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Specific Expression of an Extensin-Like Gene in the Style of *Nicotiana alata*

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cDNAs and corresponding genomic clones encoding a putative proline-rich protein (NaPRP3) were isolated from libraries prepared from *Nicotiana alata* style mRNA and genomic DNA. The predicted NaPRP3 protein is structurally similar to extensin in containing six copies of the characteristic extensin sequence Ser-Pro$_4$, but differs in being smaller (151 residues compared with >300 residues) and lacking Tyr residues. In contrast to most extensin genes, the NaPRP3 gene is not induced by mechanical wounding, and its expression is restricted to cells of the transmitting tract of the style.

INTRODUCTION

Four major classes of hydroxyproline-rich proteins (HRGPs) have been described in flowering plants: the extensins (often referred to as cell wall HRGPs), the arabinogalactan proteins (AGPs), certain solanaceous lectins, and the recently described hydroxyproline-proline-rich proteins (H/PRPs) (for reviews, see Allen, 1983; Fincher et al., 1983; Showalter and Varner, 1989; Showalter and Rumeau, 1990).

The best characterized of these proteins are extensins, which are basic glycoproteins containing high proportions of Hyp, Ser, and certain other amino acids: Lys, Tyr, Val, or His. A number of cDNA and genomic clones encoding extensin have been cloned and characterized. The sequences obtained from dicotyledonous plants all contain the characteristic repeat Ser-Pro$_4$. The Ser-Pro$_4$ repeats are usually separated by other less frequently repeated sequences such as Tyr-Lys-Tyr-Lys and Ser-Pro (for a review, see Showalter and Varner, 1989).

The expression of extensin genes is often induced by stress, such as infection or wounding. Gene expression is also developmentally regulated and tissue specific. For example, in tomato, the level of extensin mRNA is higher in roots and stems than in leaves (Showalter and Varner, 1987); in tobacco, an extensin gene is highly expressed in the cells at the tip of emerging lateral roots (Keller and Lamb, 1989), whereas in soybean stems, extensin mRNA is most abundant in cambial cells (Ye and Varner, 1991).

Although the expression of extensins in somatic tissues such as roots, stems, and leaves is relatively well studied, little is known about its expression in the sexual tissues. In this paper, we describe the isolation of a cDNA and a genomic sequence encoding an extensin-like, style-specific, proline-rich protein from *Nicotiana alata*, using the carrot extensin genomic fragment (Chen and Varner, 1985) as a probe.

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RESULTS

Isolation of cDNA Clones Encoding Proline-Rich Proteins

Two cDNA libraries were prepared from mRNA isolated from mature styles of *Nicotiana alata* plants of self-incompatibility genotypes S_2S_3 and S_6S_6. The S_2S_3 library was screened, under relatively low stringency, with a 1.8-kb XbaI fragment isolated from the genomic clone pDC5A1 encoding a carrot extensin (Chen and Varner, 1985). Eight cDNA clones were isolated and sequenced. Six of the cDNA clones were related, and a 608-bp nucleotide sequence was obtained from a compilation of sequences from the six clones. The S_6S_6 library was also screened using the same probe, and another six clones were isolated and sequenced. The longest of these was 611 bp, and the other five clones contained portions of this sequence. The nucleotide and derived amino acid sequences of this 611-bp cDNA are shown in the upper two lines of Figure 1A, and the similarity to sequence of the cDNA from the S_2S_3 library is shown in the third line of Figure 1A. The cDNAs are incomplete but encode proteins that are rich in Pro and Ser. Comparison of the two nucleotide sequences shows that they are similar but not identical, with eight single base differences between the two cDNA sequences and an additional 6-bp sequence (TTCTCC) at position 246 of the sequence from the S_2S_3 library, which corresponds to a deletion of an identical sequence at position 225. This hexanucleotide sequence encodes a Ser-Pro dipeptide, and other than this transposition the derived amino acid sequences are the same. The cDNA clone is referred to as NaPRP3 (*Nicotiana alata* proline-rich

A

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GlnLeuSerIleLeuValIleSerSerPheSerGluLeuThrAsnGlyAsp
CAACTTTCTATTTTAGTAATTAGCTCATTTTCTGAGCTCACTAATGGAGAT    51

HisGlyThrGluSerLeuProAspAlaThrLeuGlnArgPheLysPheLys
CATGGCACAGAAAGTTTGCCTGATGCAACTCTTCAGCGTTTTAAGTTTAAG   102
↑.....................................................

TrpProPhePheGlyLysSerProLysAsnSerProLysSerSerProSer
TGGCCTTTCTTTGGGAAATCTCCAAAAAATTCTCCTAAAAGTAGCCCTTCC   153
.....................................................

ArgSerProProProLysArgGluGlnProSerProProProProValLys
CGTAGCCCTCCACCTAAGCGGGAACAGCCGTCGCCACCACCACCAGTGAAG   204
.....................................................

SerProProProProSerProSerProProProProProSerPro------Pro
TCACCACCACCTCCATCACCTTCTCCACCACCTCCATCGCC------ACCA   249
...........A.....T..------.............TTCTCC....

ProProSerProSerProProProProSerProSerProProProProSer
CCACCATCGCCGTCACCACCACCACCGTCGCCGTCACCACCGCCGCCTTCT   300
.....T.....................A.............A.........

ProProAlaAspAspMetAlaProSerProSerProAlaAlaAlaProThr
CCACCAGCTGATGATATGGCACCATCTCCATCACCAGCTGCGGCACCGACA   351
.....................................................

ProProAspMetLeuLeuArgProSerAlaArgLeuAlaGlyAsnMetVal
CCACCAGATATGCTGTTGCGACCCTCAGCTCGTCTCGCTGGAAATATGGTC   402
.....................................................

IleSerGlyLeuHis***
ATTTCTGGACTTCATTAATTACTATGAAAAACAATAATAAGAAAGGAAGGG   453
........C.................G.........................

GTAATAACTTTATTAAATGATTGAGAAAGAAAATGTCCATTGTTGAGAATA   504
.....................................................

