A Symbiotic Plant Peroxidase Involved in Bacterial Invasion of the Tropical Legume *Sesbania rostrata*

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Aquatic nodulation on the tropical legume *Sesbania rostrata* occurs at lateral root bases via intercellular crack-entry invasion. A gene was identified (*Srprx1*) that is transiently up-regulated during the nodulation process and codes for a functional class III plant peroxidase. The expression strictly depended on bacterial nodulation factors (NFs) and could be modulated by hydrogen peroxide, a downstream signal for crack-entry invasion. Expression was not induced after wounding or pathogen attack, indicating that the peroxidase is a symbiosis-specific isoform. In situ hybridization showed *Srprx1* transcripts around bacterial infection pockets and infection threads until they reached the central tissue of the nodule. A root nodule extensin (*SrRNE1*) colocalized with *Srprx1* both in time and space and had the same NF requirement, suggesting a function in a similar process. Finally, in mixed inoculation nodules that were invaded by NF-deficient bacteria and differed in infection thread progression, infection-associated peroxidase transcripts were not observed. Lack of *Srprx1* gene expression could be one of the causes for the aberrant structure of the infection threads.

The interaction of rhizobia with plants of the legume family results in the formation of new root structures, the nodules, in which the bacteria fix atmospheric nitrogen for assimilation by the host. A complex signal exchange between the macrosymbiont and the microsymbiont initiates the nodulation process: Upon perception of flavonoids exuded by host roots, rhizobia switch on their nodulation genes, thus forming lipochitooligosaccharide molecules, designated nodulation factors (NFs; D’Haeze and Holsters, 2002). NFs are essential for bacterial invasion and induction of cortical cell division to form nodule organs (Geurts and Bisseling, 2002).

In the model legumes *Medicago truncatula* and *Lotus japonicus*, nodulation starts with entrapment of the bacteria in a curled root hair, followed by the formation of an infection thread (IT) that grows toward the nodule primordium and from which bacteria are released and differentiate into N₂-fixing bacteroids (Gage, 2004). ITs are tubular structures that develop after membrane invagination and further extend inward by polar growth mechanisms (Gage and Margolin, 2000; Gage, 2004). How ITs can grow against the turgor of the plant cells is still unknown, but hydrogen peroxide (H₂O₂)-driven cross-linking of root nodule extensins (RNEs) might be involved (Brewin, 2004; Gucciardo et al., 2005).

An alternative route for infection occurs as an adaptation to waterlogging and has been studied in the tropical legume *Sesbania rostrata* (Goormachtig et al., 2004a). Bacteria enter the plant tissue via cracks in the epidermis at places where lateral roots have emerged from the main root or where adventitious root primordia protrude on the stem. Intercellular bacterial microcolonies or infection pockets (IPs) are created in the outer cortex, from where ITs guide the bacteria toward the nodule primordium (Den Herder et al., 2006). However, when *S. rostrata* roots are grown under aerated conditions, invasion switches from intercellular crack-entry or lateral root base (LRB) invasion to the intracellular root hair curling (RHC) mode (Goormachtig et al., 2004b).

Oxidative burst-like phenomena have been observed as a primary response of the plant both in RHC invasion and LRB entry. Early in the interaction of *Sinorhizobium meliloti* with alfalfa (*Medicago sativa*), superoxide and H₂O₂ are produced (Santos et al., 2001). In *M. truncatula* roots, recognition of compatible NFs rapidly stimulates localized production of superoxide. This response is absent in the non-nodulating plant mutant *does not make infections1-1* (*dmi1-1*), which is impaired in the NF signal transduction pathway.
Symbiotic bacteria overcome the plant’s defense by activating antioxidant enzymes (Hérout et al., 1996; Santos et al., 1999, 2000; Sigaud et al., 1999; Jamet et al., 2003). Also, several plant genes related to protection against or production of ROS are differentially expressed during nodulation. In alfalfa roots, the enzymatic activities of catalase, ascorbate peroxidase, and glutathione reductase significantly increase upon inoculation with wild-type *S. meliloti* (Bueno et al., 2001). Moreover, a cytosolic Cu/Zn- and mitochondrial Mn-superoxide dismutase have distinct expression patterns in root nodules, suggesting specific roles for these enzymes in nodule development (Rubio et al., 2004). Finally, transcripts of a *Rhizobium*-induced peroxidase (Rip1) identified in *M. truncatula* (Cook et al., 1995) have a tissue-specific localization pattern similar to that of ROS and accumulate upon addition of exogenous H₂O₂ (Ramu et al., 2002).

