profilin in somatic tobacco tissues and during pollen development

To compare the amounts of profilin expressed in pollen and other tobacco tissues northern blot and western blot analysis was performed. Western blots containing comparable amounts of proteins from mature dry pollen, seeds and leaves were probed with a rabbit anti-tobacco profilin serum and a mouse monoclonal antibody raised against chicken gizzard actin, respectively (Fig. 3A). A normal rabbit serum which was used as a negative control gave no signals (data not shown). According to the densitometrical analysis of the bands, the profilin content of pollen proteins was a 100-fold higher than that seed or leaf proteins. Comparable results were obtained with polyclonal antisera raised against celery root profilin, against recombinant birch pollen profilin and against a synthetic peptide derived from the C-terminus of birch profilin (data not shown)

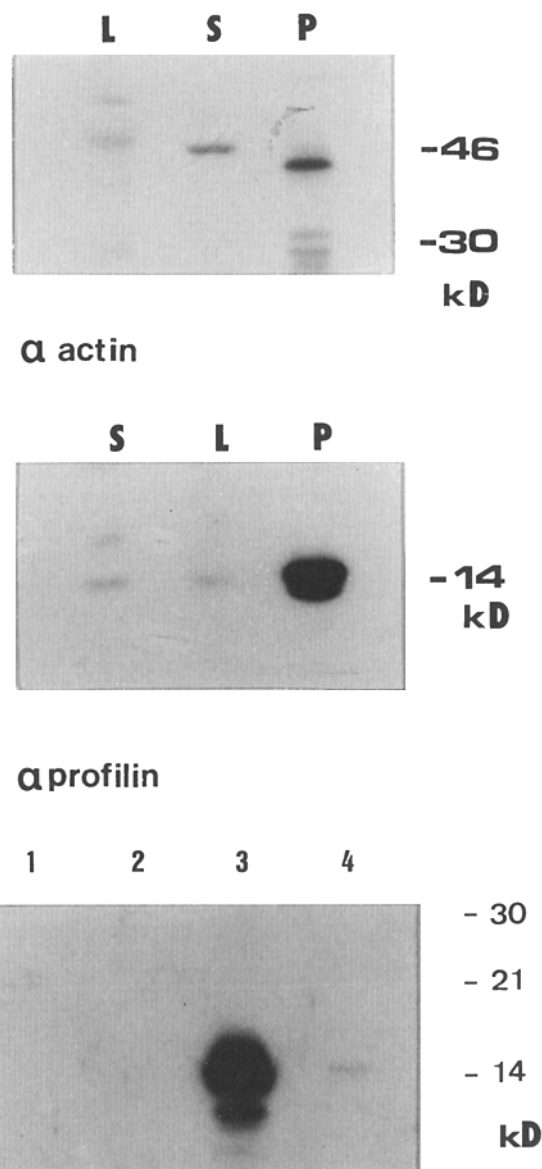


Fig. 3. A (top and middle). Detection of profilin and actin in different tobacco tissues. Comparable amounts of proteins from seeds (lane S), leaves (lane L) and pollen (lane P) were blotted to nitrocellulose. Membranes were incubated with the rabbit anti-recombinant tobacco profilin antiserum (α -profilin) and with a mouse anti-chicken gizzard actin antibody (α -actin). B (bottom). Detection of profilin in tobacco pollen during maturation and germination. Profilin could be detected in mature pollen (lane 3) and in germinated pollen (lane 4). No profilin could be found in earlier developmental stages (lane 1, microspores; lane 2, binucleate pollen). The mature pollen (lane 3) shown in B was at a comparable stage as the pollen (lane P) in A.

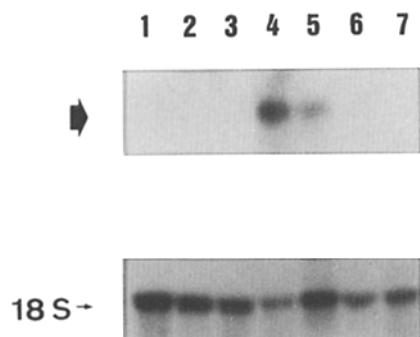


Fig. 4. Expression of the proflin transcript in different tissues and pollen of tobacco 20 μ g RNA from tobacco microspores (lane 1), mid binucleate pollen (lane 2), embryogenic pollen (lane 3), mature (lane 4) and germinated pollen (lane 5), leaves (lane 6), and ovaries (lane 7) were separated by denaturing agarose gelelectrophoresis, transferred to a nylonmembrane and hybridized with the complete tobacco pollen proflin cDNA (upper part). Hybridization with the proflin transcript was found at 700 nucleotides (indicated by an arrow) in RNA of mature and germinated pollen under stringent conditions ($0.75 \times$ SSC, 0.1% SDS, 45 °C). As shown below, the same membrane was hybridized with an oligonucleotide complementary to 18S plant ribosomal RNAs to ensure that comparable amounts of intact RNA were transferred to the membrane.

