



Pollination modulates expression of the PPAL gene, a pistil-specific β -expansin

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Abstract

Using differential screening we isolated a pistil-specific cDNA clone corresponding to a 1.2 kb mRNA and encoding a 32.5 kDa protein. The amino acid sequence shared similarity with that of group-I grass pollen allergens, which are known to have expansin activity. This clone, which later showed to share homology also with β -expansins, was named PPAL. The PPAL mRNA was specifically expressed in the secretory zone of the stigma and in the epidermal layer of the placenta. The accumulation level of the transcript increased during pollination, and the protein was secreted in the stigmatic exudate of the tobacco flower. We suggest here that PPAL is a new expansin, acting as a cell-wall-loosening agent during pollination.

Introduction

In flowering plants pollination and subsequent fertilization comprise a series of pre-programmed events that involve both the pollen, the male gametophytes and the receptive, sporophytic, tissues of the pistil. After their deposition on the stigma, the pollen rehydrate and germinate. The newly formed pollen tubes subsequently penetrate the stigma and grow through the style towards the ovules in the ovary. After penetration of the embryo sac in the ovule, the sperm cells are released and fertilization takes place. The pistil delivers a number of compounds to the pollen, for example, myo-inositol, flavonols, proteins, water and lipids which sustain hydration and germination on the stigma and growth of the pollen tubes through the style (Kroh *et al.*, 1976; Esau, 1977; Mo *et al.*, 1992; Ylstra *et al.*, 1994; Wolters-Arts *et al.*, 1998). Moreover, the pistil is thought to produce compounds that guide the pollen tubes through the stigma, transmitting

tissue and ovary towards the embryo sac (Labarca and Loewus, 1973; Cheung, 1995a). A tobacco transmitting tissue-specific protein, TTS, has been shown to attract pollen tubes and to stimulate growth (Cheung *et al.*, 1995b; Wu *et al.*, 1995).

Interactions between pollen or pollen tubes and the pistil may also determine the acceptance and rejection of pollen, either intra-specific (self-incompatibility) or inter-specific (incongruity) (Cornish *et al.*, 1988; Nasrallah and Nasrallah, 1989; Sims, 1993). In tobacco the stigma is covered with an exudate (wet stigma) that mediates the first contact between the pollen and stigma. While the proteins present in the exudate of tobacco may partake in various functions, those identified so far relate to defence proteins such as thaumatin-like proteins and β -1-3 glucanases (Kuboyama, 1997). Other pathogenesis-related proteins expressed in the pistil, such as proteinase inhibitors, osmotin, chitinases and thionins (Atkinson *et al.*, 1993; Neale *et al.*, 1990; Leung, 1992; Gu *et al.*, 1992; Karanundaa *et al.*, 1994), may also be secreted in the exudate. The need for such proteins may result from the physical pene-

The nucleotide sequence data reported here will appear in the GenBank database under the accession number AF333386.

tration and, thereby, wounding of the stigma by septic pollen tubes.

The lipidic compounds of the exudate largely determine correct hydration and germination of the pollen and penetration of the stigmatic surface by the pollen tubes (Wolters-Arts *et al.*, 1998). In species with dry stigma, i.e. from *Brassica* and *Arabidopsis*, similar functions may be exerted by the lipidic pollen coat. Ruiter (1996) showed the presence of oleosins associated with the lipid bodies in the *Brassica napus* pollen coat, and Mayfield *et al.* (2000) showed that oleosins are required for the rapid initiation of pollination in *Arabidopsis thaliana*. Similarly, other functions exerted by pollen in species with a dry stigma may be found in the exudate of species with a wet stigma.

Pollen of species with a dry stigma carry the so-called group I grass pollen allergens (Knox, 1996). These are known to cause hay fever, but their actual plant function is largely unknown. Supposedly, group-I grass pollen allergens, such as the *Zea mays* (Zea mI) pollen allergen, form a second family of expansins, the β -expansins, that may loosen the cell walls of the stigma and thus facilitate penetration of the pollen tube (Cosgrove, 1997). Grobe *et al.* (1999) found that grass group-I allergens (β -expansins) are novel, papain-related proteinases; however, unpublished data by Li and Cosgrove suggest that this may not be the case for all grass pollen allergens (Cosgrove, 2000).

Using the differential screening procedure, we isolated and characterized a pistil-specific gene whose expression is modulated or induced upon pollination and which possibly has a function in pollen-pistil interaction. The PPAL gene described herein shows homology to β -expansins and to group I grass pollen allergens.

Materials and methods

Plant material

Nicotiana tabacum cv. Petit Havana SR1 plants (Maliga *et al.*, 1973) were grown under standard greenhouse conditions. For the experiment on gene expression after pollination, plants were grown in a growth chamber maintained at 19–22 °C under a 16 h (light)/8 h (dark) photoperiod and a light intensity of 150 $\mu\text{E m}^{-2} \text{s}^{-1}$. In general, flowers at stage 5 or 11 were emasculated and left for 24 h before they were pollinated (when necessary). Pistils, non-pollinated or

pollinated, were harvested after 0, 3, 6, 12, 24, 48 and 72 h and dissected into stigma/style and ovary.

