

RESEARCH ARTICLE

Developmental Expression of Tobacco Pistil-Specific Genes Encoding Novel Extensin-Like Proteins

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We have sought to identify pistil-specific genes that can be used as molecular markers to study pistil development. For this purpose, a cDNA library was constructed from poly(A)⁺ RNA extracted from tobacco stigmas and styles at different developmental stages. Differential screening of this library led to the isolation of cDNA clones that correspond to genes preferentially or specifically expressed in the pistil. Seven of these cDNA clones encode proteins containing repetitions of the pentapeptide Ser-Pro₄, which is a typical motif found in extensins. Unlike extensin genes, the extensin-like genes described here are not induced under stress conditions. RNA gel blot hybridizations demonstrated the organ-specific expression of the extensin-like genes and their temporal regulation during pistil development. After pollination, the transcript levels of the pistil-specific extensin-like genes change relative to levels in unpollinated pistils. In situ hybridization experiments showed that at least one of these pistil-specific genes is specifically expressed in cells of the transmitting tissue. The possible roles of the extensin-like proteins in pistils are discussed.

INTRODUCTION

In angiosperms, the pistil and the stamen of the flowers are the specialized organs responsible for the reproductive processes (Esau, 1977). Generally, the pistil is composed of the stigma, style, and ovary. The sporogenous cells of the ovary lead to the production of the female gametophyte or embryo sac that contains the egg cell. The transfer of the pollen grain from the stamen to the stigma initiates the processes that can result in fertilization. Once in the stigma, the pollen germinates and the emerging pollen tube grows through the extracellular matrix of the stylar transmitting tissue toward the ovary.

In angiosperm sexual reproduction, there is an interaction between the male gametophyte (the pollen grain) and the massive sporophytic tissue of the pistil (Shivanna and Sastri, 1981). The major events during pollen–pistil interactions are the recognition and the subsequent acceptance or rejection of the male gametophyte by the pistil (Shivanna and Sastri, 1981). The pistil discriminates between the different types of pollen it receives. Usually wide intergeneric and interspecific crosses are avoided, whereas intraspecific crosses are successful except when self-incompatibility genes prevent inbreeding (Cornish et al., 1988). Molecular studies suggest that specific proteins are responsible for the postpollination behavior of the pollen in the pistillate tissue (Nasrallah and Nasrallah, 1989;

Haring et al., 1990). One of the most active areas of research related to pollen–pistil interactions is self-incompatibility. In contrast, relatively little research has been done on the molecular cell biology of pollen–pistil interactions during compatible matings (McCormick, 1991).

Despite the central importance of the pistil in the reproduction of flowering plants, there are to our knowledge only two pistil-specific genes studied so far. Gasser et al. (1989) and Budelier et al. (1990) have isolated and characterized the expression of the tomato gene 9612, whose function in the pistil is still unknown. The other example is the 1,3- β -glucanase genes specific to the stylar transmitting tissue, for which cDNA clones have been isolated from tobacco (Ori et al., 1990). The expression of these genes in the style is developmentally regulated.

Our interest is to identify and characterize genes that are specifically expressed in the pistil and to establish their possible function in pistil development, pollination, and pollen–pistil interactions. We have chosen the self-compatible species of tobacco as our model system. A stigma/style cDNA library was constructed and differentially screened, resulting in the isolation of cDNA clones corresponding to genes preferentially or specifically expressed in the pistil. Here, we describe the developmental expression pattern of pistil-specific genes encoding novel types of extensin-like proteins (PELPs).

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RESULTS

Isolation of Pistil-Specific cDNA Clones

We isolated pistil-specific cDNA clones from a tobacco stigma/style cDNA library by differential screening against seedling cDNA probes, as described by Gasser et al. (1989). Seedlings were chosen because all the major vegetative organs (roots, stems, and leaves) are represented. Most of the recombinant clones from the cDNA library were expressed both in stigma/styles and in vegetative organs. The initial screening resulted in the identification of 113 plaques that hybridized only to the stigma/style cDNA probes. Sequence analysis revealed that seven of the purified cDNA clones may encode proline-rich proteins. Based on their deduced amino acid sequence and hybridization pattern on RNA gel blots, these clones were divided into three independent classes.

Class I is represented by the cDNA clones pMG02 and pMG04 that hybridize to an mRNA of 0.95 kb. Class II includes the clones pMG08 and pMG09 and corresponds to a transcript of 1.8 kb. Class III consists of clones pMG07, pMG14, and pMG15 that are homologous to an mRNA of 1.9 kb. Figure 1 shows the homology at the level of deduced amino acid sequence between the cDNA clones of each class. Within each class, the polypeptides differ along their length by a few substitutions and by deletions and insertions of groups of amino acids. In class II, the cDNA clone pMG09 has a stop codon (UAA) in a region of high residue identity with the clone pMG08 (Figure 1B), which has a corresponding codon of CAA (glutamine). We suspect that the stop codon in pMG09 is the result of a point mutation during the cloning procedure.

The proteins encoded by all three classes contain a few repetitions of the pentapeptide Ser-Pro₄, which is predominantly located between the N-terminal region and the center of the polypeptide and is absent on the C terminus. The pentapeptide Ser-Pro₄ is a conserved repetitive sequence characteristic of the extensin, which is a protein rich in proline, serine, valine, tyrosine, and lysine, and thus highly basic (Tierney and Varner, 1987). The cDNA clones that we have isolated encode proteins that have a predicted high pI and are rich in proline, serine, valine, and lysine, but have a very low content of tyrosine. The cDNA clones of classes I and II are not full length, so the amino acid composition and pI results must be considered as preliminary. Class III shows the highest similarity to the extensins of tobacco described by Memelink (1988) and Keller and Lamb (1989); however, the residue identity is low and mainly restricted to the proline and serine residues (data not shown).

Figure 2A presents the nucleotide sequence of the class III cDNA clone pMG15 in which proline residues are mainly encoded by CCA (69 times) and serine residues by the codons TCA (16 times) and TCT (11 times). This clone carries an almost full-length cDNA; the corresponding mRNA codes for a predicted polypeptide containing 426 amino acid residues, which is equivalent to 44.3 kD. A hydropathy plot (Kyte and Doolittle, 1982) of the deduced amino acid sequence of pMG15

(Figure 2B) shows a hydrophobic N terminus characteristic of a signal peptide (Von Heijne, 1986). A potential cleavage site was located between amino acids serine-23 and lysine-24 (Figure 1C). By considering the overlap between the cDNA clones from class I (Figure 1A), it was possible to obtain an indication of the entire amino acid sequence of the corresponding protein. The predicted class I protein also carries a putative signal peptide, whereas the potential cleavage site is most likely located between the two glutamine residues at positions 22 and 23 (Figure 1A). It seems that the proteins encoded by classes I and III enter the secretion pathway and are localized extracellularly. The information available about the class II protein does not allow any suggestion about its subcellular localization.

