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Expression of Cysteine Protease Genes in Pea Nodule Development and Senescence

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Coding sequences for two cysteine proteases were amplified from cDNA derived from pea nodule mRNA using primers based on conserved regions of plant cysteine proteases. One of the amplified cDNA sequences corresponded to a previously described cysteine protease gene, *Cyp15a*, expressed in pea shoots in response to dehydration (J. T. Jones and J. E. Mullet, Plant Mol. Biol. 28:1055-1065, 1995). Inside the pea root nodule, in situ hybridization revealed that this gene is expressed strongly in the apical region and more weakly in the uninfected cortex and in the central infected tissue where nitrogen fixation takes place. The complete sequence of the cDNA corresponding to the other gene, PsCyp1, was obtained. Expression of this gene, which was studied both on RNA blots and in situ, showed good correlation with the onset of nodule senescence. In situ hybridization studies revealed that *PsCyp1* was expressed in senescent infected tissue at the base of the nodule. This signal was just detectable in normal symbiotically wild-type nodules but was much stronger in the early senescing nodules formed by a symbiotically defective mutant of *Rhizobium leguminosarum*.

Additional keywords: Pisum sativum, Rhizobium leguminosarum bv. *viciae*.

Cysteine, or thiol, proteases are very common in plants. Both genetic and biochemical evidence indicate that these proteases play an important role in cell development and cell senescence. Increased levels of protease activity are observed during leaf abscission (Wittenbach et al. 1982) and in programmed ovary senescence (Vercher et al. 1989). A cDNA coding for a cysteine protease was described among the senescence-related cDNAs isolated from the leaves of *Arabidopsis thaliana* by differential screening (Hensel et al. 1993). Elevation of cysteine protease gene expression also appears common as a result of environmental stress. It has been described in response to dehydration (Guerrero et al. 1990), mechanical wounding (Linthorst et al. 1993; Lidgett et al. 1995), and exposure to low temperatures (Schaffer and Fischer 1988). Furthermore, cysteine protease gene expression can be modulated during the circadian rhythm (Lidgett et al. 1995). These observations suggest that cysteine protease activity in plants is transcriptionally regulated and occurs whenever rapid changes in cell metabolism are required. Presumably protease activity serves to reutilize intracellular resources stored in the form of hydrolyzable proteins.

In legume root nodules, there is evidence that cysteine proteases could be involved in nodule function, in the adaptation of host cells to physiological stresses, and in the control of nodule senescence. Proteases and protease inhibitors have been identified in the cytoplasm of infected nodule cells (Pfeiffer et al. 1983; Manen et al. 1991; Pladys et al. 1991; Garbers et al. 1988; Mellor et al. 1984). A cysteine protease with an acidic pH optimum has been described in French bean nodules (Pladys et al. 1991); its activity increased markedly with the onset of senescence. Similar observations have been made in soybean (Pfeiffer et al. 1983), black gram (Lahiri et al. 1993), and alfalfa (Pladys and Vance, 1993).

Following the induction of a nodule meristem by *Rhizobium*-derived nodulation factors (lipochitin-oligosaccharides), host plant tissues and cells are colonized by infective rhizobia. The bacteria gain access to plant tissues by inducing the formation of infection threads which are tubular ingrowths of plant cell wall and plant cell membrane (Rae et al. 1992). Subsequently, the rhizobia are released into the cytoplasmic space of host cells as endosymbiotic bacteroids, enclosed by a plant-derived peribacteroid membrane. Thus, bacteria in the nitrogen-fixing stage of the *Rhizobium*-legume symbiosis inhabit a specialized compartment of the endomembrane system, an organelle-like structure termed the symbiosome. In legumes with indeterminate apical meristems, the life span of the symbiosome is not long because the central infected tissue is continually being regenerated from an apical meristem. For example, in pea and alfalfa, senescence of symbiosomes occurs in the basal part of the nodule as early as 14 days after inoculation (Truchet and Coulomb 1973; Kijne 1975) and is manifested by disintegration and collapse of the host cell and digestion of enclosed bacteria. Such processes could be regulated by the activity of proteases, particularly during nodule senescence. In legume species that form nodules with determinate meristems, the start of senescence usually correlates with the end of flowering and seed-setting, as has been shown for *Glycine max* (Pfeiffer et al. 1983), *Phaseolus vulgaris* (Pladys and Rigaud, 1985) or *Vigna mungo* (Lahiri et al. 1993). Often nodule senescence is accelerated if the symbiosis is ineffective due to a genetic lesion in the plant (Borisov et al. 1993; Kneen et al. 1990; Pladys and Vance 1993) or in the

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The complete sequence of *PsCyp1* cDNA was submitted to the GenBank database with the accession number U44947.

bacterium (Hirsch and Smith 1987; Perotto et al. 1994). It has been proposed that nodule senescence is a delayed response of the plant to *Rhizobium* as a potential pathogen, and that this response is suppressed during the productive stage of the symbiosis (Mellor 1989).