RNA isolation procedure

Seedlings, roots, stems, leaves, different flower organs and seeds were frozen in liquid nitrogen and stored at –70 °C for RNA isolation. Roots, stems and leaves were collected from 1-month old non-flowering plants. Sepals, petals, anthers, stigmas/styles, ovaries and seeds represent pools of material collected at different developmental stages.

For the developmental expression studies, stigmas/styles and ovaries were excised from flowers at stages 1–12 of tobacco flower development, as described by Goldberg (1988). Total RNA from all plant organs was extracted essentially as described by Dean *et al.* (1985). Poly(A)⁺ RNA was selected by means of a biotinylated oligo(dT) probe and streptavidin-coated paramagnetic particles (PolyAtract, Promega).

The cDNA library was constructed according to the manufacturer's protocol (ZAP-cDNA synthesis kit, Stratagene) using 5 μg of poly(A)⁺ RNA isolated from pistils 12 h after pollination. First-strand cDNA synthesis was carried out by *Xho*I-oligo(dT) priming, followed directly by second-strand synthesis. An *Eco*RI adaptor was then ligated to the cDNA. The resultant products were phosphorylated by T4 polynucleotide kinase (Stratagene). After *Xho*I digestion, the cDNA was further purified on a Sephacryl S-400 spun column (Stratagene). The resultant cDNAs had different cohesive ends (*Eco*RI at the 5' end and *Xho*I at the 3' end) and were unidirectionally ligated to the *Eco*RI and *Xho*I double-digested arms of phage λ ZAPII. The resulting phage DNA was packaged *in vitro* into phage particles with the GigapackII Gold packaging extract (Stratagene).

Differential screening

Differential screening was carried out essentially as described by Gasser *et al.* (1989) except that cDNA representing a pool of mRNAs from unpollinated pistils, mature pollen and seedlings was used as a negative probe. For the screening, about 15 000 plaque-forming units (pfu) were plated onto 15 cm diameter plates with *Escherichia coli* Sure cells for infection. Duplicated nylon replica filters (Hybond-N, Amersham) were lifted from each of the plates and treated as recommended by the manufacturer. A total of 300 000 pfu were screened. About 100 pfu were plated onto 9 cm diameter plates for plaque purification.

Hybridization probes were synthesized from 1 μ g poly(A)⁺ RNA isolated from pistils 12 h after pollination, for unpollinated pistils at the same time point, mature pollen and seedlings as described by Goldman *et al.* (1992).

Filters were prehybridized overnight in 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1% SDS and 100 μ g/ml denatured carrier DNA at 68 °C. The labelled cDNA was added to the prehybridization solution to a final concentration of 10⁶ cpm/ml and incubated at 68 °C for 36–48 h. Filters were washed at the same temperature in 6 $\times$ SSC, 0.1% SDS for 30 min and 2 $\times$ SSC, 0.1% SDS for 30 min. They were then exposed to Kodak X-Omat films for 3 h at –70 °C, washed further in 0.2 $\times$ SSC, 0.1% SDS at 68 °C for 30 min, and exposed again to the film for 7 and 24 h.

Positive-hybridizing plaques were selected and re-screened to ensure purification and the elimination of false-positives.

The cloned cDNA plaques were subcloned directly into pBluescript II SK+ plasmid by *in vivo* excision (Stratagene) for the sequence analysis.

DNA manipulation and sequence analysis

Plasmid DNA was extracted as described by Sambrook *et al.* (1989). Radioactively labelled DNA probes were prepared from gel-purified DNA fragments with a DNA labelling kit (Ready-To-Go, Pharmacia). Double-stranded DNA sequencing was performed following the dideoxy chain termination method of Sanger *et al.* (1977) with synthetic oligonucleotides homologous to the T3 and T7 RNA polymerases promoters as primers. When required, oligonucleotides (20-mers) were synthesized according to sequence information and used directly as primers for further sequencing.

Nucleotide and protein data were analysed with the computer resources provided by IntelliGenetics (Mountain View, CA) and by PC/Gene (University of Geneva, Switzerland). The homology search with the BLAST program in the GenBank was done after disabling the low-complexity filter.

RNA gel blot analyses

Total or poly(A)⁺ RNA was electrophoretically separated on a 1.5% agarose gel containing 2.2 M formaldehyde. Gels were blotted onto Hybond-N

membranes (Amersham) with 20 $\times$ SSC, followed by 2 h at 80 °C to fix the RNA to the membrane.

Hybridization was done with DNA probes or RNA probes in 5 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.5% SDS and 100 μ g/ml denatured carrier DNA at 50 or 65 °C overnight. Filters were washed in 6 $\times$ SSC for 10 min at room temperature, twice in 2 $\times$ SSC, 0.1% SDS for 15 min and twice in 0.1 $\times$ SSC, 0.1% SDS for 15 min at 50 or 65 °C.

Each filter was stripped and rehybridized with a rDNA probe as a means of controlling equal loading and RNA quantification.

