



Identification of *pronp1*, a tobacco profilin gene activated in tip-growing cells

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Abstract

In plant cells, several cellular processes depend on rapid reorganization of a dynamic network of actin cytoskeletal elements in response to internal and environmental stimuli. Profilins, ubiquitous eukaryotic actin monomer-binding proteins with highly conserved three-dimensional structures, regulate the actin cytoskeleton and are considered to link the microfilament system with signal transduction pathways. Plant profilins have been grouped into two distinct classes, gametophytic (pollen-specific) and sporophytic. Here we report the isolation of a profilin gene that seems to be activated during tip growth of specialized cells of gametophytic as well as sporophytic origin. Identification of a genomic DNA clone containing a tobacco profilin gene, *pronp1*, and analysis of the *pronp1* promoter-*uidA* fusion gene in transgenic *Nicotiana tabacum* plants revealed a prominent expression of *pronp1* in mature pollen and elongating pollen tubes and significant activity in root hairs of developing seedlings. This expression pattern was distinct from that of any other profilin gene isolated so far. *Pronp1* thus represents a unique profilin gene that is activated at the transcriptional level in two kinds of tip-growing cells, pollen tubes and root hairs, both of which require rapid organization of the actin cytoskeleton. The isolation of such a gene has fundamental importance for our understanding of modulation of the actin cytoskeleton at the molecular level.

Introduction

Remodelling of the cytoplasmic architecture in processes like cell division, differentiation, tip growth, wound repair and response to pathogen attack are mediated by fast, highly co-ordinated polymerization and depolymerization of actin filaments. This rearrangement of the actin cytoskeleton appears to be regulated by a wide variety of actin-binding proteins, of which profilin is one of the few characterized in plants (Sun *et al.*, 1995). Profilins have been suggested to have co-evolved with the actins (Meagher *et al.*, 1999). Profilins, small 12–15 kDa proteins, are ubiquitous in all

eukaryotic cells but their precise role *in vivo* is not well understood. Among the suggested functions of profilins are positive and negative regulation of actin polymerization and participation in the phosphoinositide secondary messenger signalling pathway.

In plants, profilins were originally discovered as important pollen allergens (Valenta *et al.*, 1991, 1992) and are encoded by small multigene families (Huang *et al.*, 1996; Christensen *et al.*, 1996). On the basis of sequence comparison and tissue-specific expression patterns, profilins of higher plants have been arranged into two divergent classes: constitutive or non-reproductive and pollen- or floral-specific (Christensen *et al.*, 1996; Huang *et al.*, 1996; Meagher *et al.*, 1999). Whether profilins expressed in different tissues

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have distinct, specialized biological and biochemical characteristics remains to be answered.

In this study, we report the isolation of the tobacco profilin gene *pronp1* and analysis of transgenic tobacco plants harbouring the *pronp1* promoter-GUS fusion gene construct. Detailed histochemical and quantitative examination of *GUS* gene activity revealed that the *pronp1* promoter is preferentially expressed in mature pollen and able to direct the highest levels of expression in pollen tubes. In addition, significant activity was also observed in actively growing root hairs of young developing seedlings. *Pronp1* thus represents the first profilin gene that appears to be expressed in two kinds of tip growing cells, pollen tubes as well as root hairs.

Materials and methods

Genomic library screening and sequence analysis

The *ntPro1* cDNA clone encoding a tobacco profilin (Mittermann *et al.*, 1995) was used to screen around 500 000 phage of a tobacco (*Nicotiana plumbaginifolia*) genomic λ DASH II library purchased from Stratagene (La Jolla, CA). Plaque lifts on nylon membranes (Hybond-N, Amersham) were hybridized to the [α - ^{32} P]dCTP random-prime-labelled probe in 6 $\times$ SSPE pH 7.0, 0.5% SDS, 5 $\times$ Denhardt's solution (0.1% PVP, 0.1% BSA, 0.1% Ficoll), 0.1 mg/ml herring sperm DNA for 20 h at 56 °C and membranes were washed with 2 $\times$ SSPE, 0.1% SDS at 30 °C. DNA of phage detected by the *ntPro1* probe was purified and subjected to restriction mapping and Southern blot analysis. A positive clone (*pronp1*) of 4.5 kb was chosen for further analysis and overlapping restriction fragments of the genomic insert were subcloned into pBluescript II SK⁺ (Stratagene). The complete sequence of both DNA strands was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer) with an automated DNA sequencer. DNA and protein sequence analysis was performed using BLAST search tools (National Center for Biotechnology Information).