The pea root nodule is one of the most intensely studied symbiotic models, but there is no genetic information concerning the involvement of cysteine proteases in nodule development and host cell senescence. Expression of genes for cysteine proteases in pea has been demonstrated both during senescence of ovaries (Carrasco and Carbonell 1990; Granell et al. 1992) and in response to dehydration (Guerrero et al. 1990). Here we report the isolation from pea nodules of two cDNAs that encode cysteine proteases. One of the cDNAs appears to be identical to the *Cyp15a* clone previously described in pea wilting studies (Guerrero et al. 1990); the other, *PsCyp1*, represents the coding sequence of a new gene.

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   1  ACACCAAAAA TATCCAACTA GTTCAAAGCC TAATCCACCT TTCTCTCCTT
                          M   A   S   I   L   Y   S   L   I   L   F
  51  AATTAGCCAA ACACCAATCA TGGCATCAAT ACTATACTCT TTGATCTTAT
        G   L   I   T   L   S   L   S   L   D     M   S   S     G   R   S
 101  TTGGCTTGAT CACTTTGTCA TTATCATTAG ACATGTCAAG TGGACGTAGT
        N   K   E   V     M   T   M     Y   E   K     W   L   V   K     H   Q   K
 151  AACAAGGAGG TTATGACCAT GTACGAGAAA TGGTTAGTAA AACACCAAAA
        V   Y   Y     G   L   G   E     K   N   Q     R   F   Q     I   F   K   D
 201  AGTGTATTAC GGATTAGGAG AGAAAAATCA ACGGTTCCAA ATATTTAAGG
          N   L   I     F   I   D     E   H   N   A     P   N   H     S   Y   R
 251  ATAACTTAAT ATTCATAGAT GAGCATAATG CTCCAAATCA CAGTTATAGA
        V   G   L   N     E   F   S     D   I   T     N   K   E   Y     R   D   T
 301  GTTGGGTTGA ACGAGTTTTC AGATATAACT AATAAAGAAT ATCGTGACAC
          Y   L   S     R   W   S   N     N   N   I     K   N   K     I   T   S   V
 351  GTATTTGAGC AGGTGGAGTA ATAATAATAT TAAAAACAAG ATTACCAGTG
          R   Y   A     Y   K   A     G   H   N   N     K   L   P     V   S   V
 401  TGCGATATGC TTATAAGGCA GGTCATAATA ATAAATTGCC CGTGTCTGTT
                                                          ThPa
        D   W   R   G     A   L   T     P   I   K     N   Q   G   S     C   G   A
 451  GATTGGAGAG GGGCTCTTAC TCCTATAAAA AATCAAGGAA GTTGTGGAGC
                3'-1                                    3'-2
        C   W   A     F   S   A   V     A   A   V     E   A   I     N   K   I   V
 501  GTGTTGGGCG TTTTCTGCAG TAGCGGCAGT GGAAGCTATT AACAAGATAG
          T   G   S     L   V   S     L   S   E   Q     E   L   V     D   C   D
 551  TGACAGGGAG TCTTGTGAGT TTGTCTGAAC AAGAGCTTGT GGACTGTGAT
          5'-2                                    5'-1
        R   T   K   N     K   G   C     N   G   G     N   Q   V   N     A   Y   R
 601  AGAACAAAAA ATAAAGGGTG TAATGGTGGT AACCAGGTCA ACGCGTACAG
          F   I   V     E   N   G   G     L   D   S     Q   I   D     Y   P   Y   L
 651  ATTCATCGTT GAAAATGGTG GCCTTGACTC ACAGATAGAT TATCCATACC
          G   R   Q     S   T   C     N   Q   A   K     K   N   T     K   V   V
 701  TAGGTCGGCA GAGCACATGT AATCAAGCAA AAAAAAATAC CAAGGTAGTG
          S   I   N   G     Y   K   N     V   Q   R     N   S   E   S     A   L   M
 751  AGCATCAATG GTTATAAGAA TGTCCAACGA AATAGTGAAA GTGCGTTAAT
          E   A   V     A   N   Q   P     V   S   V     G   I   E     A   Y   G   K
 801  GGAAGCTGTG GCTAATCAAC CTGTCAGCGT TGGTATTGAG GCATATGGAA
          D   F   Q     L   Y   Q     S   G   V   F     T   G   S     C   G   T
 851  AAGATTTTCA ACTCTACCAA TCGGGTGTAT TTACTGGTAG TTGTGGAACT
          S   L   D   H     A   V   V     V   V   G     Y   G   S   E     N   G   K
 901  AGCTTAGATC ATGCTGTGGT GGTTGTTGGA TATGGTTCTG AAAATGGTAA
          D   Y   W     L   V   K   N     S   W   G     T   N   W     G   E   R   G
 951  AGATTATTGG TTGGTGAAGA ACTCATGGGG GACTAACTGG GGTGAACGTG
                                        ThPb
          Y   L   K     I   E   R     N   L   K   N     T   N   T     G   K   C
1001  GTTACTTAAA GATAGAGCGT AATCTGAAAA ATACAAATAC AGGAAAGTGT
          G   I   A   M     D   A   T     Y   P   T     K   L   R   E     N   S   E
1051  GGAATCGCAA TGGATGCTAC ATACCCAACT AAGCTTCGTG AAAACTCTGA
          V   T   N     S   G   Y   E     K   L   Q     M   L   V     P   V   L   E
1101  AGTTACTAAT TCTGGTTATG AAAAACTCCA AATGCTTGTA CCAGTGCTTG
            T   P   T     N   V   A     *
1151  AAACACCTAC AAACGTTGCT TAGTATGCCT ATTGGATTAG AATATATGCC
1201  TATAGATTTT ATTACTCTTT TAATAATCAG TTTTTCAATT TCAGTGTGTA
1251  CTCTCTAAAT GTATTGGACT CAAGTGCTTG TTGTATTTTG GATTTAAAAA
1301  TAATAAACTT TTACAACCCC AAAAAAAAAA AAAAAAA
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Fig. 1. Sequence and protein translation product of the *PsCyp1* cDNA with GenBank database accession number U44947. Positions and orientations of oligonucleotides used for amplification of the cDNA fragments are shown with arrows. ThPa and ThPb are redundant oligonucleotides used in initial amplification (see Materials and Methods). The pNCyp1 sequence runs from the 5′ end of ThPa to the 3′ end of ThPb. Primers 3′-1 and 3′-2 were used in two subsequent rounds of 3′-RACE; 5′-1 and 5′-2 were used for 5′-RACE. Predicted catalytic site Cys and His residues in the protein sequence are in bold: these correspond to Cys[145] and His[281] (numbering in the complete PsCyp1 protein sequence).