In situ hybridization studies

Pollinated and unpollinated pistils were dissected from tobacco flowers, fixed with 3.7% formaldehyde, 0.1% glutaraldehyde in 100 mM phosphate buffer pH 7 for 3 h at room temperature and embedded in paraffin. The entire embedded pistil was sliced into 7–10 μ m thick sections. Hybridization was performed as described by De Block and Debrouwer (1993).

The antisense and sense RNA probes were synthesized with dioxigenin-UTP by *in vitro* transcription with the T7 and T3 polymerases of the dioxigenin RNA labelling kit (Boehringer Mannheim).

Protein isolation and immunoblot analysis

Three recombinant PPAL proteins were expressed in *E. coli* representing, respectively, amino acids 41–202 (pRF3), 41–243 (pRF4) and 41–262 (pRF6) of the deduced amino acid sequence from the full-length PPAL cDNA. Expression and isolation were carried out essentially as described by Qiagen (QIAexpression-System). After purification, recombinant protein pRF6 was sent out for polyclonal antibody production (Eurogentec).

Protein isolation from the flower organs was carried out by extracting the protein in a buffer containing 0.6% PVPP, 50 mM phosphate buffer pH 7, 10 mM EDTA, 0.1 % Triton X-100, 0.1% sarcosyl, 10 mM 2-mercaptoethanol and 25 μ g/ml PMSF.

Proteins from the exudate were isolated by rinsing the stigmas of *Nicotiana tabacum* flowers in a PBS solution (Cresti, 1986); they were then precipitated with 5 vol 100% ethanol and resuspended in a suitable volume. Protein concentrations were determined by means of a Bradford protein assay (BioRad; Bradford, 1976).

A 40 μ g portion of total protein of each sample was electrophoretically separated by SDS-PAGE on a

12.5 % gel according to Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R 250 or left unstained for electrophoretic transfer onto nitrocellulose (Schleicher and Schuell). The proteins were transferred onto nitrocellulose at 200 mA for 2 h. After the transfer, the blots were stained with Ponceau S to ensure that the transfer was complete and then washed in water to remove Ponceau S.

Non-reacting binding sites were blocked by overnight incubation of the blots in 5% powdered milk in PBS.

Western blots were incubated with polyclonal antibodies raised against pRF6 recombinant PPAL protein in a 1:1250 dilution in powdered milk for 1.5 h. The blots were washed three times for 10 min each in PBS to remove the unbound antibodies. A second antibody, alkaline phosphatase-conjugated goat-anti-rabbit IgG, was added at a 1:5000 dilution and incubated for 1 h. The blots were washed three times in PBS and once in alkaline phosphatase buffer (pH 9.5) for 10 min. The alkaline phosphatase reaction was performed by adding 300 μ l of each BCIP (15 mg/ml DMF) and NBT (30 mg/ml 50% DMF) in 10 ml of alkaline phosphatase buffer. The staining reaction was stopped in distilled water, and the blots were analysed.

Results

Isolation of a molecular marker for studying pollination

To isolate pistil-specific genes that are induced or enhanced by pollination, we constructed a tobacco cDNA library from mRNA isolated from pollinated pistils collected 12 h after pollination. Differential screening with cDNA probes made for mRNAs extracted from pollinated pistils (positive probe) and mixtures of mRNAs from equally old unpollinated pistils, seedlings and mature pollen (negative probe) resulted in the identification of ten pistil-specific clones. DNA cross-hybridization analysis showed that seven of these clones were substantially homologous, while the remaining three were clearly distinct from the first cDNA family and from each other.

We focused on one pistil-specific clone of 1064 bp. Since it hybridized to a 1.2 kb pistil mRNA, it must carry an almost full-length cDNA and may code for a 29.4 kDa protein of 274 amino acids. DNA hybridization studies using this cDNA clone as probe indicated that in the amphi-diploid *N. tabacum* PPAL

is a single gene or belongs to a small gene family (data not shown). The complete cDNA sequence was used to search for homologies in the GenBank with the BLAST program. Though no significant homologies were found at the nucleotide level, an amino acid sequence comparison showed 51% identity and 66% similarity to the Cim I (cytokinin-induced messenger) protein, a 277 amino acid, 30 kDa cytokinin-induced (glyco-)protein from soybean (Crowell, 1994). Typical stretches were found that showed homology to expansins (Cosgrove *et al.*, 1997) and, likewise, to the expansin-like group I pollen allergens from grasses, such as Lol pI (Perez *et al.*, 1990; Griffith *et al.*, 1991), Zea mI (Broadwater *et al.*, 1993) and Cyn dI (Smith *et al.*, 1996) (Figure 1A). With a few exceptions, all PPAL cysteines and tryptophans align perfectly with those of the group I grass pollen allergens and β -expansins, indicating a strong similarity in protein folding. A hydropathy plot revealed that both PPAL and the group I grass pollen allergens contain a signal peptide. Furthermore, PPAL also contains those regions found in the active sites of papain-related proteinases, similar to what has been found in the group-I pollen allergen Phl pI from timothy grass (Grobe *et al.*, 1999). Homology between antigenic epitopes of PPAL and pollen allergens was further tested using three recombinant PPAL proteins produced in the prokaryotic expression system pQE (Qiagen) and probed with the monoclonal antibody 3A2, which is specific for the carboxy-terminal domain of the group I grass pollen allergens (Smith, 1994; gift of M. Singh). Figure 1B shows that the 3A2 antibody also specifically detects the carboxy-terminal domain of PPAL. Most group I grass pollen allergens have a 15 amino acid region at the C-terminus that cross-reacts with IgE and is responsible for allergic reaction in man (Esch and Klapper, 1989). On the contrary, IgE antibodies did not cross-react with recombinant PPAL protein, indicating that this protein is not an allergen (Singh, personal communication). However, if the recombinant protein were incorrectly folded, it could still be the case that the antigenic site of PPAL is hidden by the rest of the protein.

