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RESEARCH PAPER

Nodule-enhanced protease inhibitor gene: emerging patterns of gene expression in nodule development on *Sesbania rostrata*

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Abstract

A novel marker for the early stages of nodulation of *Sesbania rostrata* was found to encode a putative member of the Kunitz family of protease inhibitors (*SrPI1*). Its expression was enhanced during nodulation, and was not up-regulated by wounding or upon infection with wide host-range pathogens. *In situ* expression patterns resembled those previously described for functions that may be implicated in delimiting infected nodule tissues from the rest of the plant. Thus, *SrPI1* may be a component of a multi-layered barrier that restrains the invading rhizobia.

Key words: Early nodulin, Kunitz family, protease inhibitors, *Sesbania rostrata*.

Introduction

Protease inhibitors from animals, plants, and microbes have a common mode of action: the proteins bind with a protease in a substrate-like manner but do not readily dissociate, thus inactivating the protease (reviewed in Laskowski and Kato, 1980). In plants, based on primary sequence data, 11 families of protease inhibitors have been recognized (Ryan, 1990; Richardson, 1991; Koiwa *et al.*, 1997). Protease inhibitors are part of the wide array of preformed defence mechanisms in storage organs, where they block the growth and development of herbivorous predators by inhibiting digestive enzymes and provide protection against pathogenic fungi and bacteria, which use hydrolytic enzymes to gain entry (Johnson *et al.*, 1989). Moreover, mechanical wounding or attack by pathogens or herbivores causes a rapid accumulation of protease inhibitor transcripts, sometimes as part of host-specific resistance mechanisms. Thus, protease inhibitors are components of both preformed and inducible defence mechanisms in plants.

These proteins also play a role in plant development. In storage tissues, they may prevent reserve proteins from premature hydrolysis by endogenous plant proteases (Richardson, 1991). Because they are abundant and resistant to extremes of heat and pH, protease inhibitors themselves may function as storage proteins that are immune to digestion until germination (Richardson, 1991). The expression of protease inhibitor genes is developmentally regulated in various plant parts, suggesting that they participate in the control of proteolysis, for instance, in stem and sieve tube development (Habu *et al.*, 1996; Valdés-Rodríguez *et al.*, 1999).

The induction of nodules on legume roots presents features of development and defence that are integrated into the construction of a unique organ that is occupied by large numbers of bacteria. *Sesbania rostrata* belongs to a small group of flooding-tolerant, tropical legumes that form nodules not only on the roots, but also on the stem at positions of (dormant) adventitious root primordia. On well-aerated roots, *S. rostrata* nodules are of the indeterminate type. However, on stems and hydroponic roots, nodules become determinate after a brief indeterminate

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Abbreviations: EST, expressed sequence tag; PCR, polymerase chain reaction; RACE, 5′ rapid amplification of cDNA ends; RT, reverse transcription; SrPI, *Sesbania rostrata* protease inhibitor.

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developmental stage. This phenotypic plasticity is mediated by ethylene, which may interfere with the persistence of the nodule meristem (Fernández-López *et al.*, 1998). Stem nodulation sites are very abundant and, upon simultaneous inoculation with a compatible microsymbiont such as *Azorhizobium caulinodans*, they develop synchronously into nodules. The system has been exploited to identify molecular markers for the early nodulation events, i.e. nodule primordium induction and bacterial invasion. Differential display has been used to compare RNA populations of uninoculated primordia with those at early time points in nodule development (Goormachtig *et al.*, 1995; Lievens *et al.*, 2001). One of the very early markers found, Srdd17, is a member of the Kunitz family of protease inhibitors. The full-length cDNA *SrPI1* has been isolated. A gene-specific probe was used to study the occurrence of *SrPI1* transcripts in various plant parts and upon wounding and pathogen attack. Transcript accumulation patterns were analysed during the development of determinate stem nodules and in mature indeterminate root nodules by *in situ* hybridization. These patterns resemble previously described expression patterns of functions that may be implicated in delimiting infected tissues from the rest of the plant.

Materials and methods

Biological material

Azorhizobium caulinodans strains ORS571 and ORS571-X15 (Goethals *et al.*, 1994) were grown as described by Goethals *et al.* (1994). *Ralstonia solanacearum* wild type (strain GMI1000; Boucher *et al.*, 1985) and mutant *hrcR* (hrp^- strain GMI1584; Van Gijsegem *et al.*, 2000) were grown in 0.5% (w/w) beef extract, 0.5 (w/w) peptone, 0.1% (w/w) yeast extract, 0.5% (w/w) sucrose, and 0.002 M $MgSO_4$. For plant inoculation, an overnight culture was centrifuged and the bacteria were resuspended in half the original volume of water. *Botrytis cinerea* spores were obtained as described in De Meyer and Höfte (1997). For inoculation, a suspension of 10^6 spores ml^{-1} was prepared in a 0.0067 M KH_2PO_4 (pH 5) buffer containing 0.02 M glucose.

Sesbania rostrata Brem seeds were surface-sterilized (Goethals *et al.*, 1989), germinated, and grown at 28 °C under a 16 h light regime at an intensity of 300–400 μmol m^{-2} s^{-1} (Goormachtig *et al.*, 1995).