ATAATCTGATGTGCATTTTCTATCGTAAGTCCAAAATTAAGACTTACGAAA   555
.A...................................................

ACAAAAAATAAGTACTAGTCTTTTACTACGAATATGTGAATGGTTCTAGAA   606
.....................................................

TTTGA                                                 611
.....ATCTCTAAATATCGTTTTCCTAAAAAAAAAAAAAAAAAAAAA
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B

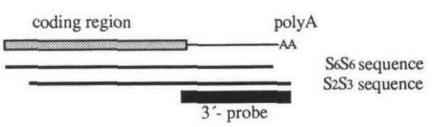

Figure 1. Nucleotide and Predicted Amino Acid Sequences of the NaPRP3 cDNA.

(A) The nucleotide and derived amino acid sequences of cDNA from plants of genotype S_6S_6 are shown in the upper lines. Similarity of the NaPRP3 cDNA clones isolated from plants of genotype S_2S_3 is shown in the lower line. Only the points of difference are shown; dots signify identity with nucleotides in the S_6S_6 sequence. Missing nucleotides and amino acids are indicated as a dash (—). The Ser-Pro_4 and Ser-Pro repeats are underlined and overlined, respectively. The two partial copies of the Ser-Pro_4 repeat (Ser-Pro-Pro-Pro and Ser-Pro-Pro) are also overlined. The putative polyadenylation signals are double underlined.

(B) The position of the HaeIII fragment within the 3′ noncoding region of NaPRP3 cDNA.

protein) cDNA. To avoid cross-hybridization with other proline-rich genes, a 252-bp HaeIII fragment corresponding to the 3′ noncoding region of the NaPRP3 cDNA plus 8 bp at the 3′ end of the coding region was used as the gene-specific probe for NaPRP3. This HaeIII fragment is referred to as the 3′ probe (Figure 1B).

DNA Gel Blot Analysis of the NaPRP3 Gene

Analysis of genomic DNA from the S-genotypes of *N. alata* using the 3′ probe of the NaPRP3 cDNA and the 1.8-kb XbaI fragment of pDC5A1 (carrot extensin) as probes is shown in Figures 2A and 2B. Digestion of *N. alata* genomic DNA with all of the five enzymes tested gave multiple bands that

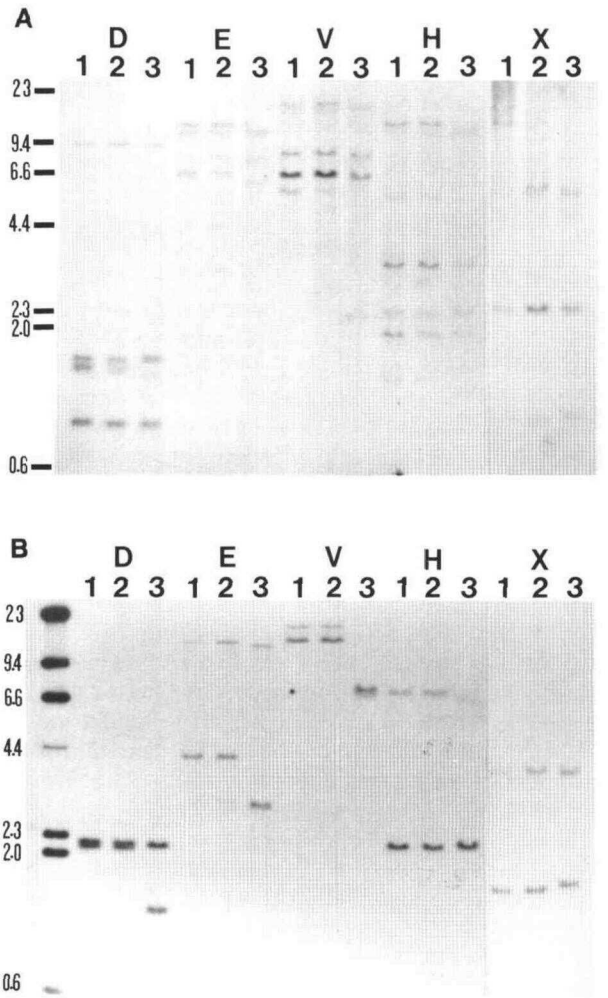

Figure 2. DNA Gel Blot Analysis of the NaPRP3 and Extensin Genes in *N. alata.*

DNA was isolated from *N. alata* (genotypes S_1S_3, S_2S_3, and S_6S_7) (tracks 1, 2, and 3, respectively) digested with DraI (D), EcoRI (E), EcoRV (V), HindIII (H), and XbaI (X), fractionated by electrophoresis on 0.9% agarose gels, and hybridized to ^{32}P-labeled probes.

(A) Autoradiograph of the DNA gel blot after hybridization with the 3′ probe of the NaPRP3 cDNA.

(B) Autoradiograph of the same blot after hybridization with the 1.8-kb XbaI fragment of the carrot extensin pDC5A1 clone under high-stringency conditions where NaPRP3 sequences were not detected. The first lane in **(B)** is λDNA digested with HindIII, and the fragment sizes in kilobases are indicated.

hybridized to the 3′ probe of NaPRP3 cDNA (Figure 2A). The carrot extensin probe detected, under stringent conditions, only one or two bands (Figure 2B) that were different from the bands homologous to NaPRP3. These results indicate the existence of another gene in *N. alata* with higher nucleotide sequence similarity to the carrot extensin gene (pDC5A1) than has NaPRP3. This gene is presumably the extensin gene of *N. alata*.

Isolation of Genomic Clones Corresponding to the NaPRP3 cDNA

An *N. alata* (genotype S_6S_6) Sau3AI genomic library in λ Dash was screened with the 3′ probe of the NaPRP3 cDNA. Two clones (NaPRP3g5 and NaPRP3g12) containing 10-kb and 20-kb inserts, respectively, were isolated. The NaPRP3g12 produced an 0.8-kb DraI, a 1.9-kb HindIII, and a 2.2-kb XbaI fragment, each of which hybridized with the NaPRP3 cDNA, as shown in Figure 3A. The 1.9-kb HindIII fragment was isolated from clone NaPRP3g12 and subcloned into pBluescript II KS+ vector for sequence analysis. The sequencing strategy is shown in Figure 3B, and the sequence of 1056 nucleotides is shown in Figure 4. The sequence matches perfectly with the NaPRP3 cDNA sequence.