Extensin-Like Genes Are Differentially Regulated

To determine if the extensin-like genes are expressed in an organ-specific fashion, RNA was extracted from roots, stems, leaves, sepals, petals, anthers, stigma/styles, ovaries, seeds, and germinating seeds. The results of the RNA gel blot analyses of these RNAs are shown in Figure 3. The class I 0.95-kb transcript is weakly detectable in sepals; present in petals, anthers, and ovaries; and abundant in stigma/styles. The isolation of cDNA clones with an expression pattern like class I can be explained by the differential screening performed against seedling cDNA probes, which do not include mRNAs of floral organs. The class II 1.8-kb transcript accumulates to a high level in stigma/styles and to a very low level in ovaries. Class III exhibits the same expression pattern as class II and corresponds to an mRNA species of 1.9 kb. These experiments show that the three classes of extensin-like genes are differentially regulated; class I is flower specific, and classes II and III are pistil specific. The existence of flower-specific and pistil-specific extensin-like mRNAs suggests that extensin-like proteins may have special roles during floral processes. Interestingly, Meeks-Wagner et al. (1989), Neale et al. (1990), and Peeters (1991) showed that extensin genes are expressed during tobacco flower formation *in vitro*.

Pistil-Specific Extensin-Like Genes Are Not Induced Under Stress Conditions

Extensin gene expression increases in response to wounding, pathogen infection, and ethylene treatment. This suggests that the corresponding gene product has a role in plant defense reactions (Cassab and Varner, 1988; Memelink, 1988; Showalter and Varner, 1989). To determine whether the expression of the genes encoding PELPs is induced in vegetative tissues under stress conditions, we extracted leaf RNA from plants subjected to wounding, tobacco necrosis virus infection, and ethylene treatment, as well as from appropriate controls (see Methods). The results of the RNA gel blot hybridizations are shown in Figure 4. None of the extensin-like genes

A class I

pMG02 MAGMNNMLMLMVAAILFCSHQVATAREVVVADDRNELQLLWPWE
 pMG04 NELQLLWPWE

47 IPCYLTWFPFWPPPPWPCPPRRPRRRPRPCP**SPPPP**RRPRPCP
 11 IPCYLTWFPFWPPPPWPCPPRRPRRRPRPCP **CP****SPPPP**RRPRPCP

93 **SPPPP**QPRRR**SPPP**
 55 **SPPPP**RRPRPCP**SPPPP**QPRRR**SPPPP**SPPPPAPSSSCSASDES

108

101 NIYRCMFNETKIDPCCPTFKSILGTSCPCYKYAENLDNQVLITIES

108

147 YCDVDSCKGVQVIKLSKEEEKKKK.

B class II

pMG08 AFTSVKILVLIQVSVLALSSFSELSFGKGISSSLDKGQHHPFST
 pMG09

47 VHLFFGKSPKSPSSPTPVNKPSPSPPPQVKSSLPPPA **KSPPPPP**
 1 **QPPATQ**RATPPP

93 AKSPPPLL**PPPP**SQPPKQPPPPPPPAKQPPSAKPPKPPSPSPAA
 11 **A** **MQRAPP** **LSQPPK**.PPPPPPPAKQPPSAKPPKPPSPSPAA

139 **QPPATQ**RATPPFAMQRA
 52 **QPPATQ**RATPPFAMQRAPPLSQPPKLP

C class III

pMG07 LPFELPPAEIP
 pMG14 LPFELPPAEIP
 pMG15 MAVIISKVLIIQLFVLVLSFSKLSHGELWLELPLPFDWPPAEIP

13 LPEIPLPFDGPTFVLPPP **SPPPPP**
 12 LPEIPLPFDGPTFVLPPP **SPPPP**PSPLP**SPPPP**SP**SPPPP**S
 47 LPDIPSPFDGPTFVLPPPSPLP**SPPPP** SP **SPPPP**SP**SPPPP**S

37

54 TLIPLIPFPGTGGFLPPLPGSKLP GLLPLIPNLPLDLPPIGGGPP
 89 T IPLIPFPGTGGFLPPLPGSKLPDFAGLLPLIPNLPLDVPPIGGGPP

37

97 VNRPKPSSSPSPVKPP**PPPP**SPCKPSPDQSTKQPP**PPPP**SKQPP
 134 VNQPKPSSSPSPVKPP**PPPP**SPCKPSPDQSAKQPPQPPPAKQ**SP**

37

143 PPP VKASSPSPAKQPPPPPPPVKSPSPSPAKQ**SPPPP**RAPSPS
 180 **PPPP**PPVKAPSPSPAKQPPPPPPPVK APSPS

58 PATQPPKQ**PPPP** **SAKSP** **PPPV**AYPPVMAFSPSPAAEPPI
 186 PATQPPKQ**PPPP** **SAKSP** **PPPV**AYPPVMAFSPSPAAEPPI
 211 PATQPPKQPPPPPRAKKSPLLPPPPVAYPPVMTSPSPAAEPPI

99 IAPFPSPTANLPLIPRRAPPVVKLPPLGKPPIVNGLVYCKSCNS
 227 IAPFPSPTANLPLIPRRAPPVVKLPPLGKPPIVNGLVYCKSCNS
 257 IAPFPSPTANLPLIPRRAPPVVKLPPLGKPPIVSGLVYCKSCNS

145 YGFPTLLNTSLLPGAVVKLVLCYNGKKTMTVQSATTDNKGEFRIIPKS
 273 YGFPTLLNTSLLPGAVVKLVLCYNGKKTMTVQSATTDNKGEFRIIPKS
 303 YGVPTLLNASLLQGAUVKLCY GKKTMTVQWATTDNKGEFRIMPKS

191 LTRADVCKKLYLVKSPNPNPNCVPTNFNGGKSGGLLKPLLPPKQPI
 319 LTRADVCKKLYLVKSPNPNPNCVPTNFNGGKSGGLLKPLLPPKQPI
 348 LTTADVCKKVLYLVKSPNPNPNCVPTNFNGGKSGGLLKPLLPPKQPI

237 TPAAVP LSDLYGVGPFI FEASSKMPCDKN.
 365 TPAAVP LSDLYGVGPFI FEASSKMPCDKN.
 394 TPAVVPVQPPMSDLYGVGPFI FEASSKMPCDKN.

Figure 1. Deduced Amino Acid Sequences of Classes I, II, and III cDNA Clones.

(A) Alignment of the cDNA clones belonging to class I according to the Genalign program (IntelliGenetics).

(B) Alignment of the cDNA clones belonging to class II according to the Genalign program.

(C) Alignment of the cDNA clones belonging to class III according to the Genalign program.

Identical amino acids are identified by vertical lines, and the pentapeptides Ser-Pro₄ are presented in boldface letters. The arrows indicate the potential signal peptide cleavage sites as determined by computer analysis (PC/Gene—program psignal), whose scores are 5.5 for class I and 7.25 for class III. Blocks of amino acid repeats are underlined, and putative N-glycosylation sites are identified by asterisks.