Heme-binding peroxidases (Dawson, 1988) are widely distributed throughout bacteria, fungi, plants, and vertebrates and can oxidize various substrates via the reduction of H₂O₂ (Dunford and Stillman, 1976). Plant peroxidases belong to a superfamily that comprises class I intracellular peroxidases of bacterial origin, secreted fungal class II peroxidases, and classical class III secretory plant peroxidases (EC 1.11.1.7). Genes encoding the latter have been duplicated many times during evolution since their appearance in the first land plants (Duroux and Welinder, 2003; Passardi et al., 2004a). Peroxidase activity can be detected throughout the whole lifespan of a plant: from germination of the seed until the final stage of senescence and death. The lack of substrate specificity, the high number of paralogous genes, and the ability to work in two catalytic cycles account for the large variety of biological mechanisms in which plant peroxidases are involved. The enzymes have been implicated in several functions of potential importance in plant defense, including direct toxicity against pathogens, production of phytoalexins, cellular growth and cell wall loosening, auxin catabolism, and plant cell wall strengthening through different mechanisms, such as lignification, suberization, and cross-linking of cell wall proteins or phenolics (for review, see Hiraga et al., 2001; Passardi et al., 2004b, 2005).

We identified a functional class III peroxidase isoform up-regulated during nodulation of *S. rostrata* (Srprx1). Expression is transiently induced, requires bacterial NFs, and is affected by H₂O₂. Transcripts accumulate along the bacterial invasion track until the ITs reach the nodule primordium and colocalize with a RNE homolog. Furthermore, in nodules occupied by NF-deficient bacteria, peroxidase transcript levels are not induced and IT progression is hampered.

**RESULTS**

**Srprx1 Encodes a Functional Peroxidase**

Differential display was used to compare gene expression in noninoculated roots and in inoculated adventitious root primordia of *S. rostrata* at different time points (Goormachtig et al., 1995; Lievens et al., 2001). A partial 185-bp cDNA clone with homology to peroxidases was up-regulated and isolated for further characterization. The full-length clone, obtained by RACE, was designated *Srprx1* (see “Materials and Methods”). Database reference searches using the BLASTX algorithm (Altschul et al., 1997) revealed an open reading frame with high homology to plant peroxidases (Fig. 1A). *Srprx1* is 70% similar to *rice (Oryza sativa)* peroxidase 131 (Passardi et al., 2004a), 70% to cationic peroxidase PNPC1 of *peanut (Arachis hypogaea)*; Buffard et al., 1990), and 72% to *Rip1* peroxidase of *M. truncatula* (Peng et al., 1996).

An N-terminal signal peptide for extracellular targeting was predicted at Ser-25 and the predicted mature protein displayed typical class III peroxidase features: a distal His (His-67) serving as a catalyst in the reaction with H₂O₂, a proximal His residue (His-195) involved in heme binding, and eight Cys forming four disulfide bridges (Cys-36-Cys-116, Cys-69-Cys-74, Cys-123-Cys-315, and Cys-22-Cys-227; Welinder, 1992; Fig. 1A). No potential N-glycosylation sites were predicted based on the known glycosylation signature Asn x (Ser-Thr) x (where x is any amino acid, except Pro; Creighton, 1993). The molecular mass of the protein without the signal peptide was estimated at 32.5 kD and the pI at 4.96, indicating that Srprx1 is an anionic peroxidase.

To demonstrate peroxidase activity, embryonic axes of *S. rostrata* were infected with an *Agrobacterium rhizogenes* strain carrying a binary vector that contained a p35S:*Srprx1* construct for constitutive *Srprx1* expression. Protein extracts from transgenic Srprx1-overproducing and control roots were subjected to native PAGE followed by in-gel 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining for peroxidase activity (see “Materials and Methods”). In each sample, several brown-colored bands corresponding to DAB-oxidizing active proteins were seen. One band was much more pronounced in the overproducing than in the control extracts (Fig. 2A). In roots harvested 2 d post inoculation (dpi) with *Azorhizobium caulinodans*, this band was also more intense than in the control roots (see below; Fig. 2A). These observations indicate that the band presumably corresponds to Srprx1 and that the native protein is able to oxidize DAB in the presence of H₂O₂. In addition, blotting of the native gel immediately followed by detection with luminol showed a band in the overproduction but not in the control extracts, indicating that Srprx1 could carry out H₂O₂-dependent oxidation of several substrates (Fig. 2B).