PPAL mRNA and protein accumulate in the pistil

To further assess the pistil specificity of the PPAL gene we determined the expression of the gene in various tobacco organs. RNA gel blots were made with poly(A)⁺ RNA extracted from roots, immature seeds, seedlings, stems, leaves, sepals, petals and a mixture

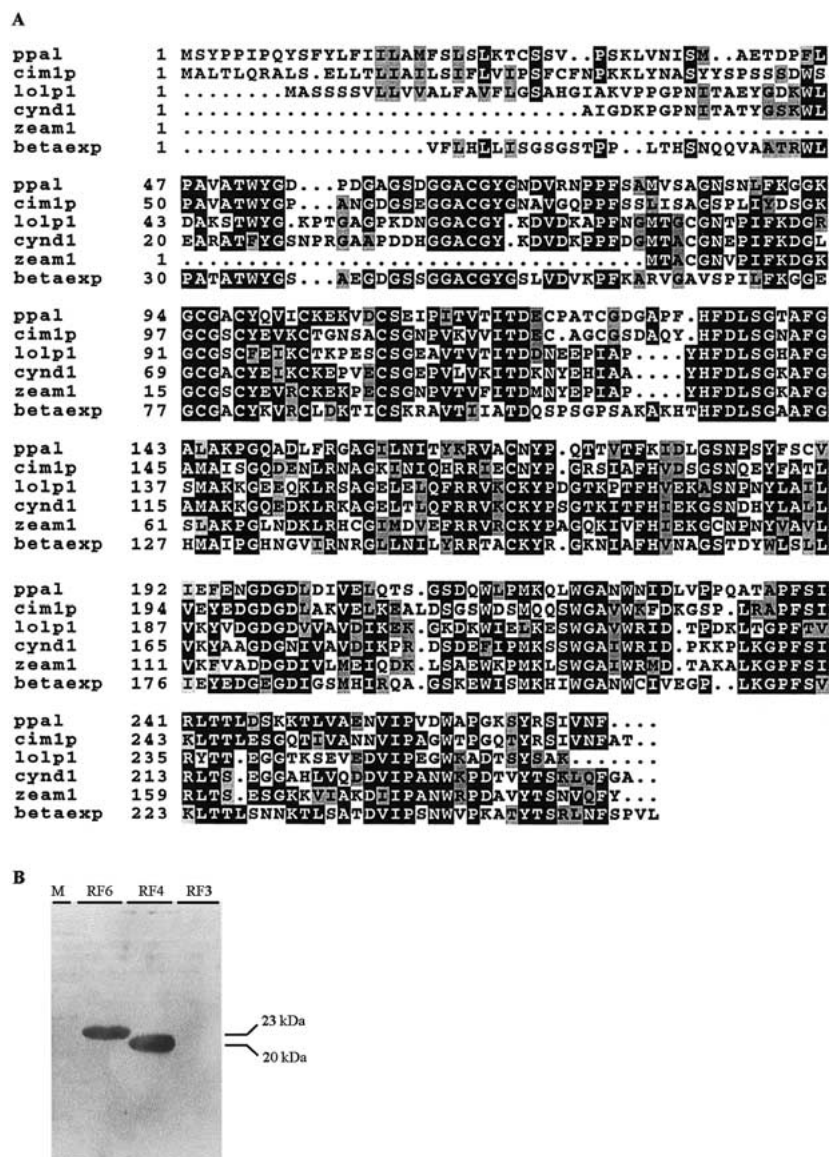


Figure 1. Comparison between PPAL, cytokinin-induced messenger, some pollen allergens and one β -expansin. **A.** Alignment of PPAL protein with different pollen allergens and expansin. Cim I (S48032) is a cytokinin-induced messenger from soybean, Lol pl (S13614) is the major pollen allergen from *Lolium perenne*, Zea mI (Q07154) is the major pollen allergen from *Zea mays*, Cyn dI (1911582) is the major pollen allergen from *Cynodon dactylon*, Beta-expansin (2224913) is from *Arabidopsis thaliana*. Identity is shown by black boxes; similarity is shown by grey boxes. **B.** Cross-reaction of a pollen allergen antibody with recombinant PPAL proteins. The three recombinant proteins pRF3, pRF4 and pRF6 (see methods) were tested for cross-reactivity with monoclonal antibody 3A2. Total protein of *E. coli* (40 μ g) was loaded in each lane. Mab 3A2 reacts to the C-terminal part of recombinant PPAL proteins RF4 and RF6 from AA 202 to AA 262.