A cDNA clone pMG15

1	GTTAACAAC TATGG CTGTTATTATTTTCATCGAAGGTCCTACTTATACA	1009	CACGACAGACAACAAAGGTGAGTTTCGGATCATGCCCAAATCTTTAAC
1	M A V I I S S K V L L I Q	334	T T D N K G E F R I M P K S L T
49	ACTTTTCGTTCTAGTACTTGGCTCATTCTCAAAGCTCAGCCATGGGGA	1057	CACAGCAGATGTTGGCAAGTGCAAGGTATATTTAGTGAAATCACCAA
14	L F V L V L G S F S K L S H G E	350	T A D V G K C K V Y L V K S P N
97	GCTGTGGCTGGAACCTCCATTGCCTTTTCGACTGGCCACCAGCGGAGAT	1105	TCCAAATTGCAATGTCCCAACAAATTTCAATGGTGGAAAATCTGGTGG
30	L W L E L P L P F D W P P A E I	366	P N C N V P T N F N G G K S G G
145	CCCATGCGCGATATCCCATCGCCTTTTCGATGGGCCTACATTCGTGCT	1153	TTTATTGAAGCCTCTCCTACCACCTAAACAACCGATTACCCCTGCCGT
46	P L P D I P S P F D G P T F V L	382	L L K P L L P P K Q P I T P A V
193	ACCGCCACCGTCACCATTTGCCATCACCACTCCGCCATCTCCATCACC	1201	TGTCCCTGTCCAACCACCCATGTCTGATTATATGGTGTGGACCTTT
62	P P P S P L P S P P P P S P S P	398	V P V Q P P M S D L Y G V G P F
241	ACCTCCGCCATCACCATCACCACTCCTCCGTCGACAATACCACTTAT	1249	TATATTTGAAGCCTCCAGCAAAATGCCATGCGATAAGAAT TGAG CTCC
78	P P P S P S P P P P S T I P L I	414	I F E A S S K M P C D K N
289	TCCTCCTTTTACCGGCGGCTTTTTCGCTCCTTTGCCCGGCTCTAAGCT	1297	TCATTAACAGAGAATACAAGCCAGAAAGATGGGAATTAATGTATGAGC
94	P P F T G G F L P P L P G S K L	1345	ATGAGTTTGTGATGGAATTTTTTTTCTTTTGTCTATAGTTTATACA
337	TCCTGATTTTGGCGGCTTTTTCGCTCTGATTCCCAACTTACCTGATGT	1393	AGGAGACAGAAAACCTTTGTACCACTATACAGAAATCAAATCAGTCGGA
110	P D F A G L L P L I P N L P D V	1441	AAATTCAAAATCGAACTTATGAAAACGAAAAA
385	GCCTCCCATAGGTGGTGGTCCACCTGTTAACCAGCCAAAGCCGTCGTC	1489	AAAAAAAAAAAAAAAAAAAA
126	P P I G G G P P V N Q P K P S S		
433	ACCATCACCCCTAGTTAAGCGCCACCACCACCACTTCACCATGTAA		
142	P S P L V K P P P P P P S P C K		
481	GCCATCACCACTGATCAGTCTGCAAGCAACCACCAACAACCTCCTCC		
158	P S P P D Q S A K Q P P Q P P P		
529	AGCAAAACAACCATCACCTCCACCTCCTCCACCACCACTTAAGGCACC		
174	A K Q P S P P P P P P P V K A P		
577	ATCTCCATCTCCAGCAAAGCAACCACCACTCCTCCACCGCCAGTTAA		
190	S P S P A K Q P P P P P P P V K		
625	GGCACCATCTCCATCTCCAGCCACTCAGCCACCTACAAAGCAACCGCC		
206	A P S P S P A T Q P P T K Q P P		
673	ACCGCCGCCACGTGCTAAGAAATCGCCTCTTCTACCTCCTCCGCCACC		
222	P P P R A K K S P L L P P P P P		
721	AGTTGCTTATCCGCCAGTTATGACACCATCTCCATCACCGGCTGCTGA		
238	V A Y P P V M T P S P S P A A E		
769	GCCACCTATTATAGCACCATTTCCATCACCAACAGCGAATCCACCCCT		
254	P P I I A P F P S P P A N P P L		
817	CATTCCTCCGTCGACCAGCACCACTAGTTAAGCGGCTTCCACCTTT		
270	I P R R P A P P V V K P L P P L		
865	GGGGAAGCCCCCAATCGTCACTGGCCTTGTATTGTAAATCCTGCAA		
286	G K P P I V S G L V Y C K S C N		
913	CAGCTATGGGGTTCCCACTCTGCTCAACGCCTCCCTACTCCAAGGAGC		
302	S Y G V P T L L N A S L L Q G A		
961	TGTTGTGAAACTAATTTGCTACGGAAAAAACAATGGTTCAATGGGC		
318	V V K L I C Y G K K T M V Q W A		

Figure 2. Analyses of the cDNA Clone pMG15 from Class III.

(A) Nucleotide sequence of the MG15 cDNA and its deduced amino acid sequence. The translation start position and the stop codon are shown in boldface letters, and the putative polyadenylation signal (AAUCAA) (Dean et al., 1986) is underlined.

(B) Hydropathy index of the 426 amino acid residues of the MG15 protein, determined according to Kyte and Doolittle (1982). The mean hydropathy plot of a window of nine consecutive amino acids is plotted against the amino acid number.

are induced in leaves under the different stress conditions tested, in contrast to the stress-inducible 1,3- β -glucanase gene (Gheysen et al., 1990), which we used as an internal control (data not shown). These experiments indicate that the PELPs

are not involved in the normal reactions of plant defense. Taken together, our results show that the extensin-like genes identified here encode novel types of proteins containing the Ser-Pro₄ motif, which are specifically expressed in flowers.