Reporter gene construct

A 1.4 kb *Pst*I-*Sma*I fragment containing the entire 5'-upstream sequences (672 bp) and the first 74 bp coding region of the genomic profilin clone, *pronp1*,

was introduced into the *Sma*I-*Xba*I sites of the β -glucuronidase (*GUS*) expression binary vector pBI101 (Clontech, USA). The resulting *pronp1-uidA* translational fusion construct was confirmed by double-stranded sequencing. The *Lat52-uidA* chimeric gene constructs was kindly provided by Dr Sheila McCormick (Twell *et al.*, 1989).

Particle bombardment and plant transformation

Bombardment of mature *N. tabacum* pollen was performed with a helium-driven DuPont Biolistic Delivery System (model PDS-1000) essentially as described by Daniell *et al.* (1990). In brief, ca. 250 μ l of a pollen suspension (40 mg/ml in liquid MS medium pH 7.0) was spread out in the middle of a nylon membrane (GeneScreen) placed on agar-solidified MS medium. Leaves, sepals, petals and stems used for bombardment were cut into small pieces of ca. 1 cm², placed on agar-solidified MS medium. After bombardment the plant material was allowed to recover for 10–12 h at room temperature before staining for GUS activity.

Tobacco (*Nicotiana tabacum* cv. W35) was used for transformation experiments. The *pronp1-uidA* chimeric gene construct was transferred into the disarmed *Agrobacterium tumefaciens* strain LBA4404 via triparental mating with an *Escherichia coli* strain that contained the mobilization plasmid pRK2013. Leaf discs of sterile-grown plants were used for transformation. Transgenic plants were selected on solidified MS medium (Murashige and Skoog, 1962) containing 100 mg/l kanamycin, transferred to soil and grown to maturity under greenhouse conditions (18 h light at 20 °C).

Genomic DNA extraction and Southern blot analysis

Genomic DNA was prepared from 0.5–1.0 g leaf tissue of transgenic plants using a DNA isolation kit (Invitrogen) according to the manufacturer's instructions. After digestion with restriction endonucleases DNA was separated in a 0.7% agarose gel, transferred onto a Hybond N⁺ nylon membrane (Amersham) and hybridized to a random-primed ^{32} P-labelled *NPT-II* probe as described by Sambrook *et al.* (1989).

Pollen germination

Pollen was germinated *in vitro* by resuspending mature pollen grains in 10% w/v sucrose, 0.1% casein hydrolysate, 1.6 mM boric acid, 3 mM Ca(NO₃)₂,

0.8 mM MgSO₄, 1 mM KNO₃ and 2.5 mM MES pH 5.9 and incubation at 28 °C for 5 h.

GUS assays

Histochemical localization

Histochemical localization of GUS activity was carried out by incubating whole organs or sectioned tissue in the appropriate staining buffers containing 0.5 mM X-Gluc (Progen) in the dark at 37 °C for 12–20 h according to the protocol of Blume and Grierson (1997). Histochemical analysis of enzyme activity in pollen was performed by incubating developing, mature or *in vitro* germinated pollen in 50 mM sodium phosphate pH 7.0, 10 mM Na₂ EDTA, 0.1% Triton-X100, 1 mM potassium ferricyanide and 1 mM X-Gluc.

Fluorometric assay

GUS enzyme activity was determined fluorometrically in crude plant extracts as described by Jefferson *et al.* (1987) with 4-methylumbelliferyl glucuronide (Sigma) as substrate. Protein concentrations in the extracts were measured with Bradford reagent (Bio-Rad) and BSA as standard. For determination of GUS activity, fluorescence of the reaction product, 4-methylumbelliferone (4-MU), was measured with a fluorometer (DyNA Quant 2000, Hoefer) at four different time points. Measurements were performed in triplicate and enzyme activity in each sample was expressed as pmol 4-MU per minute per mg protein.