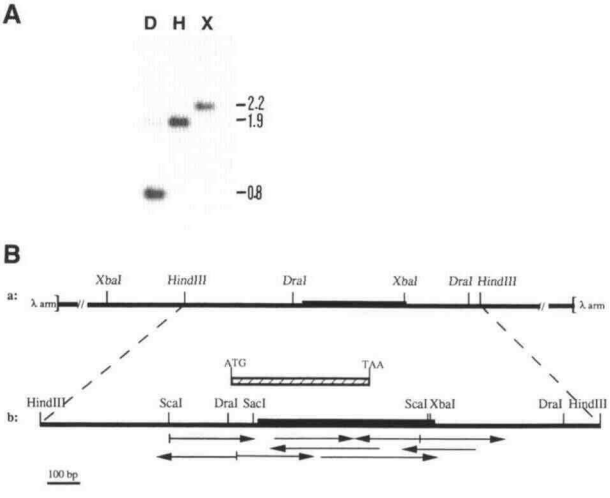

Figure 3. DNA Gel Blot Analysis and Sequencing Strategy of the NaPRP3g12 Genomic Clone.

(A) DNA was isolated from the NaPRP3g12 genomic clone and digested with DraI (D), HindIII (H), and XbaI (X) for DNA gel blot analysis using the 3′ probe of the NaPRP3 cDNA.

(B) Restriction maps of the clone NaPRP3g12 in λDash (a) and of the 1.9-kb HindIII fragment (b) are shown. The sequencing strategy for the 1.9-kb HindIII fragment is also illustrated (b). The sequence corresponding to the NaPRP3 cDNA is presented as a bold line. A partial sequence was obtained by using either specific primers (marked as single arrows) or subclones (marked as double-headed arrows).

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                                        AGTACTATTTAAGGAAACACTTATTTAAT  -143

TGTTTGTCTTAATTTAGAAGTAAAACGTTTTCTCCTAATTACCTACCTCGC                    -92

TGCGAACAAAAACCTTAAACTAAGTAAAGAAGGGTAAAAATCCTACTTCAC                    -41
                                                   1
GCTCCTATAAAATCCTTTGGCCTTTGCATTCTTTTACGCACATTATTTGA                      10
                                                   *
                                  MetAlaValAsnSerValLysAlaLeuValLeuLeu
GTTTAAATAAAAACTATGGCTGTTAATTCAGTAAAGGCCCTAGTGCTTTTA                     61
                                  ↓
GlnLeuSerIleLeuValIleSerSerPheSerGluLeuThrAsnGlyAsp
CAACTTTCTATTTTAGTAATTAGCTCATTTTCTGAGCTCACTAATGGAGAT                    112

HisGlyThrGluSerLeuProAspAlaThrLeuGlnArgPheLysPheLys
CATGGCACAGAAAGTTTGCCTGATGCAACTCTTCAGCGTTTTAAGTTTAAG                    163

TrpProPhePheGlyLysSerProLysAsnSerProLysSerSerProSer
TGGCCTTTCTTTGGGAAATCTCCAAAAAATTCTCCTAAAAGTAGCCCTTCC                    214

ArgSerProProProLysArgGluGlnProSerProProProProValLys
CGTAGCCCTCCACCTAAGCGGGAACAGCCGTCGCCACCACCACCAGTGAAG                    265

SerProProProProSerProSerProProProProSerProProProPro
TCACCACCACCTCCATCACCTTCTCCACCACCTCCATCGCCACCACCACCA                    316

SerProSerProProProProSerProSerProProProProSerProPro
TCGCCGTCACCACCACCACGTCGCCGTCACCACCGCCGCCTTCTCCACCA                     367

AlaAspAspMetAlaProSerProSerProAlaAlaAlaProThrProPro
GCTGATGATATGGCACCATCTCCATCACCAGCTGCGGCACCGACACCACCA                    418

AspMetLeuLeuArgProSerAlaArgLeuAlaGlyAsnMetValIleSer
GATATGCTGTTGCGACCCTCAGCTCGTCTCGCTGGAAATATGGTCATTTCT                    469

GlyLeuHis***
GGACTTCATTAATTACTATGAAAAACAATAATAAGAAAGGAAGGGGTAATA                    520

ACTTTATTAAATGATTGAGAAAGAAAATGTCCATTGTTGAGAATAATAATC                    571

TGATGTGCATTTTCTATCGTAAGTCCAAAATTAAGACTTACGAAAACAAAA                    622

AATAAGTACTAGTCTTTTACTACGAATATGTGAATGGTTCTAGAATTTGAA                    673
                                  ↓ PolyA
TCTCTAAATATCGTTTTCCTACCTCTGCTTTCCTAATTAATTACAAGAGGA                    724

AAATACATAAACAAACCAAAGGGAGATAATCAGACTTGTTAGAGGAATTAA                    775

TGGATGAAAATAATACATTGTTTAAGATTAAATGTTTATATTCATATCCAT                    826

GCTAAGAACCTATAATTTATAAATATATATAAGCGTATCTTATTTAGACAG                    877

TACAATTT                                                               885
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Figure 4. Nucleotide and Predicted Amino Acid Sequences of the *Genomic Clone NaPRP3g12 Encoding the NaPRP3 of N. alata.*

The first 21 amino acids, representing a putative signal sequence, are dot underlined. The potential cleavage site of the signal peptide is indicated by a downward arrow. The putative TATAAA box is shown in boldface. The putative polyadenylation signals are double underlined. The transcriptional initiation site is marked by an asterisk (*). The poly(A) addition site of the cDNA clone is marked by an arrowhead. The Ser-Pro_4 and Ser-Pro repeats are underlined and overlined, respectively.