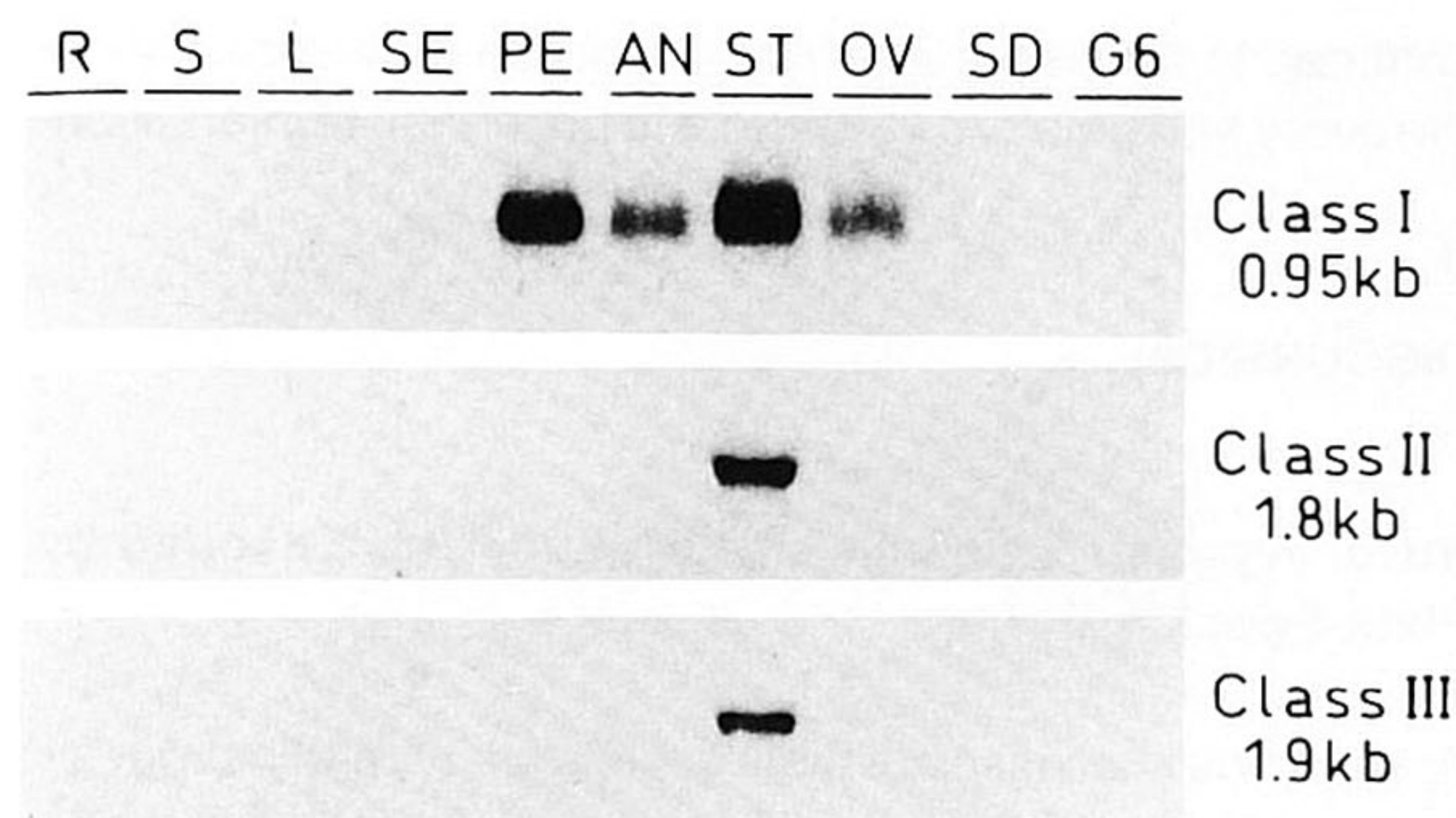


Figure 3. Accumulation of the Extensin-Like mRNAs in Different Organs.

RNA gel blot analyses of classes I, II, and III transcript levels in different organs of tobacco plants. Total RNA (10 μ g) was loaded in each lane. R, roots; S, stems; L, leaves; SE, sepals; PE, petals; AN, anthers; ST, stigmas and styles; OV, ovaries; SD, seeds; G6, germinating seeds 6 days after start of imbibition.

Extensin-Like Genes Are Temporally Regulated during Pistil Development

We have characterized the expression pattern of the PELP genes on gel blots with RNA isolated from stigma/styles at different stages of tobacco flower development as described previously (Goldberg, 1988; Koltunow et al., 1990). At stage 1, the pistil is already fully differentiated (Koltunow et al., 1990), and, at stage 12, pistil maturation and anthesis occur. Figure 5A shows that the transcript level of class I is already high in floral buds of stage 1 and reaches a maximum level at stage 5, followed by a slow decrease during further development. The mRNA complementary to class II arises in stage 1 buds, and its level gradually increases during flower development toward anthesis. The transcript corresponding to class III first appears at stage 2, and its temporal accumulation is comparable to that of class II. Both class II and class III transcripts reach the highest levels at stage 8 and remain relatively constant until stage 11. Together, these data indicate that the extensin-like genes described here are temporally regulated during tobacco pistil development.

In tobacco, stigmas from floral buds 6 days before anthesis (stages 6 and 7; Goldberg, 1988) are already receptive and capable of supporting pollen germination and tube growth (Shivanna and Sastri, 1981). Assuming that protein levels correlate with mRNA levels, the presence and abundance of the extensin-like proteins in stigmas and styles coincide with the receptivity in tobacco. Our results suggest that the PELPs of classes II and III may play a role in reproductive physiological processes in the stigma and style.

Extensin-Like Gene Expression Is Modulated by Pollination

Because pollination initiates many physiological changes in the pistil (Shivanna, 1982), we analyzed the expression of the extensin-like genes in relation to that process. Emasculated flowers at stage 12 were pollinated, and the pistils were collected 0.5, 1, 2, 3, and 6 days after pollination. As a control, pistils from emasculated but not pollinated flowers were collected at identical times (see also Methods). Figure 5B shows the RNA gel blot analyses of the collected pistils. The results indicate that class II and class III transcript levels gradually decrease after pollination, while transcript levels remain virtually constant in unpollinated pistils. The class II and class III transcripts are absent in pistils 6 days after pollination, when the stigma and style have already abscised. In contrast, the expression of class I decreases at about stage 11 in stigma and styles (Figure 5A), although it increases again 2 days after pollination. The hybridization signal observed for class I mRNA 6 days after pollination suggests that this increase is due to a higher expression in the ovary (Figure 3), which is the only part of the pistil present at this stage. In unpollinated pistils, the expression of the class I gene remains essentially constant. These results indicate that the expression of the PELP genes is modulated by the pollination process and that the class II and class III mRNAs are not necessary after successful pollination.