Results

Isolation of the genomic clone *pronp1* and sequence analysis

Pronp1 was isolated by screening a genomic library of *N. plumbaginifolia* with the cDNA clone, *ntPro1*, as a probe. This cDNA clone encodes a tobacco profilin that is highly expressed in mature and germinated pollen (Mittermann *et al.*, 1995). The 3.8 kb of the *pronp1* gene was sequenced and comparison of genomic with cDNA sequences of tobacco pollen profilins revealed identical open reading frames of 405 nucleotides, the presence of 672 bp 5'-flanking region and two introns. Within the coding region, *pronp1* displays 95–97% homology to the previously isolated pollen profilin cDNA clones (Mittermann *et al.*, 1996). Differences in the sequences could probably be due to isolation of genomic and cDNA clones from different

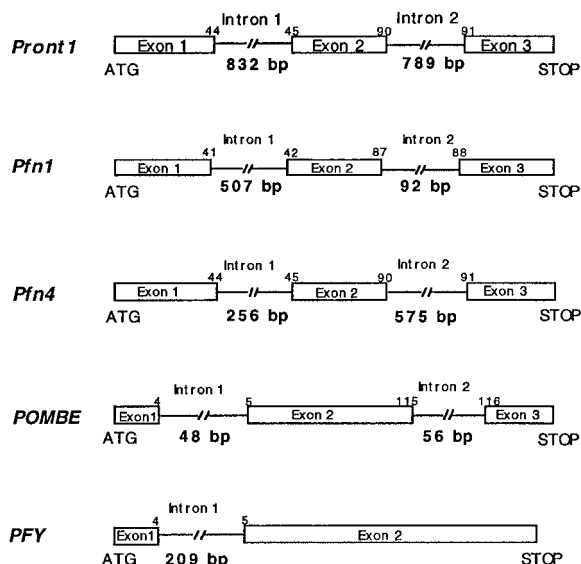


Figure 1. Comparison of intron positions and sizes of profilin genes from plants and yeast. Numbers above the gene structures indicate the codon positions, and numbers below show the size of the introns. The gene structure of *pronp1* was compared with two *Arabidopsis* profilin genes, a constitutively expressed (*Pfn1*) and a pollen-specific one (*Pfn4*), and two yeast profilin genes, *POMBE* from *Schizosaccharomyces pombe*, and *PFY* from *Saccharomyces cerevisiae*.

Nicotiana species, *N. plumbaginifolia* and *N. tabacum*, respectively. The two introns identified in *pronp1* are characteristically AT-rich, and exon-intron junctions obey the GT-AG rule. The gene structure of four (*Pfn1*, *Pfn2*, *Pfn3*, *Pfn4*) *Arabidopsis* and two yeast clones was compared. As *Pfn1* and *Pfn2* are vegetative expressed genes (89% identical at the amino acid level) and *Pfn3* and *Pfn4* (91% identical at the amino acid level) are pollen-specific one (Meagher *et al.*, 1999), one of each category is shown in Figure 1. Comparative analysis of the gene structure of *Arabidopsis* and yeast clones (Christensen *et al.*, 1996) showed that the position of the introns is conserved in plant profilin genes, but differs from the intron positions in *Saccharomomyces cerevisiae* and *Schizosaccharomyces pombe* profilin genes (Figure 1).

Analysis of the two intron regions of *pronp1* revealed within the first intron region, between bases 1250 and 1410, 80% homology to tobacco chloroplast DNA. Within the second intron, the region from 2090 to 2535 displayed high sequence similarity (75–80%) to a family of putative defective transposable elements that have been identified in several parts of the tobacco genome (Tebbutt and Lonsdale, 1993). These elements were first found and characterized in

the third intron of a pollen-specific polygalacturonase gene (*Npg1*) and upstream of an endochitinase gene (*Chn50*; Fukuda *et al.*, 1991). Due to the lack of any long open reading frames, members of this family of transposons were classified as non-autonomous transposable elements. Like most transposons, the element present in the second intron of *pronp1* is bounded by terminal inverted repeats, AAAAATAG/CTATTTTT. In addition, it appears to be flanked by small direct repeats, AAAAT/AAAAT, the so-called 'target site duplications' which are also indicative of transposable elements. Since members of the described family of defective transposons show overall sequence homology, but not identity, Tebbutt and Lonsdale (1993) suggested that the presence of these endogenous transposable elements in the tobacco genome might be due to ancient insertion events.