of the pistils (stages 1–11), according to Goldberg (1988), anthers (stages 1–11), mature pollen, stigmas/styles and ovaries at 12 h after pollination (h.a.p.). The results (Figure 2A) confirmed that the 1.2 kb PPAL transcripts were present in the pistils at only a low level, while in pollinated stigmas/styles high levels of expression were reached. The conclusion to

be drawn is that PPAL expression is pistil-specific and enhanced by pollination. To determine the exact time of PPAL expression during pistil development, we isolated total RNA from stigmas/styles and ovaries at different stages of tobacco flower development. At stage 1, the pistil is already differentiated and at stage 12 pistil maturation and anthesis occur. Figure 2B shows that

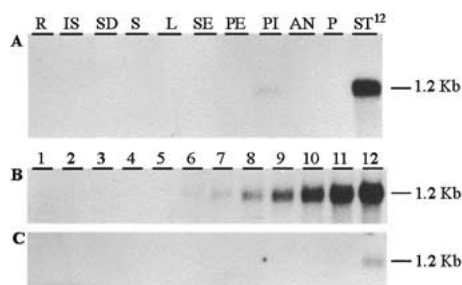


Figure 2. Tissue and temporal expression pattern of PPAL. A. RNA gel-blot analysis of PPAL transcript level in different organs of tobacco plants. Poly(A)⁺RNA (1 µg) was loaded in each lane. R, roots; IS, immature seeds; SD, seedlings; S, stem; L, leaves; SE, sepals; PE, petals; PI, pistils (stages 1–11); AN, anthers (stages 1–11); P, mature pollen; ST¹², stigmas and styles 12 h after pollination. B. Accumulation of PPAL mRNA in stigmas/styles at different developmental stages of tobacco flower (Goldberg, 1988). A 10 µg portion of total RNA was loaded in each lane. C. Accumulation of PPAL mRNA in ovaries at different flower developmental stages of tobacco (Goldberg, 1988). A 10 µg portion of total RNA was loaded in each lane.

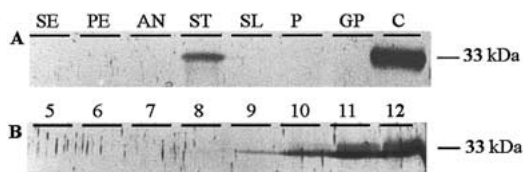


Figure 3. PPAL protein accumulation in flower organs and its accumulation profile in stigmas. A. Accumulation of PPAL protein in flower organs. Immunoblot analysis of PPAL protein in different organs of tobacco flowers. Total protein (40 µg) was loaded in each lane. SE, sepals; PE, petals; AN, anthers; ST, stigmas; SL, styles; P, mature pollen; GP, germinated pollen; C, control: PPAL protein expressed in *E. coli*. Colour reaction was performed using the alkaline phosphatase reaction. B. Developmental profile of PPAL protein in stigmas from flowers from developmental stage 5 to 12. Immunoblot analysis of PPAL protein during stigma development. A 40 µg portion of total protein was loaded in each lane. The colour reaction was performed using the alkaline phosphatase reaction.

PPAL mRNA was first present in the stigmas/styles at stage 5 and gradually accumulated during pistil maturation. The PPAL transcripts reached maximum levels at stage 12 in the stigmas/styles, while at the same time low levels of mRNA complementary to PPAL began to appear in the ovary (Figure 2C).

To study whether PPAL protein expression follows the mRNA expression pattern, we raised polyclonal antibodies against the recombinant PPAL protein for western blot analysis. Total protein was isolated from sepals, petals, anthers, stigma, style, pollen, germinated pollen and stigmas of flowers from developmental stage 5 to 12 (Goldberg, 1988). Figure 3A shows that PPAL protein was exclusively expressed in the stigma and that during pistil development PPAL

protein expression reflected the RNA expression pattern (Figure 3B). The fact that PPAL antibodies do not detect other proteins in tissues other than pistil, very strongly suggests that they were specifically raised against PPAL and do not non-specifically cross-react with other expansins.