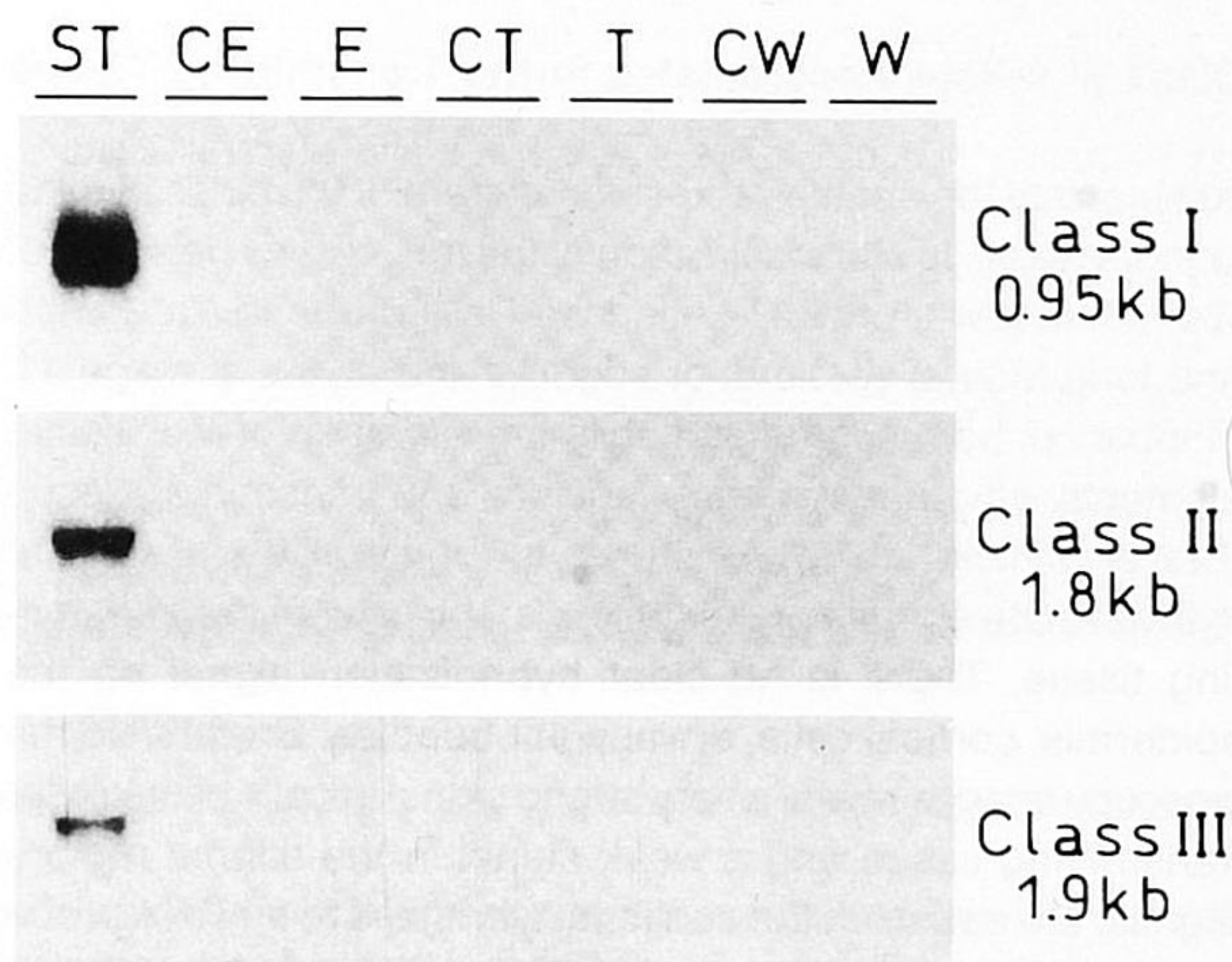


Figure 4. The Extensin-Like mRNAs Are Not Induced in Vegetative Tissues under Stress Conditions.

RNA gel blot hybridizations of classes I, II, and III cDNA clones with leaf RNA from plants subjected to different stress conditions (see Methods). Total RNA (20 μ g) was loaded in each lane, except for the positive control stigma and style total RNA (10 μ g). ST, stigmas and styles; CE, control of the ethylene treatment; E, ethylene treatment; CT, control of tobacco necrosis virus infection; T, tobacco necrosis virus infection; CW, control of wounding; W, wounding.

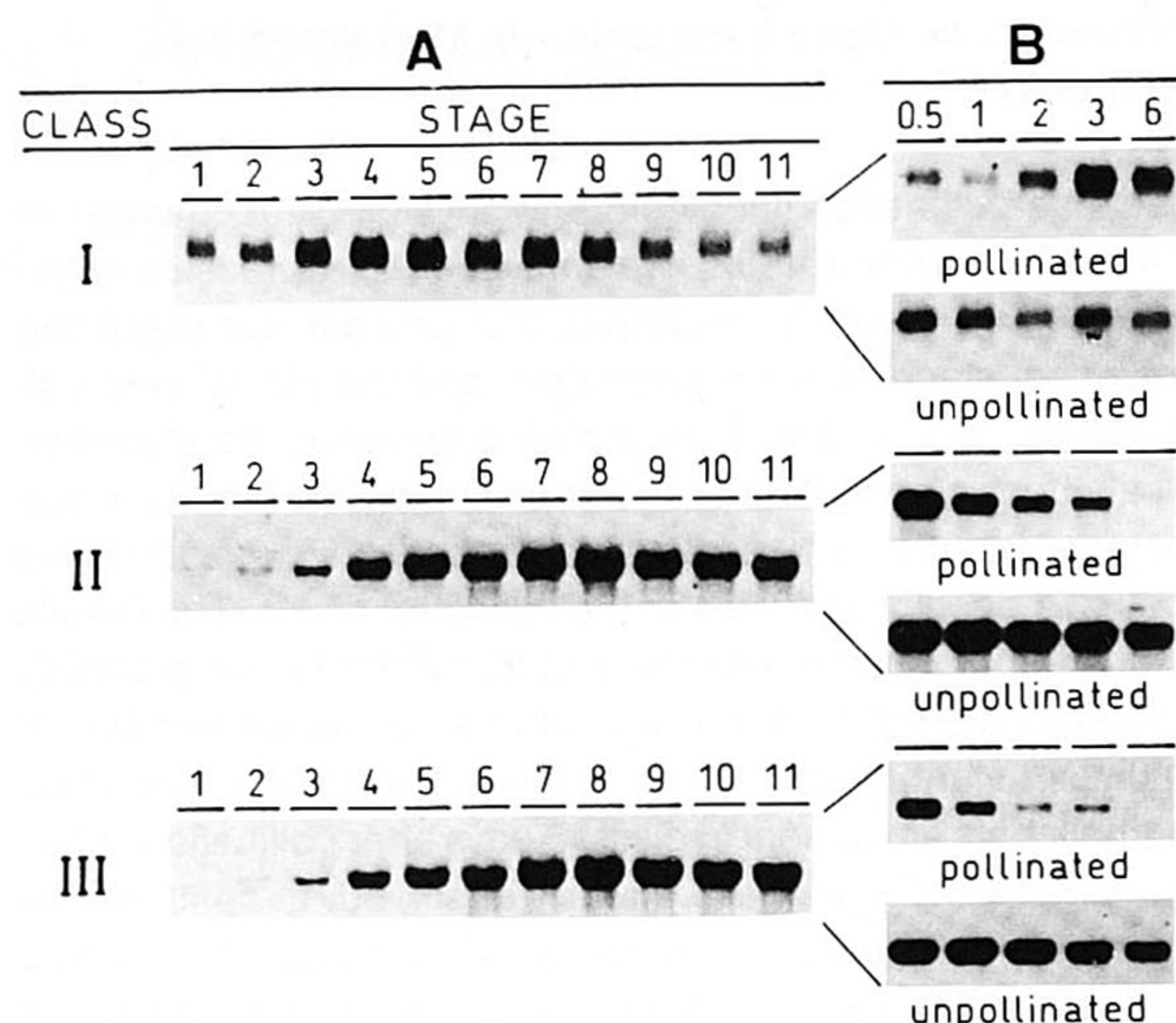


Figure 5. Accumulation of Extensin-Like mRNAs during Flower Development.