**Srprx1 Belongs to a Group of Peroxidases That Is Specific for Legumes**

To search for possible orthologs, phylogenetic analysis was performed with all known and predicted
class III peroxidases of Arabidopsis (*Arabidopsis thaliana*), *M. truncatula*, and poplar (*Populus trichocarpa*). The resulting cladogram is shown in Supplemental Figure S1. Srprx1 belongs to a group of peroxidases that form a distinct cluster in which reside all members of Arabidopsis group IV proteins (Supplemental Fig. S1; Tognolli et al., 2002). Within this cluster, Srprx1 fits into a subcluster to which five Arabidopsis proteins belong, previously also termed group B (Supplemental Fig. S1; Duroux and Welinder, 2003). The Arabidopsis and *Medicago* proteins of the latter group were selected to repeat the phylogenetic analysis (Fig. 1B). Interestingly, Srprx1 and MtRip1 cluster together in a clade to which no Arabidopsis peroxidases belong, but that contains six other *M. truncatula* peroxidases (Fig. 1B). This observation might indicate that several peroxidases in *Medicago* have evolved to exert a specialized function, for instance, during the nodulation process.

**Srprx1 Is Transiently Induced during Nodule Development**

Expression of *Srprx1* was studied by semiquantitative reverse transcription (RT)-PCR analysis. RNA was prepared from uninoculated adventitious root primordia and from developing adventitious root nodules at 4, 8, and 12 h and 1, 2, 3, 4, 5, 7, 12, and 20 dpi with *A. caulinodans*. A faint signal was observed in the uninoculated sample (Fig. 3A). Transcript accumulation started approximately 12 h after inoculation and expression was maximal from 1 to 5 d. The signal decreased to low basal levels in mature 20-d-old nodules. *Srprx1* expression analysis during LRB nodulation on hydroponic roots demonstrated similar, transient induction (Fig. 3B). The uninoculated sample had a weak basal expression level and induction appeared after 30 min of inoculation to reach a maximum after 12 h. At later stages of root nodulation, the *Srprx1* transcript level decreased. When growing plants in vermiculite, thus favoring RHC invasion, similar transient induction was observed (Fig. 3B). Developing zone I root hairs had basal expression and transcript level increased after root hair colonization to reach a maximum in developing RHC nodules. In mature nodules, transcripts dropped to the basal level. Peroxidase gene expression was not detectable by RNA gel-blot hybridization in other plant tissues, including seedlings, vegetative shoot apices, flowers, and leaves. Hence, *Srprx1* expression is very specific for the early stages of developing nodules (data not shown).

Protein accumulation was investigated by gel blotting of total protein extracts of uninoculated adventitious root primordia and from developing root primordia with *A. caulinodans*. An antibody was raised by rabbit injection of a 12-mer peptide sequence from Srprx1 coupled to a carrier protein (see “Materials and Methods”). Srprx1 protein accumulation was visible from 2 dpi on and reached a maximum at 5 dpi, after which it decreased slowly (Fig. 3C).
Figure 2. Srprx1 peroxidase activity. (A) In-gel peroxidase activity stain. Control, Root 2 dpi, and Srprx1 OE represent protein extracts from transgenic control roots, from roots 2 dpi, and from different lines overexpressing Srprx1, respectively. Protein extracts were separated by native PAGE and colored with DAB in the presence of H$_2$O$_2$ for peroxidase activity. B, Chemiluminescent detection in control (PZP 1, 2) or Srprx1-overexpressing (OE 1, 2) plant protein extracts after native PAGE and immunoblot without secondary antibody. The Srprx1 protein is marked with an arrow. [See online article for color version of this figure.]