PPAL expression in the pistil is modulated by pollination

Emasculated flowers at developmental stages 6 and 12 (Goldberg, 1988) were pollinated, and stigmas/styles and ovaries were collected at various times after pollination (Figure 4). As a control, stigmas/styles and ovaries from emasculated but unpollinated flowers were collected at identical times (see also Materials and methods). Figure 4A and B show the RNA gel blot analysis and accumulation profile of PPAL mRNAs in the collected stigmas/styles and ovaries at stage 12. Pollination caused an increase in PPAL mRNA in both stigmas/styles and ovaries, with a peak at 24 h.a.p. At that time PPAL transcript levels in pollinated pistils were about 2.5 times higher than those in unpollinated pistils. At 48 h.a.p. the PPAL mRNA levels appeared to be almost equal again. At 72 h.a.p. PPAL mRNA was no longer detectable in the pollinated pistils, while small amounts were still detectable in the unpollinated flowers. Pollination of flowers at stage 6 showed a different expression pattern (Figure 4C and D). Figure 4C shows that PPAL transcript level started to increase after 3 h with a peak at 12 h.a.p. in the pollinated pistils. At this time the PPAL mRNA level in the pollinated pistils was about 7-fold higher than in the unpollinated pistils. Later, the PPAL mRNA level in the pollinated pistil began to decrease to reach a level comparable to that found in the unpollinated ones at 48 h. Figure 4D shows that PPAL mRNA transcripts were not detectable in the unpollinated and pollinated ovaries at stage 6, but they were detectable at 48 h.a.p.. Using antibodies raised against recombinant PPAL protein we were able to detect the same expression pattern at the protein level (data not shown). These results show that pollination always induced PPAL gene expression in the stigma and ovary and that both transcripts and proteins accumulated during a short period from 12 to 24 h.a.p. In addition, PPAL transcripts were no longer present in pollinated pistils at 48 h.a.p.

Spatial expression of the PPAL gene

The strong increase in the expression level of the PPAL mRNA after pollination, together with its rela-

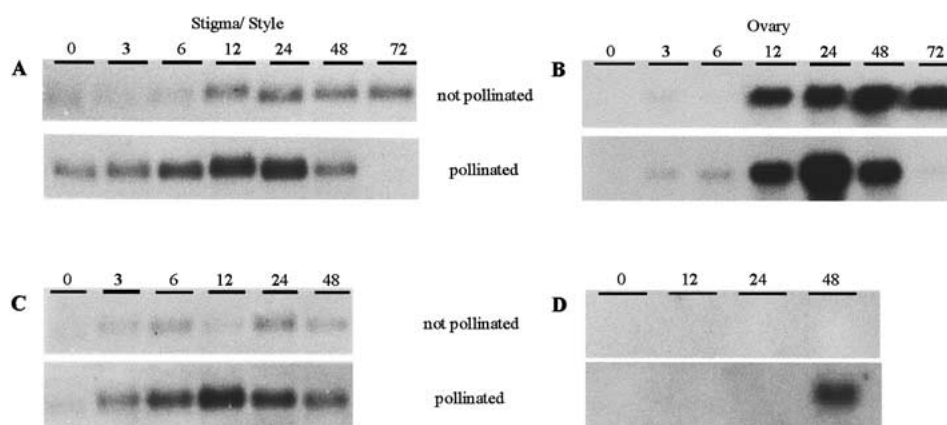


Figure 4. PPAL expression is modulated by pollination. In all these experiments flowers taken as control were emasculated but not pollinated, and stigmas/styles and ovaries were collected at the same time-points as their pollinated counterparts (0, 3, 6, 12, 24, 48 and 72 h.a.p). A. Accumulation of PPAL mRNA in stigmas/styles at stage 12 unpollinated and after pollination. A 10 μ g portion of total RNA was loaded in each lane. B. Accumulation of PPAL mRNA in ovaries at stage 12, unpollinated and after pollination. A 10 μ g portion of total RNA was loaded in each lane. C. Accumulation of PPAL mRNA in stigmas/styles at stage 6, unpollinated and after pollination. A 10 μ g portion of total RNA was loaded in each lane. D. Accumulation of PPAL mRNA in ovaries at stage 6, unpollinated and after pollination. A 10 μ g portion of total RNA was loaded in each lane.

tively low level during the early stages of pistil development, suggested that PPAL gene products may be instrumental in pollen-pistil interactions in the mature pistil. Consequently, the site of expression was precisely established. Figure 5A shows that PPAL mRNA was confined to the stigmatic secretory zone. There was no hybridization signal on the epidermis, cortical cells or vascular bundles. In the ovaries PPAL mRNA was found in the epidermal layer of the placenta (Figure 5C). Signals were absent in the section hybridized to the sense RNA probe (Figure 5B and D).

Presence of PPAL in different flowers and exudates of tobacco

Since PPAL protein is preceded by a signal peptide, we studied the presence of PPAL protein in the exudate of flowers at stages 10 and 11 (Goldberg, 1988). As a control, protein extracts were used from intact stigmas and from stigmas from which the exudate had been washed out. Figure 6 shows that PPAL protein was present in the exudate, suggesting that PPAL may be needed in the first interaction between pollen and pistil. In addition, we investigated whether PPAL- or PPAL-like proteins were expressed in related and unrelated species. Western blot analysis showed that among the stigmas of potato, eggplant, tobacco, petunia, rape seed and *Datura*, only those of tobacco and petunia appeared to contain PPAL protein. However, the petunia protein has a smaller molecular weight that may derive from post-translational modifications

present in tobacco and absent in petunia. All of the other species tested, including the other Solanaceae, did not produce in the stigma a protein cross-reacting with the PPAL antibody.