RESULTS

Amplification of putative cysteine protease sequences from nodule cDNA.

Based on the alignment of 21 plant cysteine proteases and related sequences, the degenerate sense-oriented oligonucleotide *ThPa* was constructed (Fig. 1). The nucleotide sequence of ThPa, TGY GGI HSI KGI TGG GCI TT (IUPAC ambiguity code, 'I' stands for inosine), corresponds to the protein sequence G S/R C/G WAF around the highly conserved catalytic Cys residue. The antisense oligonucleotide *ThPb* CCA ISW RTT YYY IRY IAD CCA was derived from the conserved protein sequence W I/L V/A K/R NSW found in the vicinity of the catalytic His residue. These degenerate oligonucleotides were derived from the regions that had a high degree of conservation both within plants and between plant proteins and one representative from the animal kingdom (Ishidoh et al. 1987). Oligonucleotide sequences were designed on the basis of low redundancy of the genetic code for a particular combination of residues and also because of the evolutionary conservation of particular motifs (Kamphuis et al. 1985).

Following RT-PCR, the majority of amplification products had sizes of about 500 bp (as would have been predicted from the distance between the oligonucleotide primers used). After cloning and sequencing of these products, two classes of insert were identified, one with 480-bp inserts (two clones), and another with 500-bp inserts (three clones). The representative clones were named pNCyp1 and pNCyp2, respectively. Clone pNCyp1 is a new putative cysteine protease, and we term the corresponding gene *PsCyp1*. The sequence of the pNCyp2 DNA insert was identical to that of the *Cyp15a* clone reported by Guerrero et al. (1990). This cDNA clone was initially isolated from pea shoots by screening for mRNAs that were induced in response to wilting, and its full sequence has already been determined. For convenience, we use the names *PsCyp1* and *PsCyp15a* in comparative description of the two genes whose transcripts were isolated from nodules in this study. Further analysis of the *PsCyp1* cDNA is presented here in order to describe this new cDNA sequence. In addition, both pNCyp1 and pNCyp2 DNAs were used to obtain hybridization probes to monitor gene expression in nodule tissues.

Characterization of the *PsCyp1* sequence.

Because pNCyp1 represents only a small internal fragment of the *PsCyp1* mRNA, the coding sequences missing from this clone were amplified using 3′- and 5′-RACE procedures. Oligonucleotides *3′-1* and *3′-2*, derived from the pNCyp1 sequence (Fig. 1), were used sequentially to amplify the region of the mRNA that was in the 3′ direction from the known fragment of the message sequence. Similarly, the gene-specific antisense oligonucleotides *5′-1* and *5′-2* (Fig. 1) were used sequentially to obtain the 5′-region of the *PsCyp1* cDNA. In total, four 5′-RACE clones and three 3′-RACE clones were sequenced in both orientations. The complete sequence of *PsCyp1* cDNA was submitted to the GenBank database with the accession number U44947.