Srprx1 Transcripts Do Not Accumulate after Wounding or Pathogen Infection

Extracellular peroxidases are often implicated in plant responses to wounding and pathogen infection. Wound inducibility of the Srprx1 gene was tested on leaves that were crushed with tweezers and harvested after 1, 2, 4, 8, and 16 h and 1 and 2 d. RT-PCR analysis revealed early induction of β-1,3-glucanase gene expression that served as control, indicating that strong and rapid plant reactions were triggered as a response to the mechanical damage (Fig. 4A). However, no Srprx1 expression was detected in any of the wounded leaf samples.

To determine whether Srprx1 transcripts accumulate in response to plant pathogens, the expression pattern was analyzed in S. rostrata leaves inoculated with Botrytis cinerea, a pathogenic fungus with a very wide host range (Staples and Mayer, 1995; Lievens et al., 2004). RNA from leaf samples harvested at different stages of the infection was subjected to RT-PCR. Srprx1 was not induced during the plant pathogen response that was strong and early as can be deduced from β-1,3-glucanase gene expression (Fig. 4B).

In a second pathogen assay, stem-located adventitious root primordia of S. rostrata were infected with Ralstonia solanacearum, a wide host range root pathogen (Hayward, 1991) that induces a strong defense reaction at these sites (Lievens et al., 2004). Stems were brush inoculated with either the wild-type or a non-virulent mutant strain (hrp+) and root primordia were excised after 8 h and 1, 2, 3, and 5 d. The typical brown ring at the base of the adventitious root primordia that appeared upon wild-type R. solanacearum infection was accompanied at the molecular level by accumulation of β-1,3-glucanase transcripts (Fig. 3C). In this time series, RT-PCR showed no Srprx1 induction upon pathogenesis (Fig. 4C). In conclusion, Srprx1 expression is very specific for the early stages of developing nodules, implying that the gene encodes a nodule-specific peroxidase isoform and can be considered as one of the more specific true nodulins.

Srprx1 Expression Pattern Visualized by in Situ Hybridization

To visualize the transcripts in plant tissues, expression of Srprx1 was analyzed by in situ hybridization on adventitious and lateral root nodule sections (Fig. 5). No expression above background was seen in sections of uninoculated adventitious root primordia (data not shown). At 1 dpi, transcripts were visible in cells neighboring the epidermal fissure that surrounds the base of the root primordium (Fig. 5, A and D). After 2 d, transcripts strongly accumulated in the cortical cells surrounding IPs (Fig. 5, B and E). At 3 dpi, IPs were formed that guide the bacteria to the nodule primordium and Srprx1 expression was very prominent in the cells that were flanking these IPs (Fig. 5, C and F). At 4 dpi, IPs reached the nodule primordium and traversed the newly formed cells. Interestingly, Srprx1 expression stopped abruptly once the IPs had entered the cells of the nodule primordium that would become the nodule central tissue (Fig. 5, G–I, arrows). At this stage, the signal around the fissure, the IPs, and the ITs in the outer cortex was still strong. From 6 d on, this signal gradually withdrew from the deeper cortical regions (Fig. 5J) and, at 8 d, was only faintly detectable around some remaining IPs (data not shown).

In LRBs, transcripts were visualized on butylmethyl-embedded material in which the structure is better preserved than in paraffin (Kronenberger et al., 1993). Already at 1 dpi, Srprx1 expression was observed at the LRBs, where the bacteria normally enter cortical tissue (data not shown). After 2 d, the lateral root was swollen at the base because of primordium development and IPs and ITs were observed. In both epidermal and cortical cells in direct contact with bacteria, transcripts accumulated (Fig. 5, K and L).

Srprx1 Expression Requires Bacterial NFs and Can Be Modulated by H$_2$O$_2$

To analyze whether Srprx1 transcript accumulation depends on NFs, inoculations with bacterial mutants were carried out. The A. caulinodans strain ORS571-V44 has a Tn5 insertion in a Rha biosynthesis locus, resulting in defective surface polysaccharides. Infection stops at the IP stage, but NF production is normal (Goethals et al., 1994; D’Haeze et al., 1998). Strain ORS571-V44 does not produce NFs because of a mutation in the nodA gene and is unable to provoke a nodule-related plant effect (Van den Eede et al., 1987; Mergaert et al., 1993; D’Haeze et al., 1998). Srprx1
expression was induced by ORS571-X15, but not triggered by ORS571-V44 (Fig. 6A).