which excludes the possibility that the different intensity of proflin staining might be due to an

isotype-specific reaction of the used antiserum. Pollen proteins are also richer in actin than leaf and seed proteins. According to the densitometrical analysis of the bands, mature pollen seems to contain more proflin than actin which seems not only to be due to the specificity of the antibody. It was also noted that the actin bands in pollen, leaves and seeds migrated at slightly different molecular weight which is likely explained by the expression of different actin isoforms in the tissues [28]. During early stages of pollen development no proflin was detected, whereas in mature pollen large amounts of proflin were found (Fig. 3B). Much less proflin could be detected in germinated tobacco pollen which is most likely due to a loss of proflin into the pollen germination medium. We have noted already earlier that large amounts of proflin can be found in supernatants after pollen hydration [51].

In parallel to the western blots it is demonstrated at the mRNA level that proflin transcripts could be detected only in mature and germinated tobacco pollen whereas no transcripts were found during early pollen development and in leaves or ovaries (Fig. 4). The same membrane which was probed with the complete proflin cDNA of clone

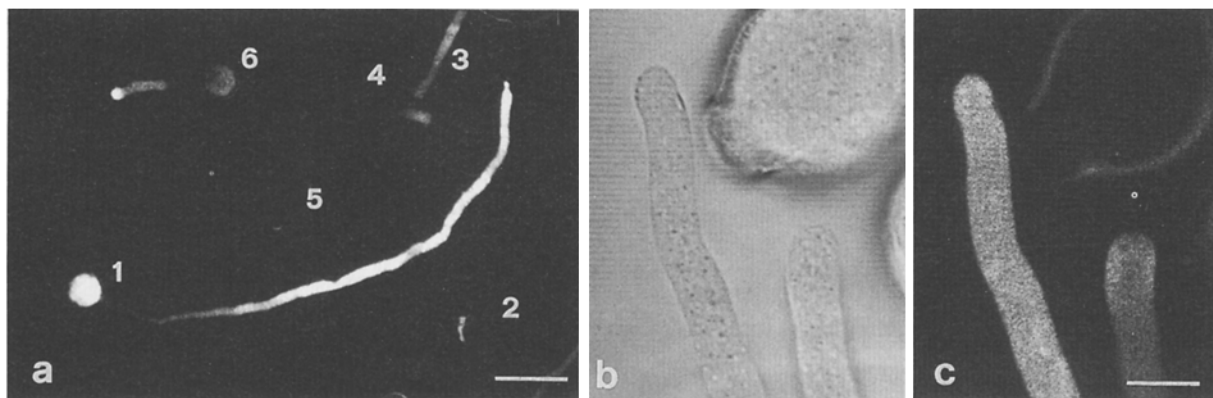


Fig. 5. Localization of proflin in resting and germinated tobacco pollen by immunofluorescence and confocal laser scanning microscopy. Illustration of the variability of the immunofluorescence labelling pattern obtained with a rabbit anti-proflin antibody on pollen tubes of *Nicotiana tabacum* grown *in vitro*. a. Cell 1 in a is strongly labelled. The tip of the pollen tube is lightly opened. Cell 3 is evenly moderately labelled. Cell 4 and 6 are predominantly stained in the apex of the tube. Cell 2 and 5 are not labelled. The bar represents 50 μ m. b. Transmission light image. c. Corresponding three-dimensional projection of optical sections of the immunofluorescence labelling of two pollen tubes and one germinated grain. The pollen tube left is evenly stained at the level of the cytoplasm, but the other pollen tube shows labelling in the tip region only. The pollen grain is unlabelled. The bar represents 10 μ m.

4 was subsequently hybridized with an oligonucleotide complementary to 18S plant ribosomal RNAs to ensure that comparable amounts of intact RNA had been transferred to the membrane.

Immunolocalization of profilin in activated and germinated tobacco pollen

Incubation of fixed *in vitro* germinated tobacco pollen tubes with two different rabbit anti-profilin antisera, RP1 and RP2 [47], and a FITC-conjugated polyclonal antibody repeatedly revealed fluorescent labelling of germinating pollen grains (after 1 h of culture) and germinated pollen grains (3 h of culture) and their pollen tube of several hundred micrometers length. The labelling was essentially at the level of the cytoplasm, but varied in intensity and distribution (Fig. 5). Some cells were evenly moderately labelled, whereas others were brightly stained, especially at the apex of the pollen tube. Part of the cell population, mostly ungerminated pollen grains, was not stained at all. Omission of the primary or secondary antibody or preincubation with serum resulted in absence of staining (data not shown). Concordant observations were obtained with pollen of *Lilium longiflorum* (E. Pierson, unpublished data).

Discussion

Profilin has been described as an actin binding protein in eukaryotes [10, 18, 33] which is also involved in the interaction with PIP₂ [13, 15, 22, 23, 24] and may have functions in signal transduction [1, 15, 27, 50]. In *Thyone* sperm profilin was described to be involved in the acrosomal reaction and it was suggested that profilin controlled actin polymerization might be involved in this process [41, 42].