A primer extension experiment was performed to determine the transcriptional initiation site, using a synthetic oligonucleotide priming at position 84 to 113 to the genomic clone NaPRP3g12, as shown in Figure 5. The primer extension products were separated on sequencing gels together with a sequencing reaction of the genomic clone NaPRP3g12 using the same oligonucleotide. The major primer extension product corresponds to the third C residue (nucleotide 1, Figure 4) in the sequence ACGCACATTAT.

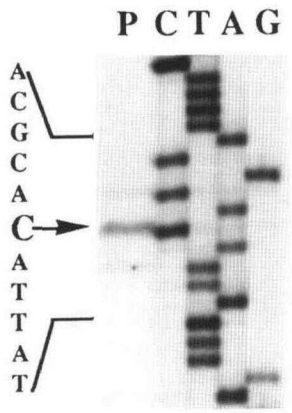

Figure 5. Determination of the Transcriptional Initiation Site.

Primer extension (P) and sequencing of the genomic clone were performed using a synthetic oligonucleotide with sequence 5′-GATCTCCATTAGTGAGCTCAGAAAATGAGC-3′ priming at position 84 to 113 of the genomic clone NaPRP3g12. The arrow indicates the position of the major primer extension product.

A sequence motif (TATAAAA) that resembles the Goldberg-Hogness (TATA) box (Efstratiadis et al., 1980) occurs at position −35 to −29. There are several putative polyadenylation signals such as AATAAT, AATAAC, AATAAG, and AATTAA in the 3′ nontranslated region.

The NaPRP3g12 genomic sequence encodes an open reading frame, which starts with an initiation codon (ATG) at position 26 and ends with a termination codon (TAA) at position 479. The potential translation initiation codon ATG is surrounded by the sequence AACTATGGC, which differs by one base from the consensus sequence (AACAATGGC) probably optimum for translation initiation in plants (Joshi 1987; Lütcke et al., 1987).

The open reading frame encodes a polypeptide containing 151 amino acids with a calculated molecular weight of 15,645 daltons. The polypeptide is rich in Pro and Ser, relatively rich in Ala, Leu, and Lys, as shown in Table 1, and has a predicted pI of 10.77. Tyr, which is often abundant in extensin, is absent, and Val and His are uncommon. This predicted amino acid sequence contains six Ser-Pro_4 and eight Ser-Pro repeated sequences, as well as a Ser-Pro_2 and a Ser-Pro_3 sequence. The Ser-Pro_4 repeats are clustered in the central part of the sequence and, in most cases, are interspersed with Ser-Pro dipeptides.

The hydropathy plot in Figure 6 of the amino acid sequence (Kyte and Doolittle, 1982) shows a hydrophobic N-terminal sequence of 21 residues. This sequence resembles a signal peptide with a potential cleavage site between Ser^{21} and Phe^{22} (Perlman and Halvorson, 1983; von Heijne, 1983).

The other clone isolated from the genomic library, NaPRP3g5, gave a DNA gel blot hybridization pattern that differed from that of NaPRP3g12 in that a 1.4-kb DraI and a 1.2-kb HindIII fragment hybridized to the cDNA probe. The 1.4-kb DraI fragment was subcloned into pBluescript KS+ vector and partially sequenced (data not shown). This clone probably represents a pseudogene, as no ATG that could act as an initiation codon and no TATA box-related sequence were present; there were also numerous nucleotide changes compared with the corresponding cDNA.

Tissue-Specific Expression of the NaPRP3 Gene

Mature styles, mature pollen grains, mature petals, young leaves, young roots with root hairs, and young stems (3 mm in diameter) were collected from *N. alata* genotype S_2S_3 plants, and mature styles were also collected from S_6S_6 plants. Equal amounts of total RNA isolated from these different tissues were loaded in the gel blot analysis and hybridized with the ^{32}P-labeled 3′ probe of the NaPRP3 cDNA. As a control, the same blot was also hybridized under high stringency with the 1.8-kb XbaI fragment from the carrot extensin clone pDC5A1 to monitor the expression of the extensin gene. The NaPRP3 cDNA hybridized strongly to a 0.7-kb style RNA from both genotypes S_2S_3 and S_6S_6, and there was no detectable hybridization after overnight exposure to RNA from stems, pollen, roots, leaves, or petals, as shown in Figure 7A. This hybridization pattern differed from that obtained with the extensin probe. Under high-stringency conditions, the extensin probe hybridized to transcripts in all tissues tested, although

Table 1. Amino Acid Composition of the NaPRP3 Protein Predicted from the Open Reading Frame of the Genomic Clone NaPRP3g12

	Composition (mol %)	
Amino acid	+SP	−SP
Pro	29.8	34.6
Ser	17.2	16.9
Leu	7.9	5.3
Ala	6.6	6.1
Lys	5.2	5.3
Val	3.9	1.5
Phe	3.3	3.8
Arg	3.3	3.8
Gly	3.3	3.8
Asp	3.3	3.8
Thr	2.6	3.0
Asn	2.6	2.3
Met	2.6	2.3
Glu	1.9	2.3
Gln	1.9	1.5
Ile	1.9	0.7
His	1.3	1.5
Trp	0.6	0.7
Tyr	0.0	0.0
Cys	0.0	0.0

Amino acid compositions including the 21-amino acid signal peptide (+SP) or excluding the signal peptide (−SP) are shown.

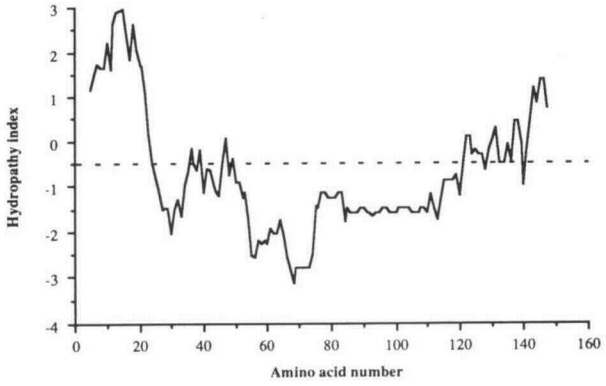

Figure 6. Hydropathy Plot of the Deduced Amino Acid Sequence of NaPRP3.