Analysis of the pronp1-uidA gene fusion by transient expression in tobacco pollen

To ascertain that the 5' non-coding region of *pronp1* represents an active promoter and to study its expression pattern, 672 bp of *pronp1* upstream sequence was fused to the β -glucuronidase (*uidA*) gene. The resulting construct was then introduced into different tobacco tissues by microprojectile bombardment. Histochemical GUS analysis revealed that the *pronp1* promoter is highly active in mature pollen. More than 60% of the tobacco pollen germinated on the agar plate after bombardment and X-Gluc staining indicating that *pronp1* is also active in germinating pollen. However, only very few blue spots as indicator of GUS activity could be detected in leaves after incubation with X-Gluc and no GUS activity was observed in other sporophytic tissues, such as stems and petals after bombardment.

The level of transient expression in mature tobacco pollen was further compared to the expression levels of *Lat52*, a pollen-specific promoter from tomato (Twell *et al.*, 1989). Chimeric constructs, *Lat52-uidA* and *pronp1-uidA*, were tested by bombarding mature *N. tabacum* pollen. Initially, *Lat52* appeared to be the stronger promoter as bombardment with *Lat52-uidA* led to the generation of dark indigo-blue pollen within 1 h of X-Gluc incubation, while first blue pollen was detected after 2 h of X-Gluc staining with *pronp1-uidA*. However, after 15 h incubation, numbers and intensity of blue pollen resulting from *pronp1-uidA* were comparable to those obtained with *Lat52-uidA*.

Spatial and temporal expression pattern of pronp1 in transgenic tobacco plants

Activity of the *pronp1* promoter was further analysed in *N. tabacum* plants stably transformed with the *pronp1-uidA* gene fusion. Of 11 independent kanamycin-resistant transformants chosen at random, 7 showed histochemical localization of GUS enzyme activity in mature pollen. Southern blot analysis of these plants revealed T-DNA insertions at 1–3 separate loci (data not shown). Most of the GUS positive plants showed 50–100% staining of mature pollen after X-Gluc treatment, and levels of enzyme activity significantly varied between individual transformants. In addition, especially in plants with more than one locus of the transgene, variation in intensity of blue staining was also observed between individual pollen grains.

Various organs and tissues of two individual transformants (with 70% blue staining pollen) and an untransformed control plant were histochemically and fluorometrically analysed for GUS activity. These plants did not show GUS staining in vegetative tissues (stem, leaf and mature root) and other parts of the flower (petals, sepals, pistils and ovaries) except for pollen. In accordance with this, quantitative analysis revealed high levels of enzyme activity only in crude extracts of mature anthers while enzyme activity in their leaves, stems, petals, sepals, pistils and ovaries was not significantly elevated above the background levels obtained in extracts of the untransformed control plant (Figure 2A, B). However, trace levels of GUS activity were detectable in crude extracts prepared from roots of mature, transformed flowering plants grown under glasshouse conditions (Figure 2A, B).

The mature pollen of transgenic plants collected just before anthesis showed high levels of GUS activity (Figure 3A). In contrast, anther tissue from which the pollen was removed did not stain blue when incubated with X-Gluc indicating that *GUS* gene expression is restricted to pollen grains. No histochemical staining was detected in pollen of the control plant (Figure 3B). GUS staining could also be observed in germinating pollen from transgenic plant whereas no staining was detected in pollen tubes of the control plant (Figure 3C and D).