To determine whether pure *A. caulinodans* NFs are sufficient to trigger *Srprx1* transcript accumulation, roots of *S. rostrata* were treated with 10^{-8} M NFs and harvested at different time points (Fig. 6B). RT-PCR analysis showed that transcripts of the peroxidase gene already accumulated 30 min after treatment and further increased to a maximum at 12 h. Later on, the signal slowly decreased.

Because H2O2 is a NF downstream signal for LRB nodulation (D’Haeze et al., 2003) and induces transcription of many different genes, *Srprx1* induction was assayed by RT-PCR in hydroponic roots of *S. rostrata* supplied with 1 mM H2O2. Expression was slightly induced 1 h after addition and reached a maximum after 24 h, whereas thereafter it decreased again to initial levels, indicating that the expression level could be modulated by H2O2 (Fig. 6C).

**Srprx1 Colocalizes with a RNE**

In a previous differential display experiment (Goormachtig et al., 1995), a nodulation-specific tag had been isolated with homology to Hyp-rich glycoproteins with typical Ser-(Pro)_{4} motifs. Although a full-length protein-encoding gene could not be isolated, the predicted amino acid sequence clearly displayed motifs of extensins and arabinogalactan proteins, characteristic for the group of RNEs found only in legumes (Brewin, 2004; Gucciardo et al., 2005). Also, several Tyr and isodityrosine residues (Tyr-x-Tyr) were present that are involved in peroxide-based protein cross-linking (Held et al., 2004; Gucciardo et al., 2005; Fig. 7A). The partial nucleotide sequence for the RNE, designated *SrRNE1*, belongs to a small gene family, as suggested by genomic DNA-blot analysis (data not shown). RT-PCR analysis showed an increase in transcript levels from 4 h after inoculation of adventitious rootlets (Fig. 7B). RNA-blot analysis after inoculation with ORS571-X15 or ORS571-V44 demonstrated that transcript accumulation depended on NF production (Fig. 7C). In situ localization of transcripts in developing adventitious root nodules revealed two major expression patterns. Two days after inoculation, transcripts accumulated around bacterial IPs and ITs (Fig. 7, E and F). At 3 dpi, expression was also visible in the nodule primordium and even more in the cells of the infection center, where ITs grew toward the open basket-shaped nodule primordium (Fig. 7, G and H). The invasion-associated pattern nicely correlated with the *Srprx1* pattern.

By using the monoclonal antibody MAC265 that is specific for RNES in pea (*Pisum sativum*; Bradley et al.,...
RNE accumulation was investigated in developing root nodules of *S. rostrata* (Fig. 7D). In uninoculated adventitious RNEs, two light bands were present around 110 and 90 kD, as similarly found in pea nodules, where immunopurified matrix glycoproteins comigrated as a doublet at 100 to 110 kD (Rathbun et al., 2002). In extracts of inoculated samples (3 dpi), these two bands were stronger and, in later stages (from 5 dpi), additional lower bands were observed, as was also the case in pea nodule extracts (Rathbun et al., 2002). Thus, RNEs accumulate in the same time frame as Srprx1 and have an overlapping expression pattern, hinting at involvement in the same biological process.

**Mixed Inoculation Nodules Do Not Induce Srprx1 Expression**

Upon coinfection with two symbiotic mutants of *A. caulinodans*, ORS571-X15 and ORS571-V44, only the NF-deficient ORS571-V44 mutant invaded cortical tissue via ITs and entered plant cells to form symbiosomes (D’Haeze et al., 1998, 2004). The resulting mixed inoculation nodules developed inefficiently and bacterial invasion was not synchronized with nodule formation. Proper IT development and growth were hampered, as thick and swollen ITs were visible (Den Herder et al., 2007). During ORS571-V44 invasion, *Srprx1* was not expressed around bacterial IPs after 6 d of coinoculation, a stage that resembled early wild-type infection (Fig. 8, A and B). Only occasionally, slight induction was observed around superficial IPs, which were occupied by the NF-producing ORS571-X15 mutants (data not shown; D’Haeze et al., 1998; Den Herder et al., 2007). Around ORS571-V44-containing ITs that reached deeper into the cortical tissue after 9 d, no *Srprx1* expression was visible (Fig. 8, C and D). These observations confirm that NFs produced locally by bacteria within the ITs are required to induce the *Srprx1* gene.