Isolation of the SrPI1 cDNA clone

5′ Rapid amplification of cDNA ends (RACE) was performed with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) to obtain the full-length clone corresponding to the partial cDNA *Srdd17*. cDNA was synthesized from RNA extracted from root primordia harvested 2 d after inoculation with *A. caulinodans* ORS571. The antisense primers sh21 (5′-GGGCACACAGAAC-ACCAGGCACAGAG-3′) and sh22 (5′-CAGCTACACCACCAG-TACTCACAAACC-3′) combined with the primers AP1 and AP2, respectively, were used for successive nested amplification steps according to the manufacturer's instructions (Clontech). The RACE products were cloned in the pGEM-T vector (Promega, Madison, WI) and the plasmid with the largest insert was designated pGEMTc173flca2. Because the clone was not full length, another round of nested polymerase chain reaction (PCR) was carried out with primers sh22 combined with AP1 and sh26 (5′-GGGTCA-CGGATAACATCAAGTGGGCATG-3′) with AP2; the largest cloned product was designated pGEMTc173fldac11. The complete open reading frame was reconstructed with Vent polymerase (New England Biolabs, Beverly, MA) in a PCR amplification reaction on the previously used cDNA template with sense primer sh33 (5′-ATGAAGGTTGCTAAGCTTCAATTCCTTC-3′) and antisense primer sh34 (5′-GCTCATTCCTGGCTTATTATTATGACCC-3′). The PCR fragment was cloned in the pGEM-T Easy vector (Promega) and designated pGEMTEasyc173flbis16. The full-length sequence obtained by joining the sequences of the different cDNA fragments was designated *SrPI1*.

DNA gel blot analysis

DNA was prepared and analysed as described by Goormachtig *et al.* (1997) and hybridized non-radioactively with the digoxigenin hybridization system (Roche Diagnostics, Brussels, Belgium). Probes were generated by PCR amplification on pGEMTEasyc-173flbis16 as template and using sense primer sl59 (5′-ACT-GGTGGTGTAGCTGGGGAC-3′) with antisense primer sl58 (5′-GCATAGACACACACACCACAC-3′), and sense primer sl107 (5′-TGGCAATTCTTGTGCCTAGTG-3′) with antisense primer sl108 (5′-TGCAATGCTCAAACCCAGA-3′) resulting in non-gene-specific and gene-specific hybridization, respectively. All procedures were done according to the manufacturer's instructions (Roche Diagnostics).

RNA analysis

RNA was prepared as described by Goormachtig *et al.* (1995). The reverse transcription (RT)-PCR analysis was performed according to Corich *et al.* (1998). For the specific amplification of a 134 bp fragment of the 3′ end of *SrPI1*, sense primer sl107 and antisense primer sl108 were used. With the same template, a ubiquitin cDNA fragment was amplified with sense primer sl14 (5′-GATT-TTTGTGAAGACCTTGACGGG-3′) and antisense primer sl16 (5′-CACAGACCCATTACACATCCACAAG-3′) as constitutive control (Corich *et al.*, 1998); a β-1,3-glucanase cDNA fragment was amplified with sense primer sl102 (5′-TAGCTATTCTGGT-AACCCTCGTG-3′) and antisense primer sl103 (5′-GTCATAT-GTAGCAGCAAATCCTCC-3′). The programme consisted of 20 cycles of amplification for 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. PCR products were detected radioactively as described by Corich *et al.* (1998) with probes generated from the cDNA fragment *Srdd17*, *Srubi1* (Corich *et al.*, 1998), and *Srglu2* (S Lievens and M Holsters, unpublished results). RT-PCR analysis was repeated at least twice with similar results.

In situ hybridization

Sections (10 μm) of paraffin-embedded root primordia, developing stem nodules, and 30-d-old indeterminate root nodules were hybridized *in situ* as described by Goormachtig *et al.* (1997). Using standard procedures (Sambrook *et al.*, 1989), the plasmid pBlueKSSrdd17 was digested with *Sac*II and *Pst*I to yield templates for ^{35}S-labelled antisense and sense probe production with T3 and T7 RNA polymerase (Amersham Biosciences, Little Chalfont, UK), respectively.

To generate specific RNA probes, the sl107-sl108 PCR product was cloned in pGEM-T (Promega). A PCR was performed with T7 and SP6 primers to amplify the insert and the RNA polymerase sites. Antisense and sense probes were obtained with the PCR fragment as template and T7 or SP6 polymerase, respectively, according to standard procedures (Sambrook *et al.*, 1989). Hybridizations with the sense probes did not result in signals above background (data not shown).

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DNA sequence analysis

DNA sequencing was carried out with universal SP6 and T7 primers. DNA sequence data were assembled and analysed using the GCG Wisconsin package (Accelrys, San Diego, CA). The percentage of identity and similarity between sequences was determined with the GAP program and alignments were produced with the PILEUP program (Accelrys).