To define the point of *pronp1* promoter activation during the process of male gametophyte development, GUS expression was assessed fluorometrically in extracts of isolated unicellular microspores and pollen of

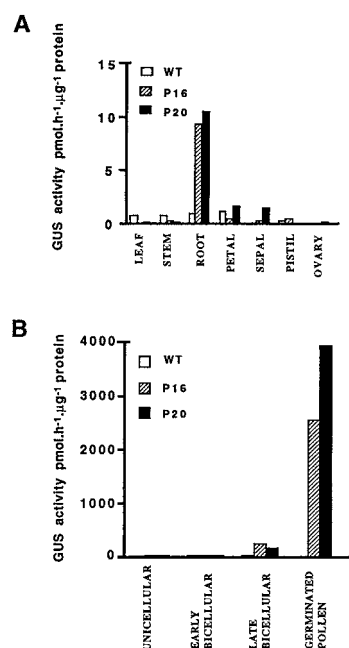


Figure 2. Fluorometric analysis of GUS activity. **A.** Fluorometric detection of GUS activity in vegetative and floral tissues of *pronpl-uidA* transformed tobacco plants. Assays were performed on extracts of mature leaves, stems, roots, petals, sepals, pistils and ovaries of a wild-type plant (WT) and two individual transformants (P16, P20). GUS enzyme activity is expressed in pmol 4-methylumbelliferone (4-MU) generated per hour per microgram of protein. **B.** Quantitative analysis of GUS activity during pollen development and pollen germination of two transgenic (P16, P20) and a wild-type tobacco plant. No GUS activity was detected in the wild-type plant. Developmental stages of microspores and pollen were defined by DAPI staining and correlated to the bud lengths. Assays were conducted on extracts of anthers containing early unicellular microspores, early bicellular pollen, late bicellular pollen and *in vitro* germinated pollen. GUS expression levels are presented in pmol 4-MU generated per hour per microgram of protein.

two successive developmental stages and in germinating pollen (Figure 2B). In unicellular microspores and early- to mid-bicellular pollen, levels of GUS activity did not exceed those in extracts of untransformed control plants. However, GUS activity started to appear during the transition from the mid-bicellular stage to mature pollen. A dramatic increase in GUS activity was observed during pollen germination and tube growth (Figure 2B) where a 20-fold increase in the enzyme activity was observed as compared to mature pollen.

In addition to pollen, dark blue GUS staining was observed in actively growing root hairs of kanamycin-resistant seedlings from selfed primary transformants (Figure 3E–H). Root hairs are tubular-shaped extensions of root epidermal cells called trichoblasts which

elongate by growth restricted to their tips. In the developing root the GUS staining first appeared in the epidermal layer where trichoblast differentiation takes place. In later stages intense GUS staining was observed in bulging and then growing root hairs (Figures 3G and H). GUS enzyme activity was clearly confined to these tip-growing cells of *pronpl-uidA* transformed plants (elongating root hairs) and could not be detected in any other part of the root.

Discussion

In this report, we describe the isolation and characterization of a unique and distinct profilin gene that appears to be activated at the transcriptional level in two kinds of tip-growing cells, pollen tubes and root hairs, both of which require rapid organization of the actin cytoskeleton. The isolated promoter sequence fused with the β -glucuronidase (*uidA*) reporter gene was used to study its temporal and spatial expression pattern in transgenic *Nicotiana tabacum* plants. Histochemical and fluorometric analysis of the transgenic plants demonstrated that the 5'-flanking sequence of *pronpl* was sufficient to direct gene expression in a cell-specific manner. GUS enzyme activity was first detectable in mid-bicellular pollen, increased progressively during further development and peaked in growing pollen tubes. This pattern of GUS activity driven by the *pronpl* promoter clearly identifies this profilin gene as a 'late pollen gene' according to classification described by McCormick (1993). Furthermore, these data of reporter gene expression corroborate previous northern blot results of tobacco profilin mRNA accumulation (Mittermann *et al.*, 1995), in which profilin transcripts were undetectable up to the early binucleate stage, but significantly increased in mature and germinated pollen.