RNA gel blot analyses of classes I, II, and III at different flower developmental stages. Ten micrograms of total RNA was loaded in each lane.

(A) RNAs extracted from stigmas and styles of flowers from stage 1 to 11 (Goldberg, 1988; Koltunow et al., 1990).

(B) Time course of pistils at different periods of time after pollination and their correspondent unpollinated pistils (see Methods).

Class III mRNA Accumulates in the Transmitting Tissue

To elucidate the spatial expression pattern of the class III gene in more detail, *in situ* localization of the mRNA was performed. We hybridized a pMG07 antisense RNA probe to transverse and longitudinal sections of stigmas and styles at stage 11. In tobacco, both stigma and style are made up of four tissue elements: epidermis, cortex, vascular, and transmitting tissue (Bell and Hicks, 1976). As shown in Figures 6A and 6C, the hybridization signals are restricted to the cells of the transmitting tissue. There is no clear hybridization signal on the epidermis, cortical cells, or vascular bundles. Bright-field microscopy images reveal a very strong signal in cells of the stylar transmitting tissue and a weak signal in the stigma region. Signals are absent in the sections hybridized to an RNA probe (see Methods) used as negative control (Figures 6B and 6D).

The result obtained by *in situ* hybridization experiments showed that the expression of the class III gene is exclusive to the transmitting tissue cells. The difference observed between the hybridization signals in stigma and style may reflect differences in class III mRNA abundance in these structures. Another possible explanation is that this difference is caused by the higher cytoplasm concentration in the cells of the stylar transmitting tissue as compared to the stigmatic cells. These results suggest that the expression of the class III gene is

confined to the tissue in which the pollen tube grows toward the ovary and points toward a role in pollen–pistil interactions.

DISCUSSION

Novel Types of Extensin-Like Proteins Are Encoded by Pistil-Specific Genes

To study the processes of pistil development, pollination, and pollen–pistil interaction at the molecular level, we identified and isolated cDNAs that correspond to genes specifically or preferentially expressed in the pistil. These genes provide molecular markers for the analysis of pistil development and can be used to study the regulation of pistil-specific gene expression. We have studied three classes of cDNA clones that are differentially regulated: class I is flower specific, and classes II and III are pistil specific.

The cDNA clones of classes I, II, and III encode proteins with a few repetitions of the pentapeptide Ser-Pro₄, which is a motif typical of extensins (Tierney and Varner, 1987). In the extensins, this motif is generally repeated several times and is present throughout the whole polypeptide backbone. The proline residues are extensively hydroxylated in the lumen of the endoplasmic reticulum and subsequently O-glycosylated in the Golgi apparatus (Cassab and Varner, 1987). The serine residues can be O-galactosylated (Smith et al., 1984; Wilson and Fry, 1986). These post-translational modifications are followed by transport of the glycoproteins to the cell membrane that may lead to secretion. In the cell wall, extensins are rapidly insolubilized, presumably through the formation of intramolecular and intermolecular covalent crosslinks between the tyrosine residues (Epstein and Lampion, 1984; Wilson and Fry, 1986). The PELPs differ from previously described extensins by a low tyrosine content, a lower copy number of the Ser-Pro₄ motif, and, overall, a less repetitive nature. If extensin insolubilization is directly related to tyrosine content, it can be inferred that the PELPs are loosely or freely localized in the cell wall and/or in the extracellular matrix. However, it remains to be shown whether these proteins are indeed secreted or retained in intracellular compartments of the endomembrane system.

Besides extensins, there is another type of hydroxyproline-rich protein, the arabinogalactan proteins (AGPs), which are present in the extracellular matrix of pistils of many species (Hoggart and Clarke, 1984; Sedgley et al., 1985; Bacic et al., 1988). It has been suggested that AGPs play a role in fertilization (Hoggart and Clarke, 1984; Sedgley et al., 1985). The relationship between the PELPs and the AGPs, if any, remains to be established.

The fact that proline-rich (or hydroxyproline-rich) proteins and glycine-rich proteins are often synthesized in an organ-, tissue-, or cell type-specific manner (Hong et al., 1989; Woessner and Goodenough, 1989; Koltunow et al., 1990; Mariani et al., 1990; Stiefel et al., 1990; Evrard et al., 1991; Salts et al., 1991; Ye