Profilin was unknown in plants until it was discovered as an important plant allergen [44, 45, 46, 49] which is capable of inducing allergic symptoms such as rhinitis, conjunctivitis and

bronchial asthma in Type I allergic patients. Meanwhile profilin is also established as an actin-binding protein in higher plants [38, 47] and evidence has accumulated that plant profilins similar to other eukaryotic profilins, can bind PIP₂ [11]. Despite the known interactions of profilins with actin and phospholipids, little is known about the biological roles of plant profilins.

Profilin was found to be present in pollen of many different monocot and dicot plants [38, 45, 46, 47]. In this study a cDNA was isolated which codes for profilin from a tobacco pollen expression library. The cDNA of clone 4 shared an average sequence identity of 75% with other plant profilins compared to a rather low average sequence identity of 35% with other profilins. Although plant, mammalian and lower eukaryotic profilins share a low sequence identity it was found that plant profilins can bind to mammalian actins. The profilin C-terminus is critically involved in this interaction [47] although the observed sequence homology between plant and mammalian profilins in this region is rather low. The analysis of the crystalline structure of profilin- β -actin confirmed these findings by indentifying the carboxy-terminal helix (H4) as a part of the primary contact region [36].

To study profilin expression in different plant tissues and during tobacco pollen development a polyclonal rabbit antiserum was raised against recombinant tobacco profilin. Although profilin could be detected in all plant tissues with this antibody and other anti-profilin antibodies the relative content of profilin was about a 100-fold higher in protein samples of mature pollen.

In other organisms evidence has already accumulated regarding a specific expression of profilin in different tissues and during development [6, 17] and for maize a tissue specific expression of profilin isoforms was demonstrated [38].

The finding that only small amounts of profilin can be detected in somatic tissues whereas large amounts can be found in mature pollen with different polyclonal antibodies which were raised against root (RP1) or pollen profilins (RP2, 5) argues for a developmental upregulation of profilin expression in mature pollen. Profilin expres-

sion is highly regulated in pollen. No profilin could be detected up to early binucleate stages but a very strong increase occurred at a late stage of pollen development. Similar results were obtained at the RNA level with a probe spanning the complete coding and non-coding region of tobacco profilin. Profilin was expressed at high levels in mature and *in vitro* germinated pollen whereas no profilin could be detected in early pollen development and only very low amounts of profilin were stained with antibodies in other tobacco tissues.

In ungerminated pollen grains of different plant species, including *Nicotiana tabacum*, rhodamine-phalloidine staining or transmission electron microscopy have revealed the presence of large amounts of microfilaments in the form of spicules, annular structures or crystalline fibrillar masses (reviewed by Pierson and Cresti [32]). It has been suggested that these aggregations constitute a storage form of actin that progressively becomes functional during pollen germination and pollen tube development, which is characterized by the rapid growth of the tube and its vigorous cytoplasmic streaming. The formation of profilactin complexes in maturing pollen may be another way through which pollen accumulates actin in anticipation of the subsequent phase of high cytoplasmic activity. This assumption meets our observation that profilin is tremendously up-regulated in maturing tobacco pollen.

By means of immunofluorescence and confocal laser scanning microscopy profilin could be detected continuously distributed in the cytoplasm of activated pollen as well as in germinated pollen grains and in pollen tubes. Some pollen tubes showed a more intensive staining at their tip. Although the staining pattern must be considered with caution because profilin represents an easily soluble protein which might be dislocated during aqueous chemical fixation [29], these results are quite consistent with studies done by immunoelectron microscopy using anhydrous fixation techniques which show a similar distribution of the protein [16, 51]. The more intense staining in tips of some of the pollen tubes might be interpreted either as accumulation of profilin there or indicate

a dissociation of profilactin complexes because all of the antibodies used cannot bind to profilin when it is complexed to actin [47].

An important role attributed to profilin is the capacity to promote actin polymerization and filament stabilization by catalyzing actin nucleotide exchange (reviewed in [1, 14]). Several arguments support the idea that profilin assumes such a function in the tip of growing pollen tubes. First, our immunofluorescence data show a high concentration of profilin in the tip of part of the cell population. Second, pollen tubes contain dense arrays of longitudinally oriented actin filaments (reviewed in [20, 31, 32, 40]), which most likely are preferentially assembled in the tip region, since pollen tubes are exclusively tip growing cells. Furthermore, the concept is in agreement with the observation that in fibroblasts profilin is markedly concentrated in those areas which display high dynamics of the actin filament system [9].

In view of this and the fact that profilin is developmentally upregulated in mature tobacco pollen it appears likely that profilactin may fulfill a specialized function before and during pollen germination, probably as a microfilament precursor. The fact that profilin is more abundant in pollen than actin and recent data which provide evidence that profilin may bind two actin molecules [36] supports however the concept that profilin may in addition to actin also interact with different other agonists such as PIP_2 and other as yet unknown partner proteins.

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