The sequence of the *PsCyp1* cDNA is presented in Figure 1. It contains 1,320 bases and has a poly(A) tail on the 3′ end. The size of the cDNA sequence is in good agreement with the size of the transcript observed on Northern blots (see below), and it is probable that it corresponds to a complete mRNA sequence because all four sequenced 5′-RACE PCR products

had the same 5′ end. In addition, it has an open reading frame that encodes a polypeptide starting with the Met residue; this polypeptide has a putative hydrophobic N-terminal signal sequence. The encoded polypeptide consists of 367 amino acid residues. Arg^{26} is predicted to be the most probable signal peptide cleavage site (von Heijne 1986) and the calculated molecular mass of the processed polypeptide is 38 kDa. Along its entire length, the predicted protein has high sequence similarity to other plant cysteine proteases (50 to 65% overall identity), and the two residues of the catalytic domain, Cys^{145} and His^{281} (numbering in the complete PsCyp1 protein sequence) are conserved. Interestingly, the two highest ranking matches for PsCyp1 protein were observed with the putative cysteine protease sequence from *Vicia sativa* (Becker et al. 1994) and the tpp sequence from *Pisum sativum* (Granell et al. 1992); the tpp sequence has been implicated in ovary senescence. High sequence conservation suggests that the *PsCyp1* probably encodes a functional cysteine protease.

Expression of the *PsCyp1* and *PsCyp15a* genes.

Inserts from the pNCyp1 and pNCyp2 plasmids were used to probe Northern blots containing RNA from different tissues of the pea plant: These included uninfected roots, stems, leaves, buds, and nodules from peas of various ages and genotypes. Corresponding mRNAs were detected in all tested tissues, but in different quantities (Fig. 2). The electrophoretic mobility of the two mRNAs detected on Northern blots was consistent with their expected size, based on the size of the corresponding cDNA clones (approximately 1,300 bases and 1,400 bases long, respectively). Under moderately stringent conditions, no significant cross-hybridization was observed between the two genes. Expression of *PsCyp1* mRNA was higher in nodules than in shoot tissues (buds, leaves, and stems), but *PsCyp15a* mRNA was found at comparable levels in all parts of the plant. Comparison of mRNA from nodules (20 days postinfection) and uninfected roots of the same age showed that expression of both cysteine protease genes was approximately twofold higher in nodules than in roots. The 13-day-old nodules had about the same level of expression of cysteine protease genes as in roots; in mature nodules there was no significant difference in signal between 30- and 60-day-old plants. However, in nodules formed by the bacterial mutant strain B661, there was a significant increase in the level of the *PsCyp1* signal. These bacteria have a defect in lipopolysaccharide (LPS) biosynthesis and form non-fixing nodules which senesce prematurely (Perotto et al. 1994). There was no statistically significant difference between the wild-type and B661 nodules with respect to the *PsCyp15a* expression (results not shown).

Analysis of cysteine protease gene expression by hybridization in situ.

To analyze tissue-specific expression of the cysteine protease genes *PsCyp1* and *PsCyp15a*, probes derived from pNCyp1 and pNCyp2 were used on median longitudinal sections of nodules, as presented in Figure 3 (with the persistent apical meristem oriented to the right). The transcript from *PsCyp15a* was expressed in uninfected tissue including cortical parenchyma (Fig. 3B), vascular tissue (Fig. 3H), and also in uninfected cells in the central tissue (Fig. 3B). In addition, *PsCyp15a* was expressed in infected cells (Fig. 3B) and here the transcription pattern was somewhat surprising. The signal was strongly expressed in the cells of the invasion zone in the apical part of the nodules, where ramification of infection threads and endocytosis of bacteria occurs (Fig. 3B). A sudden decrease of the *PsCyp15a* signal was observed at the proximal end of the apical zone on the border of the invasion zone and the early symbiotic zone, as inferred from the localization of the leghemoglobin signal in another section of the same nodule (Fig. 3D). The point of this down regulation corresponds to the transition between zone II and the interzone II-III, as described by Vasse et al. (1990) for nodules of alfalfa. A low level of *PsCyp15a* transcript was still observed in the central