Results

The SrPI1 *cDNA*

A full-length clone corresponding to the partial cDNA *Srdd17* (Lievens *et al*., 2001) was obtained after several rounds of 5′ RACE screening (see Materials and methods). The sequence contained 838 bp, including a short polyadenylation tail derived from the differential-display 3′ anchor primer. An open reading frame of 215 amino acids was significantly homologous with protein sequences in databases (Fig. 1). The highest significance score (E-score $1e^{-16}$ and 43% similarity) revealed by BLASTP searches (Altschul *et al*., 1997) was obtained with a 21 kDa cocoa seed protein (Spencer and Hodge, 1991; Tai *et al*., 1991), followed by the tumour-related protein NF34 from tobacco (E-score $1e^{-15}$ and 48% similarity; Karrer *et al*., 1998), the taste-modifying protein miraculin from miracle fruit (E-score $7e^{-15}$ and 45% similarity; Theerasilp *et al*., 1989), the protein encoded by the root-knot nematode-induced tomato gene *LeMir* (E-score $2e^{-13}$ and 51% similarity; Brenner *et al*., 1998), and the drought-repressed gene product AtDr4 from *Arabidopsis thaliana* (E-score $2e^{-11}$ and 41% similarity; Gosti *et al*., 1995). Although apparently diverse, all these proteins are members of the soybean trypsin inhibitor (Kunitz) family of protease inhibitors. Therefore, the full-length sequence was designated *SrPI1* for *S. rostrata* protease inhibitor.

In protease inhibitors, the most conserved amino acid sequence is the signature (L/I/V/M)-x-D-x-(E/D/N/T/Y)-(D/G)-(R/K/H/D/E/N/Q)-x-(L/I/V/M)-x(5)-Y-x-(L/I/V/M) in the N-terminal region (Prosite; Hofmann *et al*., 1999). The proteins also feature one or two intra-chain disulphide bonds between Cys residues at conserved positions, and they contain an N-terminal signal peptide. All these characteristics are present in SrPI1 (Fig. 1). Cleavage of the N-terminal signal peptide is predicted to occur between Ala26 and Ala27 (Nielsen *et al*., 1997).

Expressed sequence tag (EST) databases of *Medicago truncatula*, lotus (*Lotus japonicus*) and soybean (*Glycine max*) (www.tigr.org) were searched for homologous sequences. Two ESTs of *M. truncatula* (TC43418; TC48343), two from soybean (TC 132817; TC132816) and one from lotus (TC 2897) were approximately 80% similar to *SrPI1*. The two clones from *M. truncatula* and soybean were found in various cDNA libraries, indicating non-specific expression. On the other hand, TC 2897 from lotus was only found in libraries derived from nodules or nodulated roots. It is surprising that very high similarities (as high as 84% in *M. truncatula*) were found in legumes whereas in *A. thaliana* the closest homologue was only 41% similar (AtDr4). An evolutionary tree was analysed with several programs, in the hope of obtaining indications for the occurrence of a specific legume proteinase inhibitor, whose functional equivalent would be absent in *A. thaliana*. Unfortunately, this question remains unanswered, because different programs did not produce consistent results (data not shown). However, the tree studies reveal that *SrPI1* is not a functional homologue of miraculin and if there were a functional homologue of *SrPI1* in *A. thaliana*, it would be *AtDr4*, which had been found to be root-specific and repressed upon drought stress (Gosti *et al*., 1995).

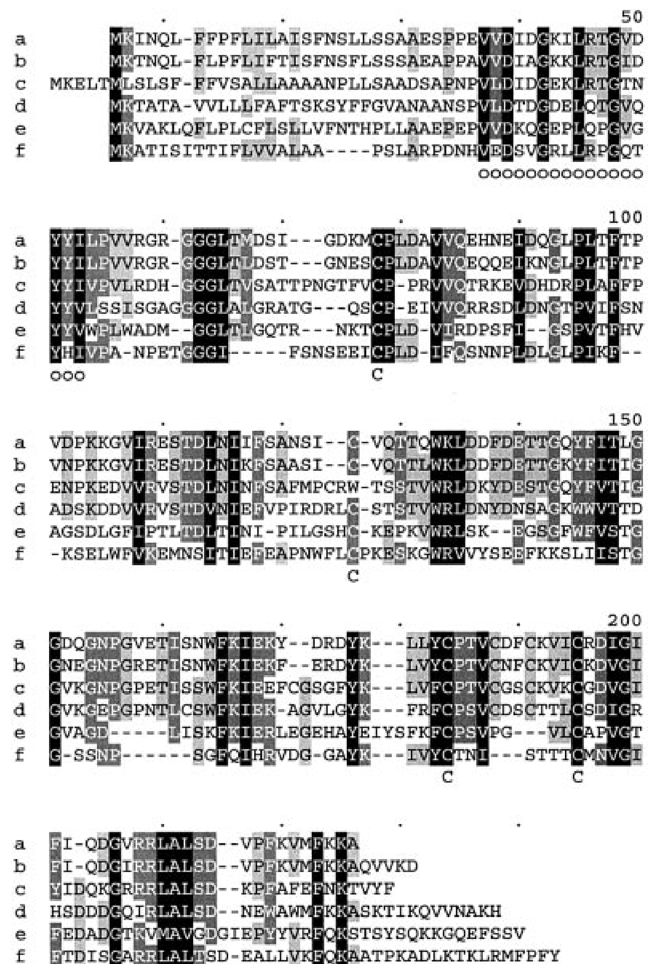

Fig. 1. Alignment of *SrPI1* to homologous proteins. The deduced amino acid sequence of SrPI1 (e; accession number AJ441323) is aligned to the *LeMir* gene product of tomato (a; accession number T07871), the tumour-related protein NF34 from tobacco (b; accession number T03803), miraculin from miracle fruit (c; accession number P13087), the 21 kDa cocoa seed protein (d; accession number P32765), and AtDr4 from *A. thaliana* (f; accession number S51480). The protease inhibitor signature pattern is indicated (o), as well as the four conserved Cys residues (C) involved in intrachain disulphide bonding. Amino acid residues are shaded according to the level of conservation.