**Figure 5.** In situ localization of *Srprx1* transcripts. Longitudinal (A–F and J–L) and transverse (G–I) sections through developing stem (A–J) and root (K and L) nodules were hybridized with a 35S-labeled antisense RNA probe and analyzed under bright-field (signal is seen as black spots [E–H and L]) and dark-field optics (signal is seen as white spots [A–D and I–K]). Successive stages of adventitious root nodule development are shown at 1 (A), 2 (B), 3 (C), 4 (G), and 6 (J) dpi with *A. caulinodans* ORS571. Images in D, E, F, H, and I are enlargements of the regions indicated by the rectangles in A, B, C, and G, respectively. K, Butyl-methyl-embedded section of a developing LRB nodule at 2 dpi. L, Enlargement of K. f, Fixation zone; fi, fissure; i, infection zone; ic, infection center; ip, infection pocket; m, meristematic zone; np, nodule primordium. Arrows and double arrows mark infection thread with or without *Srprx1* expression, respectively. Bars = 100 μm.
DISCUSSION

By screening for differentially transcribed genes during adventitious root nodule development in S. rostrata, a short cDNA fragment was isolated, whose transcript levels increased during the early stages of infection with A. caulinodans. The corresponding full-length clone contained an open reading frame with high homology to class III plant peroxidases and was designated Srprx1.

Class III plant peroxidases (EC 1.11.1.7), often referred to as the classical plant peroxidases, are targeted to the vacuole or the extracellular space. These monomeric, usually N-glycosylated proteins of approximately 300 amino acids, are structurally very similar and contain four conserved disulfide bridges. The active site consists of a heme group that is coordinated to an invariant proximal His, whereas a conserved distal His is the essential catalytic residue for binding and heterolytic cleavage of H$_2$O$_2$ (Welinder, 1992).

All these characteristics, with the exception of the N-glycosylation sites, are found in the deduced amino acid sequence of Srprx1. The presence of an N-terminal signal peptide and the absence of a vacuolar targeting sequence suggest a cell wall peroxidase. Peroxidase activity has been shown by in-gel oxidation of DAB in the presence of H$_2$O$_2$ and by luminol oxidation both in protein extracts of roots overexpressing Srprx1 and in nodule extracts after 2 d of bacterial inoculation.

Figure 6. Srprx1 expression in response to inoculation with A. caulinodans mutants, pure NFs, and H$_2$O$_2$. A. Expression after inoculation with mutants ORS571-V44 (NF deficient) and ORS571-X15 (surface polysaccharide deficient), compared to wild type as positive control. Samples were taken 8 h and 2 dpi. B. RT-PCR of Srprx1 compared to the constitutive Srubi1 after addition of 10$^{-8}$ M A. caulinodans NFs to hydroponic roots. Samples were taken from untreated roots (−) and from roots treated for 30 min (30’), and 1, 4, 8, 12, 24, and 48 h. C. RT-PCR of Srprx1 in hydroponic roots at different time points (−, 1, 2, 4, and 24 h, and 2, 3, and 6 d) after addition of 1 $\mu$M H$_2$O$_2$, with a constitutive control for loading (Srubi1).

Figure 7. Analysis of SrRNE1 during adventitious root nodule development. A. Partial protein sequence of SrRNE1 with typical extensin (orange) and arabinogalactan (green) motifs, and Tyr (yellow) and isodityrosine (underlined) residues. B. RT-PCR of SrRNE1 (top) compared to Srubi1 (bottom) on developing adventitious root nodules. C. RNA-blot analysis of SrRNE1 in adventitious root primordia 3 dpi with ORS571 (WT) and mutants X15 (surface polysaccharide deficient) and V44 (NF deficient). Equal loading was controlled by methylene blue staining (bottom). D. RNE protein detection in nodule protein extracts with MAC265 antibody. E to H. In situ localization pattern of SrRNE1 in developing adventitious root nodules at 2 (E and F) and 3 (G and H) dpi with wild-type A. caulinodans. ic, Infection center; ip, infection pocket; it, infection thread; np, nodule primordium; vb, vascular bundle. Bars = 100 $\mu$m.
Plant peroxidases are encoded by large multigene families. In the Arabidopsis genome, 73 genes have been identified, most of them expressed in roots. They account for 2.2% of root ESTs, but only a few show strict organ specificity (Tognolli et al., 2002; Welinder et al., 2002). In rice, 138 genes are distributed over all chromosomes (Passardi et al., 2004a). Also, in S. rostrata, Srprx1 is part of a large family as demonstrated by DNA gel-blot analysis and activity staining of native protein extracts. Consequently, during transcript analysis, cross-hybridization with homologous family members might occur. However, with the probe used for in situ hybridization, only one gene was detected by DNA gel-blot analysis under high-stringency washing conditions (data not shown), making it unlikely that more than one family member has been visualized.