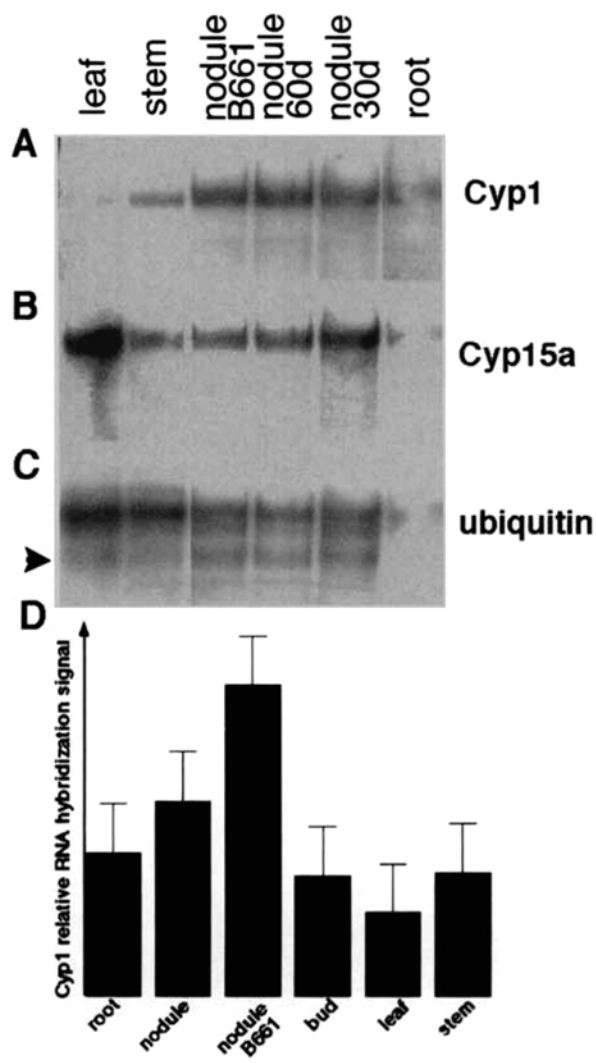
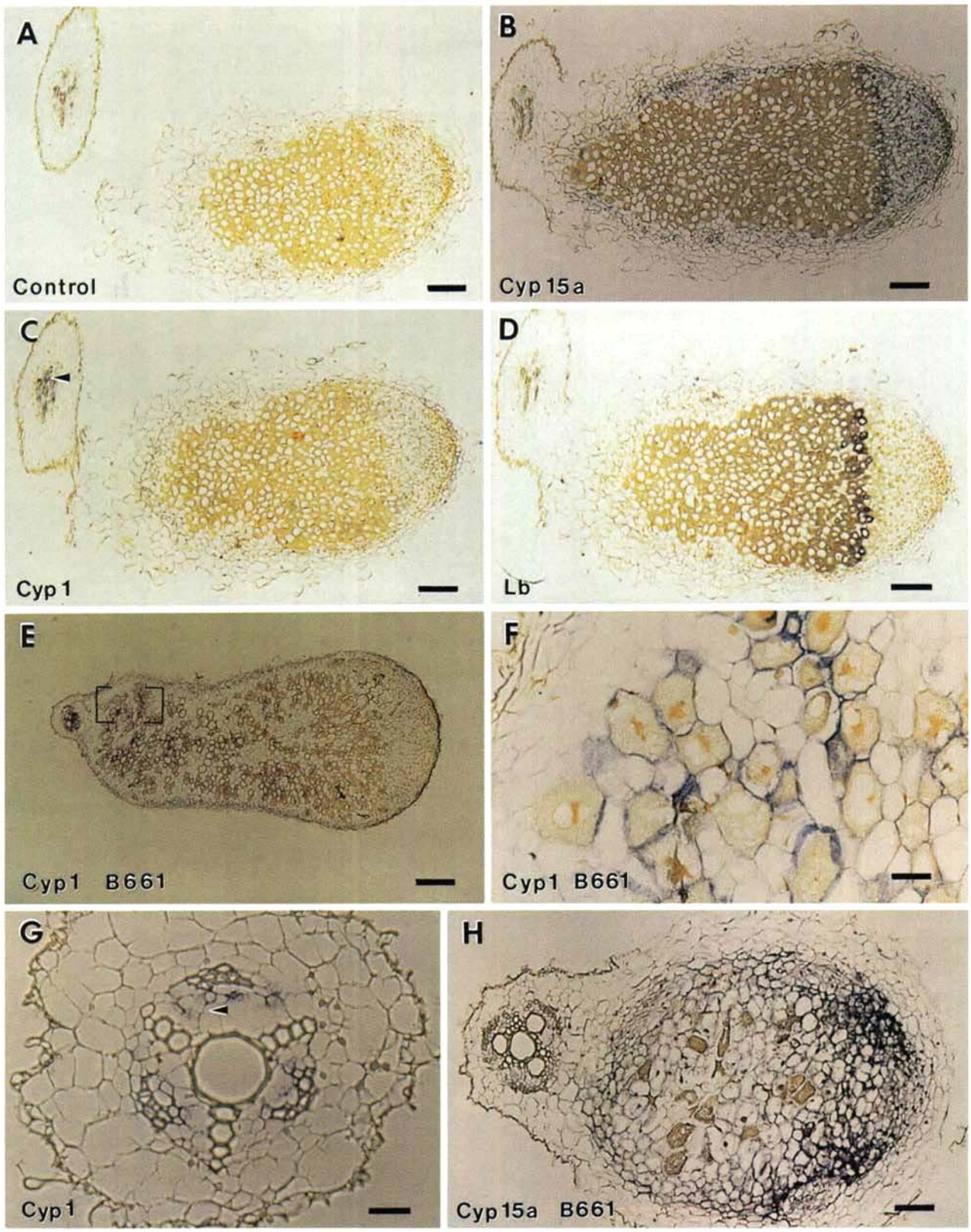