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A gel blot of *S. rostrata* genomic DNA was probed with a labelled cDNA fragment spanning the 3′-untranslated sequence and 254 bp of the open reading frame (Srdd17). When the hybridization was washed at high stringency, the autoradiogram showed one strongly hybridizing band per lane (Fig. 2). The probe hybridized less intensely with three to four additional fragments, indicative for a family of highly similar genes in the *S. rostrata* genome. Under the same stringent hybridization conditions, a probe comprising only the 3′ untranslated region of the transcript yielded a single-band pattern, corresponding to the most intense band of the previous hybridizations (data not shown).

SrPI *transcript accumulation during nodule development*

The overall expression of *SrPI1* in different plant parts and during stem nodulation was analysed by RT-PCR with the gene-specific primers (Fig. 3A, B; see Materials and methods). A low background expression was seen in uninfected root primordia; the transcript levels were up-regulated during nodule development, reaching a maximum at 2 d after inoculation and remaining high until approximately 7 d post inoculation (dpi). In 12-d-old mature nodules, levels dropped to background (Fig. 3A). Seedlings, vegetative shoot apices, flowers, leaves, and roots of *S. rostrata* contained low transcript levels (Fig. 3B).

In situ hybridizations were performed to localize the patterns of transcript accumulation in developing stem nodules (Fig. 4A–I) and in mature indeterminate root nodules of *S. rostrata* (Fig. 4J–K). During stem nodulation, bacterial invasion is initiated by a Nod factor-dependent intercellular colonization in the outer cortex after entry through epidermal fissures that occur at the base of the adventitious roots. With the gene-specific probe, *SrPI1* transcripts were detected in the dormant root meristem of the uninoculated adventitious rootlets (Fig. 4A). Only a very weak background was seen in the root cortical cells (Fig. 4A). With the unspecific probe, this background was higher (data not shown; Fig. 4F, I). This difference was the only one observed between hybridizations with the two probes. Because of the higher intensity of the unspecific probe (attributable to its larger size), the results were much clearer at later stages of nodule development and in indeterminate nodules; therefore, these data are presented in Fig. 4 (F, I–L).

Around 2 dpi, *SrpI1* transcripts were detected in the mid cortex, most strongly opposite the epidermal fissure (Fig. 4B, C). *SrPI1*-expressing cells were smaller than their cortical neighbours (Fig. 4B, C). The cells of this mid-cortical region resumed division to form the nodule primordium (Tsien *et al.*, 1983; Duhoux, 1984; Goormachtig *et al.*, 1997). At 3 dpi, a large, open, basket-shaped structure had arisen in the mid-cortex

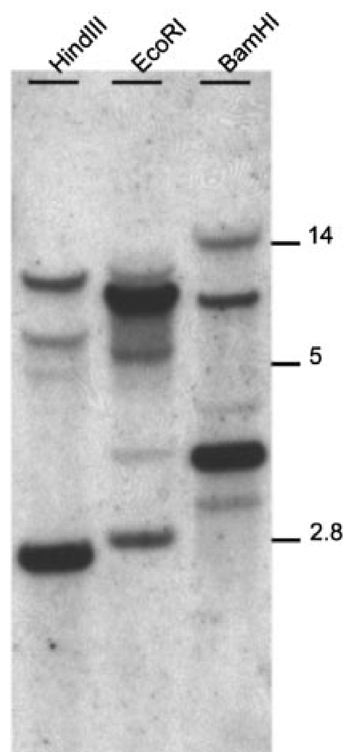

Fig. 2. DNA gel blot analysis of *SrPI1*. Genomic DNA was digested with the enzymes indicated above the corresponding lane. Numbers indicate fragment length in kb.

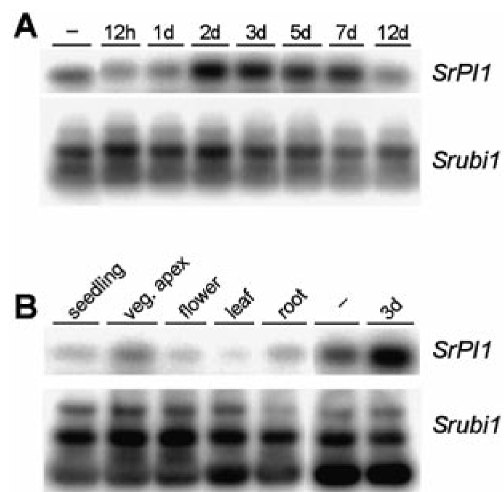

Fig. 3. *SrPI1* transcript accumulation during nodulation and in different plant parts. Expression levels were determined by semi-quantitative RT-PCR analysis using primers and probes specific for *SrPI1* (upper panels) and, as a constitutive control, the ubiquitin gene *Srubi1* (lower panels). (A) Uninoculated root primordia (–) were compared with root primordia excised 12 h, 1, 2, 3, 5, 7, and 12 d after inoculation with *A. caulinodans* ORS571. (B) *SrPI1* expression levels in seedlings, vegetative apices, flowers, leaves, roots, and, as a comparison, in uninfected root primordia (–) and developing stem nodules 3 d after bacterial inoculation.