Discussion

Using the differential screening procedure we isolated an almost full-length pistil-specific cDNA that may code for a 29.4 kDa protein (PPAL) of 274 amino acids. The observed changes in the expression of PPAL RNA and protein coincided. The PPAL protein contains a leader sequence and is secreted in the stigmatic exudate of the pistil. PPAL shows a particular pattern of temporal and spatial expression and has a strong homology to the group-I grass pollen allergens and to β -expansins.

PPAL function: similarity to pollen allergens and β -expansins

PPAL is the first group-I grass pollen allergen-like gene shown to be expressed in the pistil of dicots. Earlier, Balzer *et al.* (1996) cloned a group-III pollen allergen like gene from wheat ovaries. A number of pollen allergens have been isolated and characterized, but they have been mainly studied for their immunological properties and clinical applications, and little is known about the actual function of these proteins in plants. Although group-I grass pollen allergens

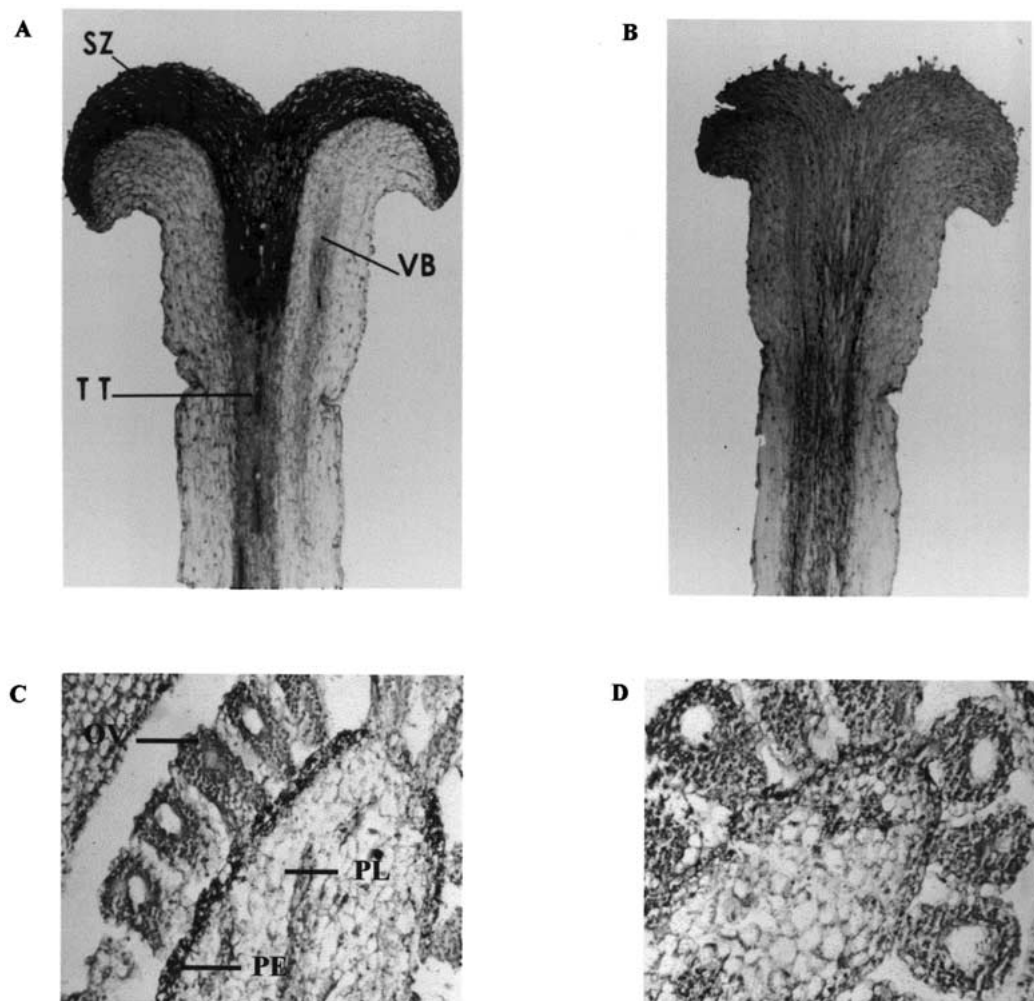


Figure 5. *In situ* localization of PPAL mRNA. A. Localization of PPAL mRNA in stigma and style a pistil at stage 11. *In situ* hybridization with an antisense PPAL dioxigenin-labelled RNA probe. Dark-purple coloration represents regions containing RNA-RNA hybrids. SZ, secretory zone; TT, transmitting tissue; VB, vascular bundle. B. *In situ* hybridization with a sense PPAL dioxigenin-labelled RNA probe on stigma and style at stage 11 (background control). C. Localization of PPAL mRNA in 24 h pollinated ovary. *In situ* hybridization with an antisense PPAL dioxigenin-labelled RNA probe. Dark-purple coloration represents regions containing RNA-RNA hybrids. OV, ovule; PE, placental epidermal layer; PL, placenta. D. *In situ* hybridization with a sense PPAL dioxigenin-labelled RNA probe on 24 h pollinated ovaries (background control).

