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-Pro-X, 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).
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 extensins, 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 pl 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 pl 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 hydrophathy 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 NAGMNRLMLMMLVAAILFCSHQVATAREVYVADORREDNE6LQLLWFWE

PMG04

47 IPCYLYWPPFFPWWTPPPPPPPFPRFRPRPRPUSHPPPPPPRPPCP

11 IPCYLYWPPFFPWWTPPPPPPPFPRFRPRPRPUSHPPPPPPRPPCP

93 SPPPQPPRPPSPPPP

55 SPPPQPPRPPSPPPPQPRPRPSPPPSPPPAPSSCSSADPES

108

101 NIVRMCNFTKDPCCPTFKSILGTCPCVYAEKPLQQLQVLLIES

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108

147 YCDVSFCKGVQQV1KLSKKEEKK.

**B class II**

PMG08 AFTSvkLvlQdSVLALSFSFELSFQG1IESSSLLQQHPHPFST

PMG09

47 VHLFFGSKSFSKPPSSPTVPKSPSSPQVQSSLLPPPASPPP

1 QPPATQHRATPPP

93 AKSPSPLLPPPQPPKQPPPPPPSPPAKQPSAPKPPSKPSPPAA

1 A MQRAPP LGQPPKPPPPPPPQPPKQPPPSAPKPPSKPSPPAA

139 QPPATQHRATPPPAMQRA

52 QPPATQHRATPPPAMQRAQPLPSQPPKLP

**C class III**

PMG07

PMG14

PMG15 MAVI8S8SSKL1QFLVFLGVSSFSKLSHGLWELPLPFDPWPPA1EP

47 LPS1FLPLPPDPFPTVFVPLPP

12 LPS1FLPLPPDPFPTVFVPLPP

97 VNRKPSFSPSVFKPQPPPPPPPQCKPSPPQDQTRKSPPPPPPSQPQPPPP

134 VNRKPSFSPSVFKPQPPPPPPPQCKPSPPQDQTRKSPPPPPPSQPQPPPP

37

54 TLIPLPPPTGQFQFLQPGSKLPGLLPLIPLNBDLPPI1QCPFP

89 T LIPLPPPTGQFQFLQPGSKLPLOFAGQLPLIPLNBDLPPI1QCPFP

37

97 VNRKPSFSPSVFKPQPPPPPPPQCKPSPPQDQTRKSPPPPPPSQPQPPPP

134 VNRKPSFSPSVFKPQPPPPPPPQCKPSPPQDQTRKSPPPPPPSQPQPPPP

37

SSPSFSQPPQPPPPOAPFPPS

143 PPP VAKASSPSAKQPPPPPPPQVSPSSPSQAKQPPPPPOAPFPPS

180 PPPPPVQAKSSPSAKQPPPPPPQVSPSSPSQAKQPPPPPOAPFPPS

58 PATQPP1KQPQP8 SAKKSP PPPPPVQVMPFSFPAEFPF

186 PATQPP1KQPQP8 SAKKSP PPPPPVQVMPFSFPAEFPF

211 PATQPP1KQPQP8 SAKKSP PPPPPVQVMPFSFPAEFPF

99 IAPFSSPTANLPLPRPAFPVPLPPLPKGPLVNLGVCXKSCN

227 IAPFSSPTANLPLPRPAFPVPLPPLPKGPLVNLGVCXKSCN

257 IAPFSSPANPLPRPAFPVPLPPLPKGPLVNLGVCXKSCN

145 YGFPTLINTLSLPGAVVLKVCYGKTMQAGTDSDNGKFR1KPS

273 YGFPTLINTLSLPGAVVLKVCYGKTMQAGTDSDNGKFR1KPS

303 YGFPTLINTLSLPGAVVLKVCYGKTMQAGTDSDNGKFR1KPS

191 LTRADVGCXKLYKVLKSNPNCVPTNFNGXSGOQLKLPFLPPQ1

319 LTRADVGCXKLYKVLKSNPNCVPTNFNGXSGOQLKLPFLPPQ1

348 LTRADVGCXKLYKVLKSNPNCVPTNFNGXSGOQLKLPFLPPQ1

237 TPAAVVP LSDLQVQFPPFEASXMPCDKN

365 TPAAVVP LSDLQVQFPPFEASXMPCDKN

394 TPAAVVP LSDLQVQFPPFEASXMPCDKN

**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 Genalignt program (IntelliGenetics).

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

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

Identical amino acids are identified by vertical lines, and the pentapeptides Ser-Pro, are presented in boldface letters. The arrows indicate the potential signal peptide cleavage sites as determined by computer analysis (PG/Genome—program psalign), 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.
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., 1988) is underlined.

(B) Hydrophathy index of the 426 amino acid residues of the MG15 protein, determined according to Kyte and Doolittle (1982). The mean hydrophathy 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-Pro motif, which are specifically expressed in flowers.
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 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-Pro, 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 Lamport, 1984; Wilson and Fry, 1986). The PELPs differ from previously described extensins by a low tyrosine content, a lower copy number of the Ser-Pro 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 arabino-galactan 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-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-µm 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; José-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 Sashti, 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 λgt10 using EcoRI linkers, as described in the cDNA Cloning System λgt10 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 synthetized from 1 μg of poly(A)+ RNA in reactions similar to the first-strand cDNA synthesis (see above). The modifications introduced were the use of 100 μCi [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 μL of 1.0 N NaOH, followed by a 10-min incubation at room temperature. The base was neutralized by the addition of 60 μL of 1.0 N HCl and 60 μL of 20 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4).

Filters were prehybridized overnight in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1% SDS, 100 μg/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 × SSC, 0.1% SDS for 30 min and in 2 × 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 × 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 λgt10 clones were isolated by EcoRI digestion and subcloned in the EcoRI site of pGEM1 (Promega). Radioactively 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-32P-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 dideoxyxynucleotide 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 PCGene (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 wound 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 µg/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 × 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 × SSPE, 5 × Denhardt’s solution, 0.5% SDS, and 100 µg/mL denatured carrier DNA at 50°C overnight. Filters were washed twice in 2 × SSPE, 0.1% SDS for 10 min at room temperature, once in 1 × SSPE, 0.1% SDS for 15 min, and once in 0.1 × 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 digoxigenin RNA labeling kit (SP6/T7) from Boehringer Mannheim. The negative control was 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).

ACKNOWLEDGMENTS

We thank Jurgen Denecke, Marc Cornelissen, Enno Krebbers, and Kevin O’Brien for critical reading of the manuscript; Jurgen Denecke and Allan Caplan for stimulating discussion; Marc De Block for teaching us the in situ hybridization protocol; Dirk De Brouwer and Barend De Graaf for helping in the in situ hybridization experiments; Dominique Van Der Straeten for advice on the ethylene treatment; Frank Meulewaeter for providing the tobacco necrosis virus infected leaves; Godelieve Gheysen for providing the 1.3-β-glucanase clone; and Karel Spruyt and Vera Vermaercke for preparing the figures. M.H.S.G. is supported by the Brazilian Research Council (CNPq), Fellowship No. 200746/88.0; M.P. was supported by a fellowship from the Consiglio Nazionale delle Ricerche.

Received May 29, 1992; accepted July 15, 1992.
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