Histochemical detection of GUS activity demonstrated novel sites of expression in tissues not identified previously and allowed a precise localization in specific cells. It was interesting to see reporter gene expression in actively growing root hairs of young, developing seedlings from selfed primary transformants. The polarized growth of root hairs and pollen tubes is similar in several respects. In both cases plant cell expansion is limited to a single growing point and leads to the formation of a tubular-shaped cell (Schnepf, 1986; Steer and Steer, 1989; Heath, 1990). Ultrastructural studies of pollen tubes and root hairs have revealed a similar characteristic organization of the

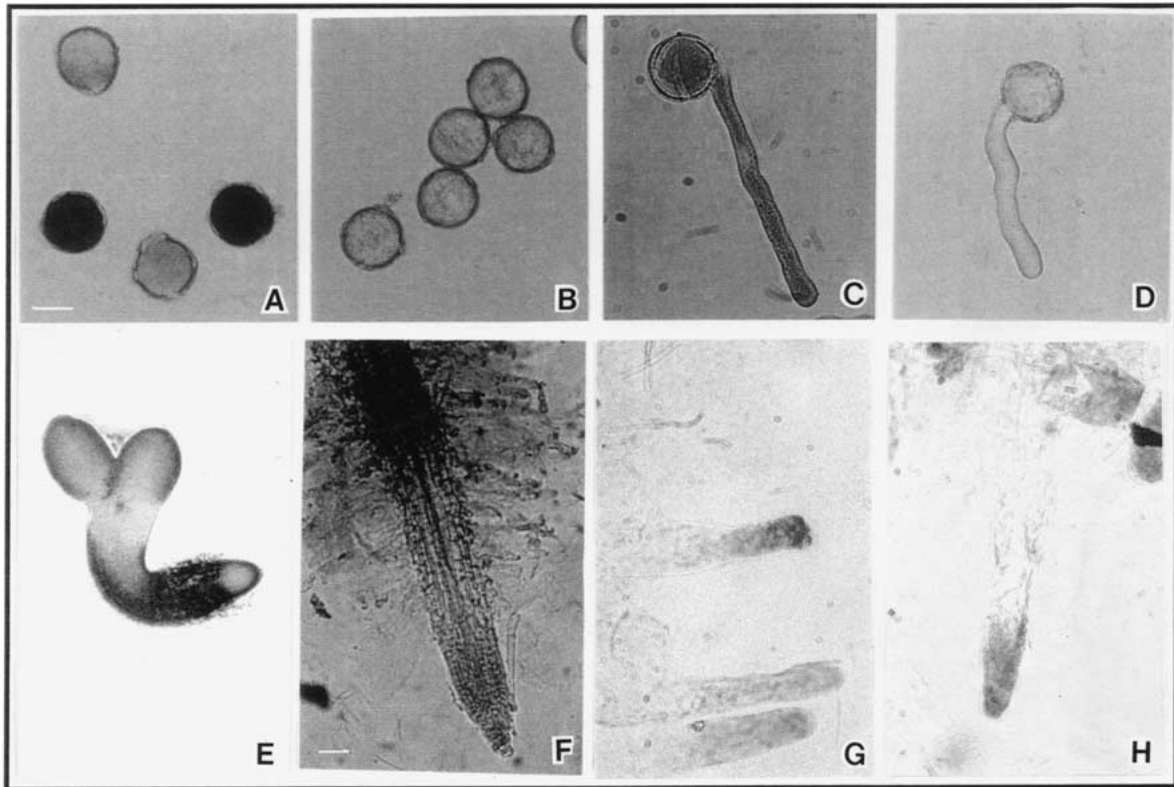


Figure 3. Histochemical localization of GUS activity in *pronp1-uidA* transgenic tobacco plants. A. Mature pollen of transgenic plant showing staining for GUS enzyme activity. Bar scale = 14 μm . B. Mature pollen from untransformed control plants after incubation with X-Gluc. C. *In vitro* germinated pollen of transgenic tobacco showing GUS activity. D. *In vitro* germinated pollen of untransformed control plant after incubation with X-Gluc. E. Young seedling from selfed primary transformant showing GUS staining in root hairs. F. GUS staining in elongating root hairs of young seedlings from selfed primary transformant. Bar scale = 160 μm . G and H. Close-up of GUS-stained root hairs (75 $\times$).