Both in adventitious and hydroponic LRB nodule development, Srprx1 transcripts and proteins accumulated transiently during the early stages of the interaction, with a difference in time frame that corresponds to the faster nodule development on hydroponic roots. Transient induction also occurred during RHC invasion, suggesting a basic function in nodulation. Srprx1 expression is remarkably specific for nodulation: Srprx1 is rapidly induced by NFs, the main bacterial morphogens that control nodule development; no transcripts have been detected in other plant tissues; and the expression level did not increase upon pathogen attack, a trigger that activates various other peroxidase genes (Harrison et al., 1995; Chittoor et al., 1997; Curtis et al., 1997; Liu et al., 2005).

The temporal expression profile of Srprx1 is somewhat reminiscent of that observed for rip1 in M. truncatula. The latter gene is maximally induced in roots in the preinfection period preceding bacterial infection, but is still up-regulated after nodule primordia can be observed (96 h) to drop to basal levels afterward (Cook et al., 1995). However, in situ hybridizations indicated that Srprx1 and rip1 are expressed at different sites during nodule formation. The rip1 transcripts have been localized to epidermal cells in the differentiating root zone, but also to the nascent nodule primordium (Cook et al., 1995; Peng et al., 1996). In contrast, Srprx1 expression is tightly linked to the presence of invading rhizobia. Srprx1 is exclusively expressed in cells that are in direct contact with NF-producing bacteria, such as those flanking the epidermal fissure, IPs, and ITs, but is restricted to the invasion preceding entry in the nodule central tissue. Thus, based on the expression data, Rip1 and Srprx1 cannot be true orthologs. Phylogenetic tree analysis indicated that Srprx1 and Rip1 cluster together in a clade that consists exclusively of Medicago and Sesbania peroxidases and that is related to the group IV peroxidases of Arabidopsis (Tognolli et al., 2002), suggesting that several peroxidases have evolved to exert a specialized function during nodule formation. The presence of seven members in M. truncatula points to functional redundancy or to subfunctionalization (Adams, 2007).

Class III peroxidases often use H2O2 as a substrate for oxidizing various biological substrates. In S. rostrata, H2O2 has been localized at the sites of Srprx1 induction, namely, in the walls of the cells neighboring the epidermal fissure early after inoculation of root primordia, in cortical cells that will collapse to form IPs, and in intercellular and intracellular ITs (D’Haese et al., 2003). H2O2 has also been shown to be a NF downstream signal for LRB invasion and to modulate Srprx1 expression.

A putative substrate of the peroxidase could be RNEs, whose expression profile coincides with that of Srprx1. RNEs accumulate at stages similar to those of peroxidase and induction also depends on NF production. RNEs are Hyp-rich glycoproteins characterized by interspersed motifs typical for extensin and arabinogalactan proteins (Brewin, 2004; Gucciardo et al., 2005). The extensin motif with contiguous Hyp residues, such as SFPFP, is predicted to carry small Ara glycosylations, whereas clustered noncontiguous blocks of Hyp are sites for addition of large arabinogalactan polysaccharides built around a 1-3, β-linked Gal backbone (Kieliszewski, 2001; Tan et al., 2004). These matrix glycoproteins are found only in legumes and are encoded, at least in pea, by a family of genes of different length, but with very similar molecular structures (Rathbun et al., 2002).