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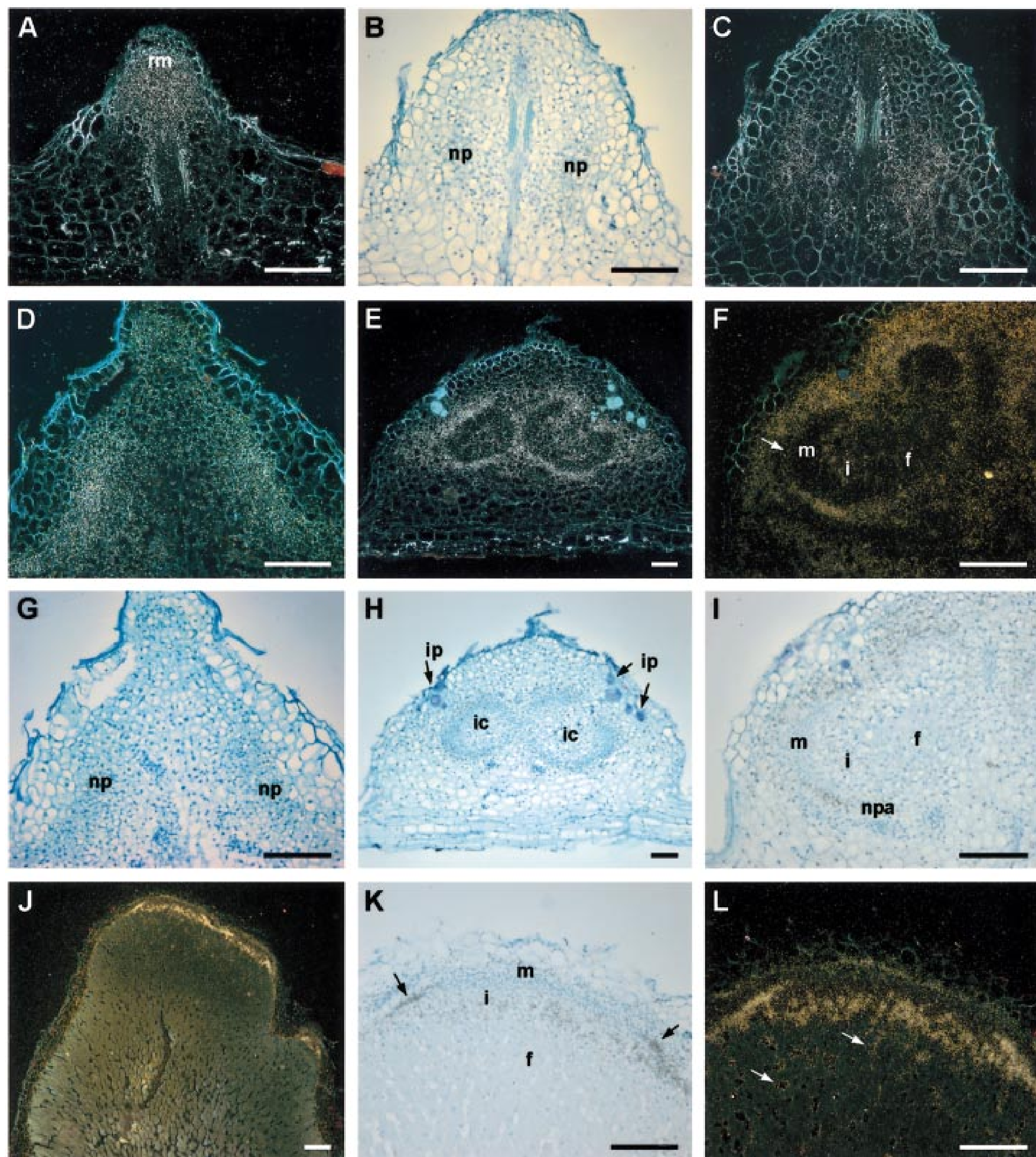

Fig. 4. *In situ* localization of *SrPI1* transcripts. Longitudinal sections (10 μm) through developing stem and root nodules were hybridized either with a specific antisense RNA probe only spanning the 3′ untranslated region (A–E, G, H) or an antisense RNA probe corresponding to the *Srdd17* fragment (F, I–L) and visualized under dark-field (A, C–F, J, L; signal seen as white spots) and bright-field (B, G, H, I, K; signal seen as black spots) microscopy. (A) Uninfected root primordium. (B, C) Developing stem nodule, 2 d after inoculation. (D, G) Root primordium, 6 d after inoculation with *A. caulinodans*, *X15* mutant. (E, H) Developing stem nodule, 3 d after bacterial inoculation. (F, I) Developing stem nodule, 4 d after inoculation. Arrow indicates the expression in the outer cells of the meristem. (J–L) Mature indeterminate root nodule (K, L enlargements of J). Arrows in (K) and (L) indicate expression in the nodule parenchyma and the uninfected cells, respectively. f, fixation zone; i, infection zone; ic, infection centre; ip, infection pocket; m, meristem; np, nodule primordium; npa, nodule parenchyma; rm, root meristem. Bars=100 μm.