Fig. 2. Northern blot of pea tissues probed with insert DNA from pNCyp1 and pNCyp2 to reveal transcripts from *PsCyp1* (A) or *PsCyp15a* (B), respectively. The same blot was sequentially hybridized with the cysteine protease probes and with ubiquitin (C). The signal was completely removed from the blot before each subsequent hybridization. Cumulative data from a number of similar blots for the *PsCyp1* gene are displayed quantitatively in (D). The relative strength of the hybridization signal was compared in different tissues, using as a reference the invariant ubiquitin band marked with an arrowhead. Data from different filters were brought to the same relative scale using reference samples common to several filters and assuming linear regression. Error was calculated as sample standard deviation from regression for reference samples.


A
Control
B
Cyp 15a
C
Cyp 1
D
Lb
E
Cyp1 B661
F
Cyp1 B661
G
Cyp1
H
Cyp15a B661

infected tissue and, in addition, the signal was detected in the senescent zone (zone IV). The signal for *PsCyp15a* in the basal part was increased in the non-fixing, early senescing nodules formed by the B661 mutant. However, because of the much higher hybridization at the apical region in both Fix$^-$ B661 nodules (Fig. 3H) and wild-type nodules (Fig. 3B), it is probable that the inability to detect any gross increase in the level of *PsCyp15a* signal on Northern blots for B661 nodules was due to the 'masking' of this senescence-related signal by the more dominant signal derived from the apical region.

In sections taken from wild-type nodules, the hybridization signal for the *PsCyp1* probe was extremely difficult to detect and was scarcely visible in counterstained specimens such as that illustrated in Figure 3C. This weak signal was confined to a few cells close to the base of the nodule, as might be expected for a gene probably associated with senescence. In addition, there was slight expression of *PsCyp1* in parenchymal cells within the stele section of roots near the nodule tissue (Fig. 3G). Expression of *PsCyp1* was much stronger in the senescent zone of non-fixing nodules induced by the LPS-defective mutant strain B661 (Fig. 3E,F). In this case, transcript was apparently localized in both infected and uninfected cells of the central tissue.

DISCUSSION

In pea, there are examples of cysteine protease genes which are up-regulated during ovary senescence (Granell et al. 1992), or in response to water stress (Guerrero et al. 1990). We have investigated the possibility that cysteine protease genes may also be involved in pea nodule development and senescence. By using primers derived from conserved regions of plant cysteine proteases, we were able to amplify segments of two mRNAs corresponding to genes *PsCyp1* and *PsCyp15a* that are expressed in nodules and show high homology to cysteine proteases. One of these mRNA sequences has not been described previously, while the other was identified in a collection of cDNAs obtained from mRNA that was up regulated in pea shoots in response to dehydration (Guerrero et al. 1990).

In situ hybridization experiments revealed a high level of gene expression for *PsCyp15a* in the apical part of the nodule and in the nodule cortex. This gene has previously been shown to be strongly expressed in leaves and stems when plants were dehydrated until turgor was lost (Guerrero et al. 1990). In mature nodules, gene expression for *PsCyp15a* is high throughout the area where ramification of infection threads and cell invasion occurs, but is much reduced in the more proximal regions of the nodule (Fig. 3B). In view of the observed correlation of *PsCyp15a* gene expression with wilting and osmotic stress in pea shoots (Guerrero et al. 1990; Jones and Mullet 1995), it is possible that strong expression of this gene in the nodule apex and invasion zone may indicate that these cells also experience some form of water deficit or change of turgor.

Polyclonal antibodies specific for the *PsCyp15a* gene product have been used in immunogold staining of pea stem cortical cells (Jones and Mullet 1995): These studies revealed that the protein is targeted to the extracellular matrix. It is therefore possible that the functioning of this gene is an important component of infection thread development during the invasion of nodule tissues by *Rhizobium*. Furthermore, the low but significant level of transcript that was detected in the central infected tissue (Fig. 3B) suggests that the Cyp15a protease may also function within the symbiosome compartment.