The hydropathy values of each amino acid have been determined by using an interval of nine amino acids according to the weight system of Kyte and Doolittle (1982). Values above the dotted line indicate hydrophobic regions, and values below the dotted line represent hydrophilic regions.

the expression in different tissues varied both quantitatively and qualitatively. The level of the extensin mRNA is highest in roots, relatively high in styles, and much lower in pollen, petals, and leaves. The number and size of the extensin transcripts also differed in different tissues. Pollen contains two large transcripts (2.5 and 3.0 kb); in contrast, there is only one smaller (1.8 kb) dominant transcript in other tissues (Figure 7B).

The 3′ probe of the NaPRP3 cDNA was hybridized to longitudinal sections of mature pistils (genotype S_6S_6), as shown in Figure 8A. Strong hybridization of the cDNA probe to the cells of the transmitting tissues was obtained, with no or little hybridization to stigmatic, cortical, or epidermal cells (Figure 8B). As a control of the hybridization specificity, a cDNA fragment encoding a stigmatic proteinase inhibitor (A. Atkinson, unpublished data) was used under identical conditions. In contrast to the NaPRP3 cDNA, this cDNA probe bound mainly to the cells of the stigmatic papillae (Figures 8C and 8D).

Developmental Regulation of the NaPRP3 Gene

To examine the expression of the NaPRP3 gene during development of the style, equal amounts of total RNA isolated from developing style tissues [small buds (2 to 3 cm), fully elongated buds (5 to 6 cm), 1-day-old and 2-day-old flowers] were subjected to gel blot analysis using the ^{32}P-labeled 3′ probe of the NaPRP3 cDNA as a probe. The level of hybridizing transcript in the style increased dramatically from the small bud stage to a maximum level in the fully elongated bud stage, as shown in Figure 9. This increase correlated with the physical elongation and physiological maturation of the style. The level of hybridizing transcript decreased from fully elongated buds to 1-day-old and 2-day-old flowers (Figure 9). In contrast, the level of extensin transcripts was constant during these four developmental stages of the style (data not shown).

Response of the NaPRP3 Gene to Wounding

The RNA gel blot analysis in Figure 10A shows that the level of the NaPRP3 transcript decreased rapidly in excised, wounded styles, as was also found in intact styles (Figure 9). There was no NaPRP3 transcript detected in either stems or leaves before and after wounding (Figure 10A). In contrast, wounding resulted in a marked increase in expression of the extensin gene in all the tissues examined (Figure 10B). The increase in expression was apparent 2 hr after wounding in both styles and stems but was relatively delayed in leaves, with no increase detectable until 8 hr.

DISCUSSION

Expression of HRGPs in various vegetative tissues, such as roots, stems, and leaves, has been studied by several groups of investigators, but apart from studies of style AGPs (Gleeson and Clarke, 1979; Sedgley et al., 1985), there have been no reports on studies of style HRGPs. In this study, we describe a gene, NaPRP3, encoding a putative HRGP from *N. alata*. This gene was obtained by screening style cDNA libraries under relatively low stringency with the carrot extensin genomic

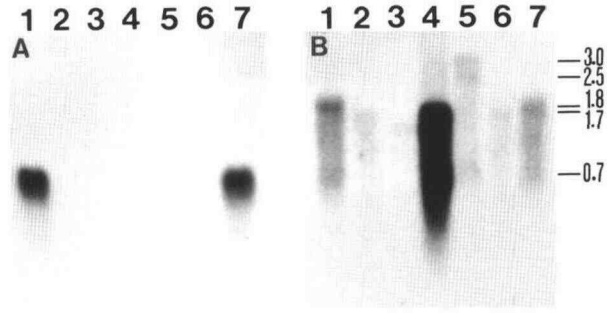

Figure 7. Tissue-Specific Expression of the NaPRP3 and Extensin Genes.

Total RNA was isolated from *N. alata* (genotype S_2S_3) (1) styles, (2) stems, (3) leaves, (4) roots, (5) pollen, and (6) petals and from *N. alata* (genotype S_6S_6) (7) styles. Equal amounts (8 μg/lane) of RNA were fractionated on formaldehyde agarose gels, transferred to Hybond-N membranes, and hybridized with ^{32}P-labeled probes.

(A) Autoradiograph of the RNA gel blot after hybridization with the 3′ probe for the NaPRP3 cDNA.

(B) Autoradiograph of the same blot after hybridization with the 1.8-kb XbaI fragment of the pDC5A1 clone. The size of RNA transcripts in kilobases is indicated at the right.

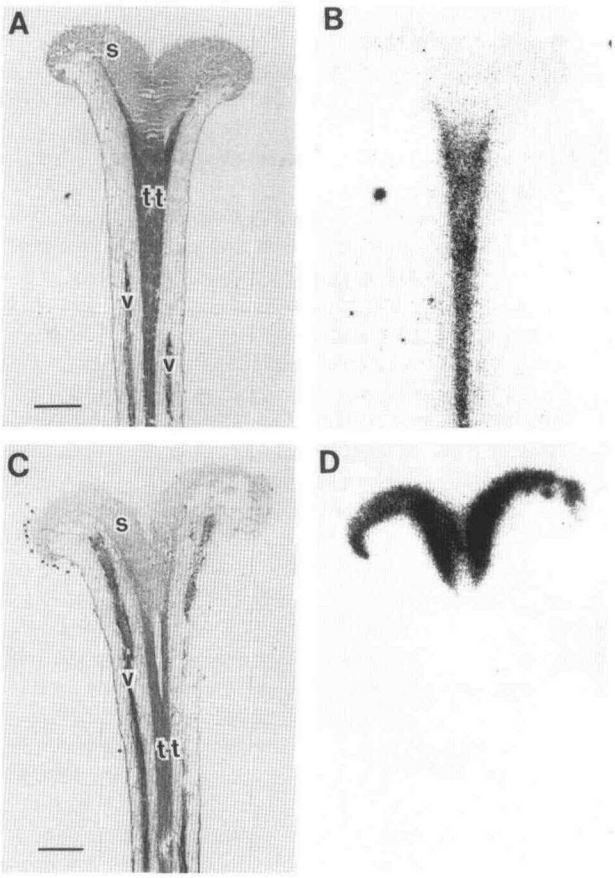

Figure 8. In Situ Hybridization of the NaPRP3 cDNA and a Proteinase Inhibitor cDNA to Sections of *N. alata* Style.