(Fig. 4E, H). *SrPI1* transcripts were found in the outermost cell layers of the nodule primordium, completely surrounding the open-basket structure. Expression was also associated with the bacterial invasion track. The azorhizobia first proliferated in infection pockets (Fig. 4H, ip), located in the outer and inner cortex, then they moved towards the nodule primordium through inter- and intracellular infection threads, and formed an infection centre

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(Fig. 4H, ic). Whereas no expression was observed in the outer cortical region adjacent to superficial infection pockets, expression was strong in the infection centre (Fig. 4E, H). Around 4 dpi the bacteria started penetrating into host cells and differentiated into nitrogen-fixing bacteroids. The differentiated central tissue of the nodule had now the zonated appearance of an indeterminate nodule (Goormachtig *et al.*, 1997). *SrPI1* gene expression was observed in scattered cells of the infection zone and of the young fixation zone (Fig. 4F, I). Besides this new expression pattern, *SrPI1* transcripts were still found in cells encircling the nodule central tissue, in the nodule parenchyma (npa), and in the outermost cell layer of the meristem (arrow) (Fig. 4F, I). In stem nodule development, meristem activity ceased approximately 8 dpi and maturation started to yield a 'determinate' nodule. In such a nodule at 10 dpi, transcripts could no longer be detected (data not shown).

On hydroponic roots, determinate nodules are formed, following the same process as for stem nodulation (Ndoye *et al.*, 1994). However, under specific conditions, such as on roots grown in a well-aerated environment, mature root nodules of *S. rostrata* remain indeterminate (Fernández-López *et al.*, 1998). A differential pattern of *SrPI1* expression was seen on sections through 30-d-old indeterminate root nodules (Fig. 4J–L). Strong transcript accumulation was observed in many (but not all) cells of the infection zone immediately adjacent to the nodule meristem. In the youngest part of the fixation zone protease inhibitor transcripts were detected in the uninfected cells (Fig. 4L, arrows), whereas expression was absent in the older nitrogen-fixing nodule tissue (Fig. 4J–L). In addition to this central tissue expression, transcripts also accumulated in the young, distal parts of the nodule parenchyma (Fig. 4K, arrows).

SrPI1 *expression in response to nodulation mutants of* A. caulinodans

Nod factors are essential to trigger the early responses of nodulation. Strain ORS571-V44 produces no Nod factors and fails to nodulate (Van den Eede *et al.*, 1987; Mergaert *et al.*, 1993; D'Haeze *et al.*, 1998). No transcripts were enhanced when ORS571-V44 was applied to roots (data not shown). Mutant strain ORS571-X15 produces normal Nod factors, invades the outer cortex, and elicits Nod factor-related plant responses such as the initiation of cell divisions. However, an altered surface polysaccharide composition prevents this mutant from penetrating more than superficially into the plant's cortical tissue (Goethals *et al.*, 1994; D'Haeze *et al.*, 1998). Sections of 6-d-old pseudonodules were hybridized with the specific *SrPI1* probe (Fig. 4D, G). Transcripts were detected in the outer cortical cells that restarted division and formed a nodule primordium. More superficial infection pockets appeared in the outer cortex, and the primordium was much broader than after wild-type inoculation. Because of invasion arrest, the nodule primordium did not develop further and, eventually, *SrPI1* transcripts disappeared (data not shown).

Wound and pathogen induction

To test whether *SrPI1* was induced upon mechanical damage, *S. rostrata* leaves were harvested at different times between 1–48 h after wounding (see Materials and methods). RT-PCR analysis showed that the *SrPI1* gene was not induced, in contrast with a β-1,3-glucanase gene (*Srglu2*) that is a molecular marker for wound and defence-related plant responses (S Lievens and M Holsters, unpublished results) (Fig. 5A).

Pathogen response was investigated by infecting leaves with spores of the pathogenic fungus *Botrytis cinerea*. After 48 h of spore application, macroscopic lesions were visible (data not shown). β-1,3-Glucanase gene expression was strongly induced from 4 h after inoculation on. However, no *SrPI1* induction was detected by RT-PCR analysis (Fig. 5B).