Expression of both genes *PsCyp1* and *PsCyp15a* increased as nodules matured. Whereas the 13-day-old nodules, which had just started to fix nitrogen, displayed the same level of mRNA as uninfected roots, the amount of transcript for both cysteine protease genes had doubled in nodules by day 18 postinfection. Expression of the *PsCyp1* gene increased twofold in early-senescing nodules formed by the LPS mutant of *Rhizobium* compared to expression in wild-type nodules of the same age. A similar correlation between the level of the *PsCyp1* gene expression and cell senescence was observed by in situ hybridizations. Although a twofold increase in the extractable nodule RNA hybridization signal of the *PsCyp1* gene is modest, the in situ hybridization data showed that this expression was confined to a small subset of cells in the central tissue of the nodule. Although the data presented here do not provide a causative link between cysteine protease gene expression and cell senescence in nodules, the identification of a gene (*PsCyp1*) opens a way of studying this relationship.

MATERIALS AND METHODS

Microorganisms.

Recombinant plasmids were propagated in *E. coli* JM101 (Yanisch-Perron et al. 1985). For inoculation of peas, *Rhizobium leguminosarum* bv. *viciae* wild-type strain 3841 was used (Wood et al. 1989). Strain B661 is its LPS-defective derivative produced by Tn5 transposon mutagenesis (Kannenberg et al. 1992): when inoculated onto pea seedlings, this strain gives rise to small non-fixing nodules that senesce prematurely (Perotto et al. 1994).

Plant material.

The pea seedlings (*Pisum sativum* cv. Wisconsin Perfection) were germinated in conical flasks on agar medium con-

Fig. 3. In situ hybridization analysis of cysteine protease gene expression. Median longitudinal sections of "Sprint2" pea nodules infected with wild-type *Rhizobium* strain 3841 (A,B,C,D,G) and sections of nodules from "Wisconsin Perfection" infected with symbiotically defective *Rhizobium* strain B661 (E,F,H) were hybridized with riboprobes containing digoxygenin. Hybridization signal was detected as blue following immunoperoxidase-based staining with BCIP and NBT as substrates: tissue sections were counterstained with Auramine O (yellow) which highlights the central infected tissue. **A,** *Cyp15a* sense strand (negative control), showing background staining with Auramine O. **B,** *Cyp15a*, showing strong labeling in the nodule apex, and weaker labeling in outer uninfected parenchyma and central infected tissue. **C, G,** *Cyp1*, showing faint labeling in root vascular tissue (arrowhead) in a transverse section of root tissue adjacent to a nodule (C) and at greater magnification in another root section (G). **D,** Leghemoglobin *Lb* (positive control), showing strong signal in interzone region and lower level of signal in central infected tissue of symbiotic zone. **E,F,H,** Sections of Fix$^-$ nodules, showing extensive expression of both cysteine proteases. **E,** *Cyp1*; **F,** enlargement of boxed region from E showing expression of *PsCyp1* in host cells with senescent bacteroids; **H,** *Cyp15a*, showing strong expression in invasion zone and weaker expression in central tissue of nodule occupied by lipopolysaccharide-defective bacteria. Fix$^+$ nodules of "Wisconsin Perfection" were similar to those of "Sprint2" (data not shown). Scale bar represents 20 μm for A,B,C,D; 40 μm for E; 5 μm for F,G; 10 μm for H.

taining mineral salts but lacking a nitrogen source, and were inoculated and harvested as described elsewhere (Bradley et al. 1988). Nodules were usually harvested from 25- to 30-day-old plants (18 to 23 days postinfection); samples of other plant organs were collected at the same time, unless stated otherwise. Pea cultivar "Sprint2" was a gift from I. Tikhonovich (St. Petersburg).

Plant RNA purification.

Total RNA was prepared from plant tissues using the hot phenol extraction procedure described by de Vries et al. (1982). Poly(A)$^+$ RNA was prepared from total RNA using paramagnetic oligo(dT) beads (Dynal, UK) following the manufacturer's recommendations. Typically, 100 μg of total RNA (as measured by the OD_{260} absorbance) was used with 100-μl suspension of beads to produce an estimated 1 to 5 μg of mRNA.

Amplification of cDNA fragments by RT-PCR.

For the synthesis of the first strand of cDNA for PCR amplifications, 2 to 3 pmole of oligo$(dT)_{15\text{-}18}$ was annealed to 1 to 2 μg of poly(A)$^+$ RNA from 4-week-old nodules in 15 μl of 60 mM KCl, 60 mM Tris-HCl, pH 8.3, by cooling from 90°C to 45°C over 30 min. To the annealing mixture was added 10 μl of 20 mM $MgCl_2$, 20 mM DTT, 60 mM KCl, 60 mM Tris-HCl pH 8.3, 2 mM dNTP, 5 U of AMV reverse transcriptase (Pharmacia, Uppsala). After incubation for 30 min at 45°C, the reaction mix was diluted to 1 ml, and 1 μl was used as template for the PCR amplification with oligonucleotide primers. Amplification reactions contained 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM Mg_2Cl, 0.01% gelatin, 125 μM dNTP, 10 pmole of oligonucleotides in 50 μl total volume. Amplifications were performed for 40 cycles with Taq polymerase (Perkin-Elmer) at 94°C 30 s 50°C 1 min, 72°C 2 min, and final extension for 10 min. at 72°C. Products of the PCR amplification reaction were cloned without size selection in a Bluescript vector, and the inserts were subjected to DNA sequencing.