(A) and **(C)** Longitudinal cryostat sections of a mature style of *N. alata* (genotype S_6S_6) stained with toluidine blue, showing the stigma (s), the transmitting tissue (tt), and vascular bundles (v).
(B) Autoradiograph of the same section as **(A)** after hybridization with the ^{32}P-labeled 3′ probe of the NaPRP3 cDNA, showing localization of the NaPRP3 mRNA in cells of the transmitting tissue.
(D) Autoradiograph of the same section as **(C)** after hybridization with the ^{32}P-labeled proteinase inhibitor cDNA, showing that the proteinase inhibitor gene is specifically expressed in cells of the stigma.
Bars = 500 μm.

clone (Chen and Varner, 1985) as a probe. We also demonstrate expression of an extensin gene in styles and pollen, as well as in other tissues.

The Protein Sequence Derived from NaPRP3 Is Similar to Extensin

The protein encoded by NaPRP3 is similar to extensin in having a high proportion of Pro and Ser residues and the characteristic Ser-Pro_4 repeats. The six Ser-Pro_4 repeats in NaPRP3 are tandemly reiterated with or without a Ser-Pro dipeptide between the repeats. The same arrangement of Ser-Pro_4-Ser-Pro-Ser-Pro_4 is present in extensin sequences deduced from cDNA and genomic clones of several plants such as tomato, petunia, and bean (Showalter and Varner, 1989). The peptide sequence Ser-Hyp_4-Ser-Hyp-Ser-Hyp_4 was also found in tomato extensin through peptide sequencing by Smith et al. (1986). Like many extensins, NaPRP3 contains a putative signal peptide, suggesting that NaPRP3 may also be a secreted cell wall protein. Similar signal peptides have been identified in many cloned extensin sequences.

There are, however, differences between the NaPRP3 protein and the extensins. The polypeptide deduced from the NaPRP3 genomic clone is much smaller than any reported extensin in that it contains only 151 amino acid residues, in contrast to the polypeptides encoded by the tomato and carrot genomic extensin clones that contain 372 and 306 amino acids, respectively (Chen and Varner, 1985; Showalter and Varner, 1987). Whereas the lengths of the nonrepeating sequences at both the N terminus and C terminus in NaPRP3 protein are about the same as in other extensins, the proportion of these sequences in the whole molecule is higher in the NaPRP3 protein because of its overall smaller size.

A second difference is that the NaPRP3 protein lacks Tyr, which many extensins contain, although the tomato extensin encoded by the genomic clone Tom5 only contains one Tyr residue. Tyr residues have been proposed to be involved in cross-linking the molecules to form a network in cell walls (Fry, 1982; for a review, see also Wilson and Fry, 1986). The lack of Tyr residues may allow secretion of NaPRP3 through the wall into the transmitting tract matrix.

NaPRP3 is also different from hydroxyproline-proline-rich proteins, which contain characteristic repeats such as Pro-Pro-Val-Tyr-Lys (Showalter and Rumeau, 1990). It also differs from the solanaceous lectins in its lack of cysteine residues.

Regulation of Expression of the NaPRP3 Gene Is Different from That of the Extensin Gene

RNA gel blot analyses showed that the NaPRP3 gene is highly expressed only in the style of *N. alata* and that it is developmentally regulated. In situ hybridization experiments indicated that expression of the NaPRP3 gene is mainly in the cells of the transmitting tissue of the style, although we cannot rule out weak expression in other tissues. In contrast, the extensin gene was expressed in all tissues tested in *N. alata*, although its expression in different tissues differs, being highest in roots, relatively high in styles, and much lower in pollen, petals, and leaves. These data are consistent with the observations of Showalter and Varner (1987), who showed that roots have the highest levels of extensin mRNA, whereas leaves contain very low levels. The number and size of the extensin transcripts are also different in various tissues of *N. alata*. Pollen contains two large transcripts (2.5 and 3.0 kb); in contrast, there is one dominant smaller (1.8 kb) transcript in other tissues. Similar

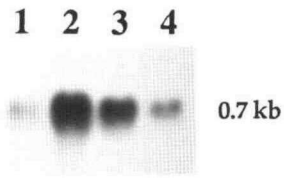

Figure 9. Expression of the NaPRP3 Gene during the Style Development.

Total RNA was isolated from styles of (1) small buds (2 to 3 cm), (2) fully elongated buds (5 to 6 cm), (3) 1-day-old flowers, and (4) 2-day-old flowers. Equal amounts (8 μg/lane) of RNA samples were fractionated on a formaldehyde agarose gel, transferred to a Hybond-N membrane, and hybridized with the ^{32}P-labeled 3′ probe of the NaPRP3 cDNA. The size of the RNA transcript is indicated at the right.

expression patterns for the extensin gene were also noted for soybean (Hong et al., 1989).

Wounding resulted in a dramatic increase in the expression of the extensin gene in *N. alata* styles, stems, and leaves, similar to that reported in other dicotyledonous species such as carrot, bean, and tomato (Chen and Varner, 1985; Corbin et al., 1987; Showalter and Varner, 1987). In contrast, the NaPRP3 gene in styles was not induced after wounding but decreased, as also occurs with aging of intact styles. No NaPRP3 transcript was induced after wounding in stems or leaves. It is interesting to note that the tobacco extensin gene HRGPnt3, the expression of which was restricted to the cells at the tip of emerging lateral roots, was not induced in mature stems and leaves by wounding (Keller and Lamb, 1989).