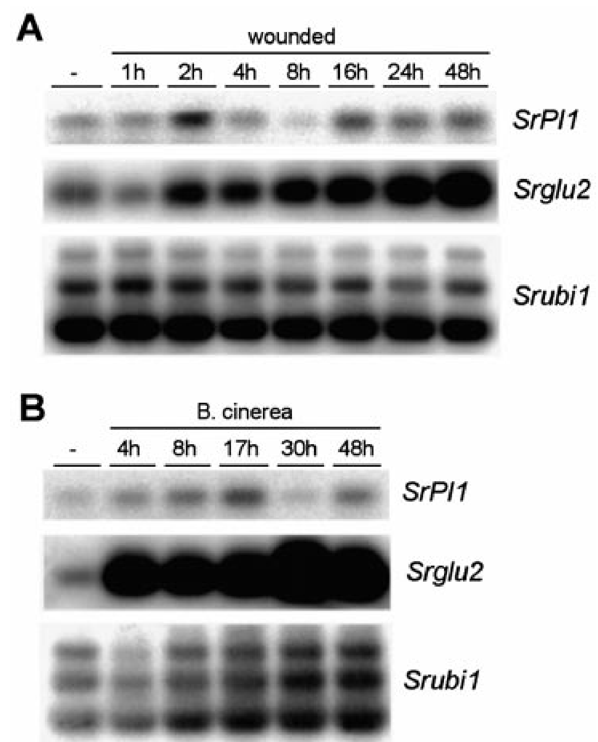

Fig. 5. *SrPI1* expression in leaves upon wounding and infection with *B. cinerea*. Expression levels were determined by semi-quantitative RT-PCR using primers and probes specific for the gene indicated at the right of the panels, including a ubiquitin gene (*Srubi1*) and a β-1,3-glucanase gene (*Srglu2*; accession number AJ441324) as a constitutive and positive control, respectively. (A) Control leaves (–) compared with leaves sampled at 1, 2, 4, 8, 16, 24, and 48 h after wounding. (B) Control leaves (–) compared with leaves harvested 4, 8, 17, 30, and 48 h after inoculation with a *B. cinerea* spore suspension.

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In another assay, a bacterial pathogen, *Ralstonia solanacearum,* was applied to the dormant root primordia on the stem. Wild-type bacteria provoked browning at the base of the root primordia from approximately 3 dpi on (Fig. 6C, F). A hrp⁻ non-virulent mutant strain *hrcR* did not elicit any response (Fig. 6B, E). RT-PCR pointed out that no *SrPI1* transcripts accumulated at any time point from 8 h to 5 dpi (Fig. 6). By contrast, the β-1,3-glucanase gene was induced (Fig. 6) upon wild-type infection, but not after application of the hrp⁻ mutant strain.

Discussion

A new early marker for nodule initiation on *S. rostrata* is a member of a large and diverse family of protease inhibitors. *SrPI1* has an N-terminal signal peptide and the typical hallmarks of the soybean trypsin inhibitor (Kunitz) family of protease inhibitors (Ryan, 1990; Richardson, 1991): one or two intra-chain disulphide bonds between Cys residues at conserved positions and a typical signature in the N-terminal region. In *S. rostrata* several homologous genes occur, forming a small family. Different members of multicopy families of plant protease inhibitor genes are often differentially expressed (Jofuku and Goldberg, 1989). By using a gene-specific probe, *SrPI1* expression has been shown to be enhanced during nodulation and is not up-regulated by wounding or upon infection with two wide host-range pathogens.

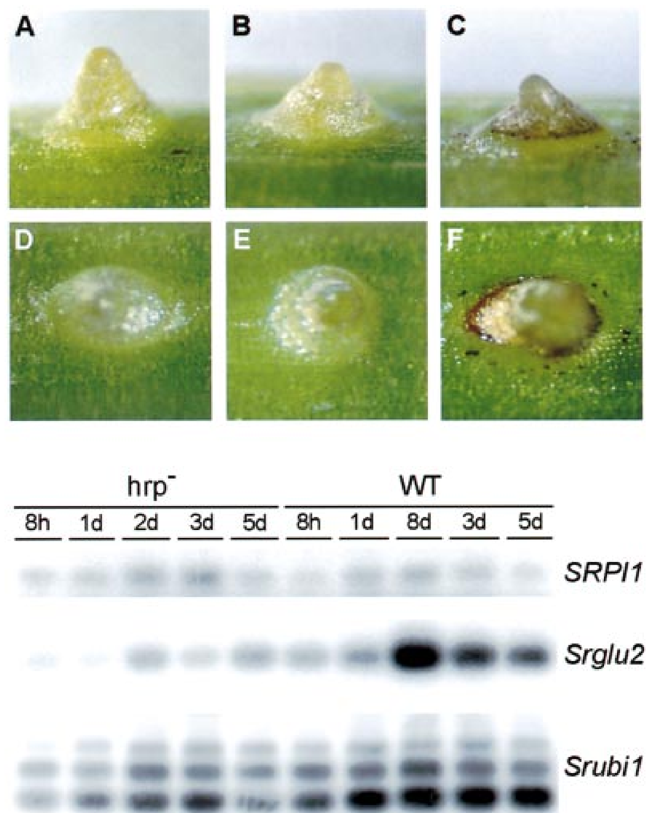

Fig. 6. Phenotypic view of root primordia upon infection with a wild-type and hrp⁻ *R. solanacearum* strain and the corresponding *SrPI1* expression analysis. (Upper panel) Side views (A–C) and top views (D–F) of uninfected (A, D) and root primordia 3 d after inoculation with *R. solanacearum* wild-type (C, F) or the hrp⁻ strain *hrcR* (B, E). (Lower panel) RNA levels were analysed in root primordia excised 8 h, 1, 2, 3, and 5 d after inoculation with either *R. solanacearum* (WT) or the hrp⁻ strain *hrcR* (hrp⁻). Expression levels were determined by semi-quantitative RT-PCR using primers and probes specific for the gene indicated at the right of the panels, including a ubiquitin gene (*Srubi1*) and a β-1,3-glucanase gene (*Srglu2*) as a constitutive and positive control, respectively.