cytoplasmic contents, with exocytotic Golgi vesicles and ER elements at the tip, large organelles, like Golgi bodies, ER and mitochondria, in the subapical zone, and a central vacuole at the proximal part of the cells (Schnepf, 1986). Furthermore, it has been shown that the growth of both cells is associated with a continuous influx of Ca^{2+} at the apex and an internal Ca^{2+} gradient (Reiss and Herth, 1979; Clarkson *et al.*, 1988; Schiefelbein *et al.*, 1993). In view of these similarities, it is expected that genes exist whose products are required for the tip growth of both cell types. Schiefelbein *et al.* (1993) reported identification of an *Arabidopsis thaliana* mutant *tip1* in which a single mutation leads to abnormal cell expansion in the root hairs and pollen tubes. It was suggested that *TIP1* gene product plays an important role during tip growth in both root hairs and pollen tubes but neither the gene or its product has been identified. The profilin gene *pronp1* reported in the present study appears to be one

such gene which may play an important role during tip growth in both types of tip growing cells.

The actin cytoskeleton has been reported to be essential for the control of local growth in root hairs and pollen tubes (Cai *et al.*, 1997; Kropf *et al.*, 1998; Bibikova *et al.*, 1999). Actin-binding proteins such as profilin could thus play an essential role in regulating the organization of actin filaments. Furthermore, it has been conclusively shown that profilins regulate actin filament assembly in the acrosomal process of *Thyone* sperm (Tilney *et al.*, 1983) and actin-dependent tip growth in other eukaryotic systems, like fibroblast cells (Giuliano and Taylor, 1994) and neuronal growth cones (Wills *et al.*, 1999). It is quite likely that profilin encoded by *pronp1* is involved in regulating actin filament dynamics in both pollen tubes and root hairs.

On the basis of sequence comparison and tissue-specific expression plant profilins have so far been arranged into two broad groups: constitutive or non-reproductive, and pollen-specific (Christensen *et al.*,

1996; Huang *et al.*, 1996; Meagher *et al.*, 1999). A phylogenetic tree constructed by comparing the amino acid sequences of eighteen plant profilins (Yu *et al.*, 1998) indicated that profilin genes isolated from vegetative organs grouped together in one cluster, whereas those isolated from pollen or strongly expressed in pollen formed another cluster. In case of *Arabidopsis*, pollen-specific as well as constitutive profilin genes have been isolated (Christensen *et al.*, 1996; Huang *et al.*, 1996), and it has been shown that the pollen-specific members are significantly more closely related to pollen-specific profilins from distant dicot and monocot species than to constitutive members in the same organism. Analysis of pollen-specific profilins had so far demonstrated that their expression is restricted to mature and germinating pollen grains (Christensen *et al.*, 1996; Yu *et al.*, 1998). However, analysis of plant profilin evolution and comparison with profilins from other eukaryotes points to a higher complexity of the multigene family and suggests the presence of additional groups and subgroups beside the two main plant profilin classes (Huang *et al.*, 1996). Recently, Kovar *et al.* (2000) reported two classes in profilin gene family with distinct biochemical and live-cell properties suggesting that the maize profilin isoforms perform distinct functions in the plants.

In this study, we present evidence that *prnpl1*, a profilin gene that is preferentially expressed in tobacco pollen and, according to sequence analysis, falls into the group of pollen-specific profilin genes, is also activated in root hairs. We therefore propose that *prnpl1* represents a hitherto not described subgroup of profilin genes specifically expressed in tissue types which require the presence of large amounts of profilin such as pollen tubes and root hairs. Since the cellular function of profilin in regulating actin polymerization appears to be highly conserved in distantly related organisms it seems unlikely that profilins from different tissues perform different functions. Instead, we would like to propose that expected demands for profilin in different cell types is met by cell-specific transcriptional regulation. *Pronpl1* appears to be one such gene which is developmentally expressed in tip-growing cells to meet high profilin levels needed to sustain their specialized rapid growth rate.

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