3′- and 5′-RACE PCR.

The 3′-RACE protocol that was used has been described elsewhere (Kardailsky et al. 1996). For 5′-RACE, a modification of the original protocol (Frohman et al. 1988) was developed which involved synthesis of the complete cDNA template for the amplification reaction on a solid support. Briefly, nodule poly(A)$^+$ RNA from 50 μg of total RNA was prepared using 50 μl of paramagnetic oligo (dT) beads (Dynal, UK), and the cDNA synthesis was performed without dissociation of RNA from the solid support, thereby linking the first strand of the cDNA covalently to the paramagnetic beads. Superscript reverse transcriptase was used according to manufacturer's recommendations (BRL), with reaction for 2 h at 42°C, and then 30 min at 50°C. RNA was removed by alkaline hydrolysis at 65°C for 2 min in 0.1 M NaOH, and the beads carrying cDNA were washed three times with TE buffer. Oligo(dA) tails were added to the 3′ ends of the cDNA strand using 5 units of terminal deoxynucleotide transferase (Pharmacia) with 40 μM dATP at 37°C for 1 h. The primer sequence for the amplification was then added to the created oligo(dA) tails using Klenow fragment of DNA polymerase I, 0.2 mM each dNTP and 0.33 μM of the oligonucleotide CTCGAGGATCCGCGGCCGC$(dT)_{16}$ (which contains multiple cloning sites) with incubation for 30 min at 37°C. The cDNA on beads was washed three times at 65°C in TE buffer to remove unincorporated reaction components and partially synthesized second strand of cDNA. The beads were resuspended in 50 μl of TE buffer and stored at 4°C. The first strand of cDNA on the solid support with the defined sequence on its 3′ end can be repeatedly used as a convenient template for 5′-RACE for any gene expressed in a tissue from which RNA was extracted. Oligonucleotide GTGACGCCAGAATTAGC with the multiple cloning sites and the gene-specific sequential antisense oligonucleotide primers were used essentially as in the 3′-RACE reaction but with 1 μl of the cDNA bead suspension as a template. Products of 3′- and 5′-RACE reaction were subcloned into a Bluescript SK vector. DNA sequences of individual independent subclones were determined on the ABI automated sequencer with the PRIZM kit and the consensus sequence assembled using the Staden package. On average, each base in the entire sequence was read in 4.5 gel runs.

Northern blotting.

For the quantification of gene expression, approximately equal amounts of poly(A)$^+$ RNA were loaded on the agarose-formaldehyde gels, and transferred to nylon filters (Hybond N, Amersham, UK) according to the manufacturer's instructions. Hybrization probes were used under standard conditions, with $[Na^+]$ = 0.2 M, 40% formamide at 42°C. The final wash was in $[Na^+]$ = 0.016 M at 60°C. Filters were exposed with the PhosphoImager plates (Fujix, Tokyo). Image scanning, handling, and quantification of radioactivity present in individual bands were performed using the software provided with the scanner (BAS1000, Fujix). For quantification of gene transcription levels, filters were reprobed with a pea ubiquitin cDNA fragment (courtesy of F. Watts): The level of gene expression was measured relative to the ubiquitin signal for each individual sample, using as a reference the two smallest bands in the pea ubiquitin RNA profile (~1,200 and ~1,300 bases) which reflect most accurately the quantity of loaded RNA (Christensen and Quail 1989; Binet et al. 1991; Callis and Vierstra 1989; Kardailsky et al. 1996). Data from different filters were brought to the same relative scale using reference samples common to several filters. Normalization to the same scale assumed linear regression, and error was calculated as sample standard deviation from regression for reference samples.

In situ hybridization.

Digoxigenin-labeled riboprobes were synthesized from linearized plasmids using T7 or T3 promoters of the pBluescript SK(+) vector. The pea leghemoglobin clone was kindly provided by T. Bisseling. Labeling was carried out essentially as recommended by the supplier (Boehringer, Mannheim) with minor modifications. Nodules were fixed in 4% formaldehyde, dehydrated, embedded in wax, and sectioned following standard protocols (De Block and Debrouwer 1993; Wilson et al. 1994). Slides with sections were hybridized with riboprobes and signal was visualized as alkaline phosphatase (AP) activity using bromochloroindophenol (BCIP) and nitrobluetetrazolium (NBT) as substrates. The incubation time in the AP reaction ranged from 2 days to 4 h, depending on the strength of the signal. Sections were counter-stained with Auramine O and photographed on a Zeiss Axiophot microscope.

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