A few nodulation features of *S. rostrata* are quite peculiar. *S. rostrata* belongs to the group of legumes with indeterminate nodules (Pueppke and Broughton, 1999) and, indeed, indeterminate nodules are formed on well-aerated roots. However, on stems and on hydroponic roots, nodules become determinate after a short, indeterminate stage. This phenotypic plasticity is mediated by the plant hormone ethylene (Fernández-López *et al.*, 1998). Because stem nodulation is well accessible, it is much easier to study than root nodulation, especially for very early aspects of nodule initiation and bacterial invasion. The primary bacterial invasion at the basis of adventitious root primordia is intercellular. Superficial and deeper infection pockets are formed in a Nod factor-dependent process that involves cell death (D'Haeze *et al.*, 1998). From infection pockets, infection wicks grow towards the developing nodule. *SrPI1* transcripts accumulate early after bacterial inoculation, well in advance of the start of nitrogen fixation. At earliest stages of nodule development, *SrPI1* is expressed in the nodule primordium. Later on, transcripts are found in a region that encircles the developing central nodule tissue, i.e. the nodule parenchyma and the outermost cells of the nodule meristem. Also inside the central tissue the gene is expressed in scattered cells of the infection zone and in uninfected cells of the young fixation zone, as clearly illustrated on the sections of indeterminate nodules. In addition, an infection-related pattern can be seen, located in the infection centre. In mature nodules that lack an active meristem, expression fades out. A corresponding expression pattern has been observed in indeterminate root nodules. Clearly, expression is associated with development and infection and depends on the presence of Nod factor-producing bacteria. Upon inoculation with the bacterial mutant ORS571-X15, defective in surface polysaccharides, the process is arrested at the level of superficial infection pockets and nodule primordium, implying that deeper infection is required to drive the growth of the primordium and to direct the switch from primordium to developing nodule.

In the literature, as far as is known, there is only one report of a legume gene that encodes a Kunitz-type protease inhibitor produced during nodulation (Manen

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et al., 1991). The gene from winged bean is induced during the senescence of infected cells. The protein may control a protease of bacterial or plant origin that is necessary for the maintenance of symbiosis. It will be interesting to continue studying similar functions in the growing set of data arising from the *M. truncatula* and lotus genetics/genomics efforts.

What could be the role of a protease inhibitor in the nodulation process? The spatial expression pattern of *SrPI1* during stem nodule development is intriguingly similar to the patterns of *Srchi13* and *Srchr1*, two other early nodulin genes from *S. rostrata* (Goormachtig *et al.*, 1998, 1999). *Srchi13* encodes a class III chitinase that has been proposed to play a role in controlling the spread of bacteria and their Nod factors. A similar function has been suggested for the *S. rostrata* gene, *Srchr1* (Goormachtig *et al.*, 1999), which encodes an early nodulin that is similar to chalcone reductases. Also this gene is expressed in the uninfected cells of the central tissue where it may be involved in the synthesis of antibiotic phytoalexins that could prevent bacterial entry. Similarly, expression of the nodule-associated protease inhibitor gene has been seen in plant cells that remain uninfected by the microbial symbiont, but that are in close contact either with bacteria (in the infection centre) or with infected cells (the infection zone, the uninfected cells in the central tissue and the nodule parenchyma). In the assumption that SrPI1 itself is a secreted protein (it has an N-terminal signal peptide), it could play a role in the control of proteases during development and, thus, build a protective barrier to prevent the escape of bacteria. The large number of rhizobia in the central nodule tissue represent a potential hazard because the uncontrolled spread of rhizobia could have pathogenic effects. The target proteases could be of bacterial origin. However, to date, no secreted proteases have been identified from rhizobia. Therefore, it is more likely that the inhibitor activity of SrPI1 is involved in the control of plant proteases related to nodulation, a function similar to that suggested for protease inhibitors in storage tissues or in sieve tube development (Richardson, 1991; Habu *et al.*, 1996). SrPI1 could inhibit a plant protease that facilitates bacterial invasion of *S. rostrata* tissues or cells.

Serine proteases, the target of Kunitz-type protease inhibitors, have been identified in legume nodules (Panter *et al.*, 2000). In actinorhizal nodules a cDNA encoding a serine protease of the subtilisin family (*ag12*) is expressed in infected cells of the infection zone and the fixation zone (Ribeiro *et al.*, 1995), a pattern that is more or less complementary to the central tissue-specific expression observed with the *SrPI1* probe. In conclusion, SrPI1 could be another component of the mechanism that regulates invasion during nodule development, protecting the rest of the plant by a multi-layered barrier against escape of bacteria.

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