share some immunologically similar characteristics, like binding Mab 3A2 and human IgE antibodies, the suggestions made so far on their function have been based on homologies to other known genes. For example, the major birch pollen allergen Bet vI was shown to be similar to pathogenesis-related proteins (Swoboda *et al.*, 1996). The pollen allergens Amb aI from ragweed and Cry jI from Japanese cedar are believed to be pectate lyases (Knox *et al.*, 1996). Zea m1 from *Zea mays* was shown to have expansin activity and may have a role in loosening the cell walls of the stigma and style to allow penetration of the

pollen tube through these tissues. The soybean Cim I, a vegetative homologue of pollen allergens, is also believed to have a cell-wall-loosening role, consistent with the induction of its expression by cytokinin, which stimulates cell proliferation and growth in soybean cell cultures. Since Zea mI/Cim I share a limited sequence homology to expansins, Cosgrove *et al.* (1997) suggested that this group should be denoted as β -expansins, which are believed to act on different cell-wall components than the members of the original α -expansin family. Lol pI from ray grass and Cyn dI from Bermuda grass can also be placed in this new

group of expansins on the basis of their homology. The overall similarity between PPAL and these group-I grass pollen allergens ranges from 55% to 66%, which is very high taking into account the evolutionary distance between grasses and solanaceous species. α -Expansins, β -expansins, group-I grass pollen allergens and PPAL share key domains important for folding and the active site important for expansin action. These regions include a series of conserved Cys residues that are likely involved in disulfide bridges within the protein, a series of Trp residues that may function in protein-polysaccharide binding and a His-Phe-Asp motif. The latter motif and the Cys residues are also highly conserved in members of the family of K-cellulases (Cosgrove, 1997, 1998). Interestingly, PPAL, like the group-I grass pollen allergens (β -expansins) and α -expansins, contains the functionally important papain-like sequence motifs. Grobe *et al.* (1999) showed that the pollen allergen Phl pI from timothy grass has papain-like proteinase activity. The possible target for papain like activity could be the Tyr-Lys-Tyr sequences found in extensins, which function in cell-wall strengthening in the cell walls of dicots and some monocot plants. The expansin activity found in group I grass allergens and possibly in PPAL, could therefore be exerted by cleaving extensins endoproteolytically, which results in a weakening of the walls of the tissues mediating plant cell-wall growth. However unpublished data by Li and Cosgrove suggest that this may not be the case for all grass pollen allergens (Cosgrove, 2000).

Phylogenetic analysis of the amino acid sequences of α -expansins, β -expansins and group I grass pollen allergens revealed that PPAL is closely related to the latter (data not shown). On the basis of this homology and consequent structural similarity, PPAL should be regarded as a new expansin. In conclusion, PPAL is an expansin-like protein found in the pistil of tobacco; it could function in pollen-tube growth by facilitating pollen adhesion, pistil penetration and growth over its natural track.

PPAL in pollen-tube growth and fertilization

RNA and protein levels of PPAL in the pistil increase during development in flowers at stage 5 until maturity and are strongly enhanced by pollination. Expression then decreases to an undetectable level 72 h.a.p., when fertilization normally is completed. However, PPAL is still expressed in unpollinated pistils. The PPAL transcript is present in the stigmatic secretory zone of

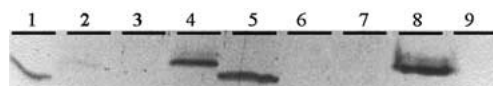


Figure 6. Accumulation of PPAL protein in stigma and exudate of tobacco and other species. Immunoblot analysis. A 40 μ g portion of total protein was loaded in each lane. The colour reaction was performed with the alkaline phosphatase reaction. Proteins were extracted from stigma of *Petunia* line W115 (1), tobacco stigma from which the exudate was removed (2), stigma of egg-plant (3), tobacco stigma on which exudate was still present (4), stigma of *Petunia* line V5 (5), stigma of *Brassica* (6), stigma of *Datura* (7), exudate of tobacco (8) and stigma of potato (9).

the pistil but also in the epidermal layer of the ovary, although in the latter it is only expressed at anthesis after pollination. Thus, PPAL accumulation seems to correlate with those sites (such as the exudate and ovary cavity) where pollen tubes could grow without physical constraints. Such a distribution indicates that PPAL could play some role in both the stigma and ovary. In this respect, it is interesting that the expression of PPAL always increases with pollination. Moreover, the PPAL transcript appears in the ovary of young flowers exclusively after pollination, at a time point corresponding to stage 8 of flower development. The increase in PPAL expression in stigmas and ovaries after pollination indicates the presence of a signalling route between pollen and pistil. The increased expression in mature ovaries especially proves that the signal moves ahead of the growing pollen tubes, as at 12 h.a.p. the pollen tubes are only half-way along the style of tobacco. This increase in PPAL expression in the ovary may be part of a more extensive response, as shown by Chandra Sekhar *et al.* (1995). The ovaries of tobacco express at least 11 newly synthesized proteins, all of which precede the arrival of the pollen tubes. The actual nature of the signals and their routes through the tissues, however, are still unknown.

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