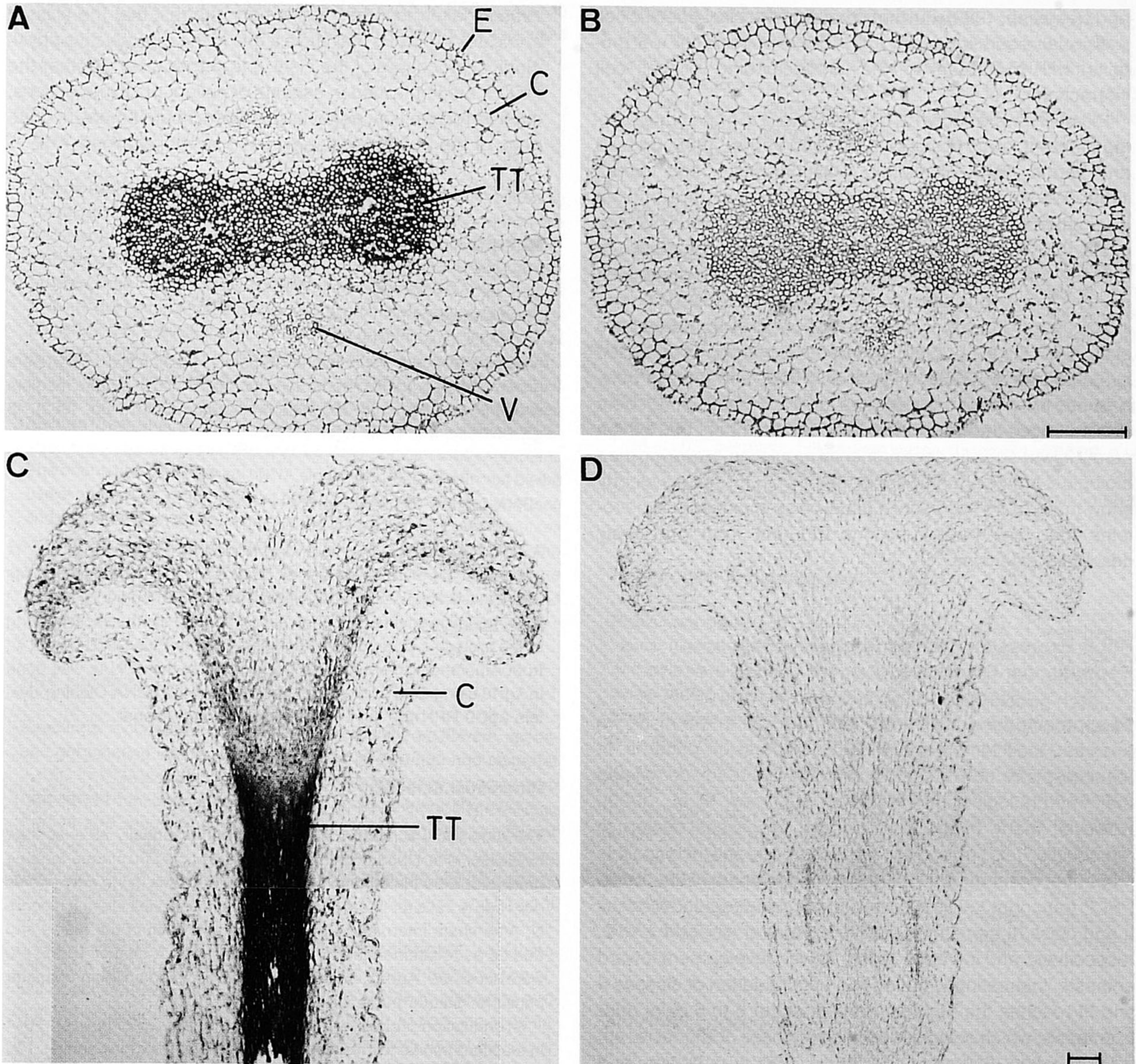


Figure 6. Localization of Class III mRNA in Stigmas and Styles of Stage 11 Pistils.

Stigmas and styles were fixed, embedded in paraffin, sliced into 10- μ m sections, and hybridized with single-stranded dioxigenin-RNA probes, as outlined in Methods. Photographs were taken by bright-field microscopy.

(A) and (C) In situ hybridization of a pMG07 (class III) antisense RNA probe. Purple or blue coloration represents regions containing RNA/RNA hybrids. (B) and (D) In situ hybridization of an antisense RNA probe made to the *neo* gene (see Methods), used here as a negative control.

E, epidermis; C, cortical cell; TT, transmitting tissue; V, vascular bundles. Bars = 0.1 mm; the scale is the same for (A) and (B), and the other scale is the same for (C) and (D).

and Varner, 1991; Ertl et al., 1992; Josè-Estanyol et al., 1992; Wyatt et al., 1992) suggests that these proteins do not necessarily have merely a structural role in the cell wall. Cell wall proteins may have additional roles in signal recognition and signal transduction between cells. It is postulated that pistil

proteins are responsible for the recognition of the pollen (Dumas et al., 1984), allowing pollen hydration, germination, and tube growth. The ultimate fate of a pollen grain is dependent on a series of events involving cell-cell recognition, followed by signal transduction and cellular response (Nasrallah

and Nasrallah, 1989). Further studies of the pistil-specific cell wall proteins and, in general, other tissue-specific cell wall proteins will contribute to the understanding of cell-cell interactions.

Extensins were shown to be synthesized at higher rates during different forms of stress (Chen and Varner, 1985; Cassab and Varner, 1988; Memelink, 1988; Showalter and Varner, 1989). We showed that PELP transcripts do not accumulate in vegetative tissues subjected to stress conditions. This suggests that PELPs have a biological function unrelated to defense reactions. Interestingly, some pathogenesis-related proteins have been shown previously to be implicated in flowering processes in tobacco (Fraser, 1981; Lotan et al., 1989; Neale et al., 1990; Ori et al., 1990). One of the explanations proposed by Lotan et al. (1989) and Ori et al. (1990) is that these polypeptides are part of a larger gene family containing members with pathogen-inducible characters and members that exhibit purely flower-specific developmental regulation. However, their biological function in flowers is unknown. Our results clearly show that PELPs represent a novel class of extensin-like proteins that may have functions different from previously described extensins.

PELP Expression Profiles Suggest a Role in Reproductive Processes

To approach the question of PELP function in the pistil, we analyzed their temporal and spatial expression patterns. In tobacco, pistils from stage 1 flowers have already completed their morphological differentiation (Koltunow et al., 1990). However, many of the physiological processes related to reproduction (e.g., receptivity, maturation, and self-incompatible response) occur during the further development of the pistils. PELP transcript levels are developmentally regulated: class II and class III transcripts start to appear at stages 1 and 2, respectively, and increase during flower development toward anthesis. Interestingly, the mRNA accumulation of classes II and III reaches the highest levels at about 5 to 6 days prior to anthesis, which coincides with the increase of stigma receptivity to pollen (Shivanna and Sastri, 1981; Kandasamy and Kristen, 1987). After pollination, class II and class III transcript levels gradually decrease, in contrast to unpollinated pistils in which they remain constant. These results indicate that the expression of the PELP genes is modulated by the pollination process. At present, we cannot distinguish if the decrease of the expression levels of class II and class III genes is a direct effect of signals produced during successful pollination and fertilization, or an indirect effect caused by the senescence of the stigma and style triggered by the pollination (Singh et al., 1992). However, our data show that the mRNAs of classes II and III are no longer required after effective pollination. In addition, class III mRNA is localized specifically in cells of the transmitting tissue. The spatial expression pattern of class III may explain the low transcript level observed in ovaries, where there is a part of the transmitting tissue in connection with the

ovules. Taken together, our results suggest that the class II and class III PELPs may have a role in the reproductive physiological processes of the pistil. It is not yet clear whether the PELPs are implicated in aspects of pollen-pistil interactions, such as recognition, adhesion of pollen, nourishment, or guidance of the pollen tubes.

METHODS

Plant Material

Nicotiana tabacum 'Petit Havana' SR1 plants (Maliga et al., 1973) were grown under standard greenhouse conditions and in culture medium containing half-strength MS salts (Murashige and Skoog, 1962), 15 g/L sucrose, and 0.6% agar, pH 6.0.

cDNA Library Construction

A cDNA library from stigma and style poly(A)⁺ RNA (stages 3 to 11 of tobacco flower development, as described by Goldberg, 1988) was constructed. First-strand cDNA synthesis was carried out by oligo(dT) priming, followed directly by the second-strand synthesis, according to the protocols of the cDNA Synthesis System Plus RPN1256Y/Z kit from Amersham International. The cloning of the cDNAs was done in λ gt10 using EcoRI linkers, as described in the cDNA Cloning System λ gt10 RPN1257 kit from Amersham International.