Possible Function of the NaPRP3 Gene

The fact that expression of the NaPRP3 gene is restricted to cells of the transmitting tissue and reaches a maximum level in fully elongated buds suggests a correspondingly specific function for this protein. Transmitting tissue cells in styles of *N. alata* have thin primary walls, and the cells are separated at maturity by extracellular, densely staining, amorphous material (Bell and Hicks, 1976; Sedgley et al., 1985). During pollination, the transmitting tissue serves as the pathway through which pollen tubes grow to the ovary. The transmitting tissue cells might be expected to encounter strong mechanical pressure caused by the penetration of pollen tubes. The presence of a putative signal peptide suggests that NaPRP3 may be a secreted, extracellular protein, and the structural similarity of the NaPRP3 with extensins suggests that the NaPRP3 protein may play a structural role in cell walls: deposition of this protein may reinforce walls in cells of the transmitting tissue. In this context, an extensin gene (HRGPnt3), which was highly expressed only in the pericale and endodermis in cells destined to form the tips of the emerging lateral root, has been described by Keller and Lamb (1989). A specific structural role in strengthening the cell walls of the new roots to withstanding mechanical forces was also suggested for its HRGPnt3 protein.

Apart from providing a physical pathway for the pollen tubes, transmitting tissue cells are also involved in providing appropriate physiological conditions for germination and growth of compatible pollen. Members of one of the three major classes of hydroxyproline-rich glycoconjugates in plants, the arabinogalactan proteins, are major components of the extracellular matrix of the transmitting tissue of several species (Hoggart and Clarke, 1984). It has been suggested that AGPs may serve as glues or provide nutrients for growing pollen tubes (Gleeson and Clarke, 1979), and similarly a function for the NaPRP3 proteins may be in guidance or nutrition of the growing pollen tubes. Lastly, during maturation of the style, transmitting cells secrete sucrose and amino acids into the extracellular medium and onto the surface of the stigma, and these may act as nutrients for potential pathogens. The maximum level of the expression of the NaPRP3 gene is correlated with the maturity of the style. In this study, we have demonstrated that the extensin gene is expressed in the style of *N. alata* consistent with a protective role for extensin in the style, and NaPRP3 may also have a similar defense function in the transmitting tract. The question of NaPRP3 gene function will be addressed through a study of the properties and location of its protein product.

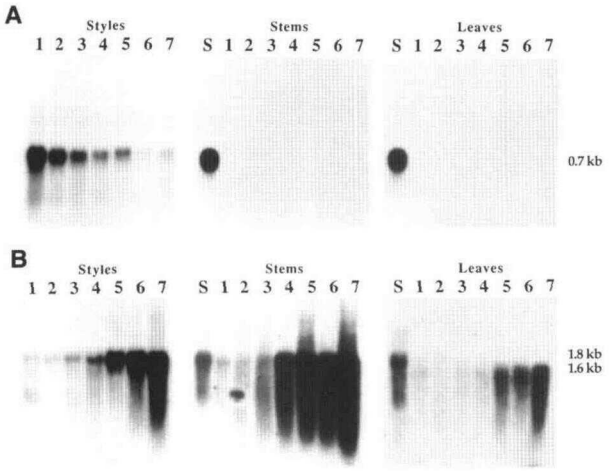

Figure 10. Response of the NaPRP3 and Extensin Genes in Styles, Stems, and Leaves after Wounding.

RNA was isolated from tissues (1) 0, (2) 1, (3) 2, (4) 4, (5) 8, (6) 12, and (7) 24 hr after wounding. Equal amounts of total RNA (8 μg/lane for style RNA and 10 μg/lane for stem and leaf RNA) were fractionated on formaldehyde agarose gels, transferred to Hybond-N membranes, and hybridized with ^{32}P-labeled probes. RNA (10 μg) from unwounded styles (S) was included in the blots of stem and leaf RNA for reference.

(A) Autoradiograph of the RNA gel blots after hybridization with the 3′ probe of the NaPRP3 cDNA.

(B) Autoradiograph of the same blots after hybridization with the 1.8-kb XbaI fragment of the pDC5A1 clone. The size of RNA transcripts is indicated at the right.

METHODS

Plant Materials

Four *Nicotiana alata* plant lines of different self-incompatibility genotypes (S_1S_3, S_2S_3, S_6S_6, and S_6S_7) (Anderson et al., 1986) were, as available, used in the present study. In each experiment, the S-genotype of the plant material used is stated, although there is no reason to expect any relationship between S-genotype and the proline-rich protein genes studied. Seed of incompatibility genotype S_1S_3 and S_2S_3 was a kind gift from Dr. K. K. Pandey (Division of Scientific and Industrial Research, Palmerston North, New Zealand). S_6S_6 and S_6S_7 plants were grown from seed kindly provided by Dr. G. Breidemeijer (Stiching Ital, Wageningen, The Netherlands). All of the plants were maintained under glasshouse conditions.

For experiments on gene expression after wounding, styles were wounded by cutting into fragments 6- to 8-mm long. Young stems (3 mm in diameter) were cut into approximately 2-mm sections. Leaf tissues were wounded by fragmenting the leaf into rectangular sections approximately 3 × 10 mm. The wounded tissues were incubated in a moist chamber at 25°C for 1 to 24 hr. The tissues were then frozen in liquid nitrogen and stored at −70°C prior to analysis.

Extensin Probe

Plasmid pDC5A1 containing a fragment of genomic DNA encoding carrot extensin was kindly made available by Drs. J. Chen and J. E. Varner (Department of Biology, Washington University, St. Louis, MO). An XbaI 1.8-kb fragment that encodes 25 Ser-Pro_4 pentapeptide repeats was isolated and used as a probe.

Isolation of RNA and DNA

Total RNA was extracted with guanidinium thiocyanate, followed by centrifugation in caesium chloride solutions according to Maniatis et al. (1982). Polyadenylated RNA was selected by spun columns containing oligo(dT)-cellulose (Pharmacia). Genomic DNA was isolated from leaf tissues according to the procedure of Bernatzky and Tanksley (1986).

Construction of cDNA and Genomic Libraries

The construction of the S_2S_3 cDNA library in λgt10 from mRNA isolated from mature *N. alata* S_2S_3 styles was reported previously (Anderson et al., 1986). To construct the S_6S_6 cDNA library, 5 μg of polyadenylated RNA isolated from mature styles of *N. alata* plants of genotype S_6S_6 was used to synthesize cDNA using a cDNA synthesis kit (Pharmacia). After ligation of EcoRI linkers to the cDNA, the cDNA was ligated to phage λZAP arms (Stratagene). The resulting phage DNA was packed in vitro into phage particles using Gigpack II packaging extract (Stratagene) and plated with *Escherichia coli* XL1-blue.