Differential Screening

The differential screening was carried out essentially as described by Gasser et al. (1989). For the screening, about 2000 plaque-forming units (pfu) were plated onto 14-cm-diameter plates using *Escherichia coli* NM514 cells for infection. Duplicate nylon replica filters (Hybond-N; Amersham International) were lifted from each of the plates and treated as recommended by the manufacturer. A total of 40,000 pfu were screened. Approximately 100 pfu were plated on 9-cm-diameter plates for plaque purification.

Hybridization probes were synthesized from 1 μ g of poly(A)⁺ RNA in reactions similar to the first-strand cDNA synthesis (see above). The modifications introduced were the use of 100 μ Ci ³²P-dCTP for the first hour of reaction and the use of random hexanucleotides as primers. Unlabeled dCTP was then added to a final concentration of 0.5 mM, and the reaction was continued for 30 min. The labeled cDNA/RNA hybrids were purified by chromatography over Bio-spin 30 (Bio-Rad). In preparation for hybridization, the probes were denatured and the RNA was hydrolyzed by the addition of 60 μ L of 1.0 N NaOH, followed by a 10-min incubation at room temperature. The base was neutralized by the addition of 60 μ L of 1.0 N HCl and 60 μ L of 20 \times SSPE (1 \times SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4).

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, 100 μ g/mL denatured carrier DNA at 68°C. The labeled cDNA was added to the prehybridization solution to a final concentration of 10⁶ cpm/mL and incubated at 68°C for 36 to 48 hr.

Filters were washed at the same temperature in $6 \times$ SSC, 0.1% SDS for 30 min and in $2 \times$ SSC, 0.1% SDS for 30 min. Filters were exposed to Kodak X-Omat films for 3 hr at -70°C , washed further in $0.2 \times$ SSC, 0.1% SDS at 68°C for 30 min, and exposed again for 7 and 24 hr.

DNA Manipulations and Sequence Analyses

Phage DNA as well as plasmid DNA were extracted as described by Sambrook et al. (1989). Inserts of the selected cDNA λ gt10 clones were isolated by EcoRI digestion and subcloned in the EcoRI site of pGEM1 (Promega). Radioactive labeled DNA probes were prepared from gel-purified DNA fragments using the random-primed DNA labeling kit (Boehringer Mannheim). Oligonucleotide probes were labeled by phosphorylation of 5' termini with γ - ^{32}P -ATP as described by Sambrook et al. (1989). The majority of the sequence analysis was performed according to the method of Maxam and Gilbert (1977). The shortest cDNA clones were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977), using as primers synthetic oligonucleotides homologous to the SP6 and T7 RNA polymerases promoters. The orientation of the partial cDNA clones has been confirmed by the use of riboprobes in RNA gel blot hybridizations. Nucleotide sequence and protein sequence data were analyzed using the computer resources provided by IntelliGenetics, Inc. (Mountain View, CA) and by PC/Gene (University of Geneva, Switzerland). The sequence data reported here are available in the EMBL data bank under accession numbers Z14014 to Z14020.

RNA Isolation

Seedlings, roots, stems, leaves, different organs from flowers, seeds, and germinating seeds were frozen in liquid nitrogen and stored at -70°C for RNA isolation. Roots, stems, and leaves were collected from 1-month-old nonflowering plants. Sepals, petals, anthers, stigma/styles, ovaries, and seeds represent pools of material collected at different developmental stages. Seeds were germinated for 6 days in shaking liquid cultures at 24°C in culture medium containing MS (Murashige and Skoog, 1962) salts, 10 g/L glucose, and 0.5 g/L MES and brought to pH 5.8.

For the analysis of the plants under stress conditions, at least three individual 1-month-old plants were used for each treatment. The effect of ethylene was examined placing the plants in glass jars and flushing continuously at a rate of 90 mL/hr with 10 ppm ethylene for 24 hr. The control plants were placed in glass jars under the same conditions, but flushed continuously with water. For wounding experiments, leaves were extensively punctured with a forceps and collected after 24 hr. The virus infection was performed by dusting the leaves with carborundum (BDH Chemicals, Poole, UK), inoculating with purified virus, which was diluted to 1 $\mu\text{g/mL}$ with inoculation buffer (10 mM sodium phosphate, pH 7.0), and rinsing with water. These plants were further grown for 12 days in a growth chamber at 23 to 27°C and with 16-hr light/8-hr dark periods. The controls for the wounding and for the virus infection experiments were plants kept respectively at the same conditions as the treated plants.

For the developmental expression studies, stigmas and styles were excised from flowers at stages 1 to 11 of tobacco flower development, as described by Goldberg (1988) and Koltunow et al. (1990). To analyze the expression patterns in relation to pollination, the immature anthers from stage 11 flowers were removed manually. One day later, the mature pistils were pollinated and were collected 0.5, 1, 2, 3, and

6 days after pollination. Control flowers at stage 11 were also emasculated, but not pollinated. The unpollinated pistils were collected at identical time points as the pollinated pistils. Total RNA from all plant organs was extracted essentially as described by Dean et al. (1985). Poly(A)⁺ RNA was purified by chromatography over oligo(dT)-cellulose according to Ausubel et al. (1987).

RNA Gel Blot Analysis

Total RNA was electrophoretically separated in a 1.5% agarose gel containing 2.2 M formaldehyde and transferred to Hybond N⁺ using $20 \times$ SSC and alkaline fixation, as described by Amersham. Because the clones encoding extensin-like proteins are very GC rich, small DNA fragments or oligonucleotides were used as probes on RNA gel blots to minimize the possibility of cross-hybridization. Hybridization was performed with DNA probes or oligonucleotide probes in $5 \times$ SSPE, $5 \times$ Denhardt's solution, 0.5% SDS, and 100 $\mu\text{g/mL}$ denatured carrier DNA at 50°C overnight. Filters were washed twice in $2 \times$ SSPE, 0.1% SDS for 10 min at room temperature, once in $1 \times$ SSPE, 0.1% SDS for 15 min, and once in $0.1 \times$ SSPE, 0.1% SDS for 10 min at 50°C .

In Situ Hybridization Studies

Pistils were dissected from stage 11 tobacco flowers, as described previously (Goldberg, 1988; Koltunow et al., 1990). Small pieces of stigma and style were fixed with 1% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7, as described by Cox and Goldberg (1988). The fixed stigmas and styles were dehydrated, embedded in paraffin (Paraplast Plus; BDH Chemicals), and sliced into 10- μm sections, essentially as outlined by Cox and Goldberg (1988). The antisense RNA probes were synthesized using dioxigenin-UTP by in vitro transcription with SP6 and T7 RNA polymerases of the diogenin RNA labeling kit (SP6/T7) from Boehringer Mannheim. The negative control used was an antisense RNA probe made to the gene encoding neomycin phosphotransferase (*neo*). The techniques used for the hybridization and detection of the RNA/RNA hybrids will be described in detail elsewhere (M. De Block and D. De Brouwer, manuscript in preparation).

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