A genomic library in λDash (Stratagene) was constructed by Dr. S.-L. Mau from *N. alata* (genotype S_6S_6) DNA partially digested with Sau3AI to yield fragments of 10 to 23 kb in size.

Isolation of cDNA and Genomic Clones

The S_2S_3 and S_6S_6 style cDNA libraries were screened by hybridization (Maniatis et al., 1982) with the 1.8-kb XbaI fragment from the pDC5A1 plasmid. The hybridization was performed at 42°C overnight in 5 × SSC, 50% deionized formamide, 5 × Denhardt's solution, 0.1% SDS, 50 mM Na phosphate, pH 6.8, 1 mM Na pyrophosphate, and 0.5 mg/mL denatured salmon sperm DNA. Filters were washed twice in 2 × SSC, 0.1% SDS at 42°C for 30 min and twice in 1 × SSC, 0.1% SDS at 42°C for 30 min.

The genomic library was screened with the 3′ probe of the NaPRP3 cDNA labeled by random priming (Feinberg and Vogelstein, 1984). Filters were prehybridized at 68°C for 2 hr in a solution containing 1.5 × SSPE, 1% SDS, 0.5% defatted dried milk (Blotto), and 0.5 mg/mL denatured salmon sperm DNA. Hybridization was performed at 68°C for 12 to 16 hr in the same buffer plus 10% dextran sulphate and ^{32}P-labeled probes. Filters were rinsed with 2 × SSC at room temperature and washed twice in 2 × SSC, 0.1% SDS at 68°C for 30 min. A final 30-min wash was performed in 1 × SSC, 0.1% SDS at 68°C. Positively hybridizing plaques were selected and rescreened to ensure purification and elimination of false positives. DNA inserts in λgt10 or λDash were cut and subcloned into either pGEM-3Z or pBluescript II KS+ plasmid vectors, whereas cDNA inserts in λZAP were subcloned directly into pBluescript II SK+ plasmid by in vivo excision (Stratagene) for the sequence analysis.

DNA Sequence Analysis

Double-stranded DNA sequencing was performed using the dideoxy chain termination method of Sanger (Sanger et al., 1977), following either the Klenow fragment protocol (Amersham International) or the sequenase protocol (United States Biochemical Corp). When required, oligonucleotides (20-mers) were synthesized according to sequence information and used directly as primers for further sequencing.

Primer Extension

A 30-mer synthetic oligonucleotide of sequence 5′-GATCTCCATTA-GTGAGCTCAGAAAATGAGC-3′, priming at position 84 to 113 of the genomic clone NaPRP3g12, was end-labeled with [γ-^{32}P]ATP (Amersham International) and T_4 polynucleotide kinase (Promega). Total RNA (10 μg) was mixed with 0.1 pmole gene-specific radioactive primers in 10 μL of 40 mM Pipes (pH 6.0), 1 mM EDTA, and 0.4 M NaCl. The mixture was heated at 80°C for 5 min and incubated at 37°C overnight. The RNA/primer mixture was precipitated by ethanol and resuspended in 20 μL of reverse transcription buffer containing 50 mM Tris-HCl (pH 8.3), 60 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 20 U RNasin, and 50 U AMV reverse transcriptase. After 1-hr incubation, the reaction was stopped by addition of EDTA. After RNase digestion, phenol/chloroform extraction, and ethanol precipitation, the primer extension product was analyzed by polyacrylamide gel electrophoresis.

RNA and DNA Gel Blots

Total RNA was fractionated in a 1.2% agarose gel containing 2.2 M formaldehyde according to Maniatis et al. (1982). The amount of RNA and the integrity of ribosomal RNA were confirmed by ethidium bromide staining of the gels. Gels were blotted to Hybond-N membranes

(Amersham International) using 20 × SSC as transfer buffer, followed by 3-min treatment with UV light to fix the RNA to the membrane.

DNA samples (5 μg) were digested with the appropriate restriction endonuclease. The restriction fragments were separated by electrophoresis in 0.9% agarose gels and blotted onto a Zeta-Probe membrane (Bio-Rad) using 0.4 M NaOH as the transfer buffer.

RNA and DNA gel blots were prehybridized (2 hr) and hybridized (12 to 16 hr) at 68°C in a buffer containing 1.5 × SSPE, 1% SDS, 0.5% Blotto, 0.5 mg/mL denatured salmon sperm DNA and 10% dextran sulphate. Blots were rinsed with 2 × SSC at room temperature and washed twice in 2 × SSC, 0.1% SDS at 68°C for 30 min. A final 30-min wash was performed in 1 × SSC, 0.1% SDS at 68°C.

DNA gel blots that were to be reprobed were washed in 0.4 M NaOH for 10 min to release bound probe, the filters were neutralized by washing in a solution containing 0.1 M Tris-HCl, pH 8.0, 0.1 × SSC, and 0.1% SDS for 15 min and then were washed with 0.1 × SSC, 0.1% SDS for 10 min. The filter was then prehybridized and hybridized as above with a different probe.

In Situ Hybridization

In situ hybridization was performed according to Cornish et al. (1987). Hybridization of the ^{32}P-labeled cDNA probes to the sections was carried out at 40°C for 12 to 16 hr in the hybridization buffer containing 5 × Denhardt's solution, 5 × SSC, 50 mM Na phosphate buffer, pH 6.8, 1 mM Na pyrophosphate, 5 mM EDTA, 50% formamide, and 0.5 mg/mL herring sperm DNA. The sections were washed successively in 2 × SSC at room temperature, 1 × SSC at 40°C for 30 min, and twice in ethanol. Cronex video imaging films (Du Pont) were exposed for the appropriate time. Photographs of the sections (stained with 0.05% toluidine blue) and the autoradiographs were taken under a light microscope.

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