The occurrence of a specific subgroup of Hyp-rich proteins and peroxidases in legumes and the very localized and transient induction of Srprx1 during early nodulation stages are in agreement with a specialized role in nodulation. Peroxidative cross-linking of RNEs might have a function in the initiation of ITs.
by isolating the bacteria enclosed in the curled root hair, thereby counteracting the turgor pressure of the host plant cell and driving IT growth (Brewin, 2004). A fluid-to-solid transition in the outer cortex by peroxide-driven insolubilization of RNPs might also be required during crack entry in *S. rostrata*, possibly until the ITs enter the nodule central tissue. A functional knockout of the *Srprx1* gene might clarify these issues. Unfortunately, RNA silencing in transgenic roots yielded no nodulation phenotype (J. Den Herder, unpublished data). This outcome is not surprising because several related nodule-enhanced peroxidase gene tags have been found back in *S. rostrata* nodulation (W. Capoen and M. Holsters, unpublished data), strongly hinting at the possibility for functional redundancy. In mixed inoculation nodules that are invaded by the non-NF-producing mutant ORS571-V44 (D’Haeze et al., 1998) after initial complementation by the NF-producing, noninvasive strain ORS571-X15, *Srprx1* expression was not detected and IT progression was seriously hampered, with many bulged threads and IP-like structures in the infection center. In addition, electron microscopic analysis revealed a rim of low electron-dense material at the borders of the IT matrix that was continuous with the exopolsaccharide layer of the bacteria (Den Herder et al., 2007). The spreading of the exopolysaccharide in these ITs might be caused by changes in the physicochemical properties of the matrix. In conclusion, NF-induced functions—among them *Srprx1*—play a role in proper IT progression. Moreover, *Srprx1* is a molecular marker that will be of great use in unraveling the plasmid form, according to the manufacturer’s protocol (Stratagene). The plasmid with the largest insert was sequenced and designated pSrExT1.

### Protein Analysis and Activity Assay

A polyclonal antibody was raised by several rabbit injections of a 12-mer peptide sequence (LYKQSYSPYEAF) of *Srprx1* with high antigenicity and low hydrophobicity (as predicted by the PeptideStructure program in the GCG Wisconsin package; Accelrys), coupled to the keyhole limpet hemocyanin protein with the Imject Maleimide Activated Immunogen conjugation kit (Pierce) via an extra Cys residue. Serum was taken 63 d after the first injection and used for protein analysis.

Plant protein extracts were prepared by grinding developing adventitious nodules in liquid nitrogen and addition of 1 vol of extraction buffer (25 mM Tris-Cl, pH 8.0, 5 mM EDTA, 15 mM MgCl₂, 85 mM NaCl, 0.1% [v/v] Tween20, and protease inhibitor cocktail tablets [1/10 mL; Complete mini; Roche Diagnostics]). After 2 h of rotation at 4°C, proteins were separated from the remainder by centrifugation at 10,000g and 4°C for 30 min. Protein concentration of the supernatant was determined with the Dc Protein assay (Pep2#17_63d) at 4°C (1/10,000). After washing, the secondary antibody (anti-rabbit-IgG-HRP; GE Healthcare) was incubated for 1 h at room temperature (1/10,000) and detected with a chemiluminescence kit according to the manufacturer’s instructions (Perkin-Elmer). Detection of *SrRNE* occurred with 1% (v/v) of MAC265 hydridoma culture supernatant (Bradley et al., 1988) and anti-rat-IgG-HRP (1/10,000) as secondary antibody. For the activity assay, extracts (prepared without Tween20) were separated on a native PAGE in Tris-Gly buffer without prior denaturation of the samples. Afterward, the gel was equilibrated for 30 min in 20 mM sodium citrate buffer (pH 5.5) before addition of 0.03% (w/v) H₂O₂ and 1 mM DAB. Replacement of the reagent mix by water stopped the reaction and the gel was dried in a gel air dryer (Bio-Rad).

### Isolation of Full-Length cDNA Clones

5'-RACE was performed with the Marathon cDNA amplification kit (CLONTECH) to obtain the full-length clone corresponding to the partial cDNA *Srdd15*. cDNA was synthesized from RNA extracted from root primordia harvested at 2 dpi with *A. cauliformis* ORS571. Antisense primer sh18 (5'-CCCTGAGTCAACACCTGCATTCTTTG-3') in combination with the API primer provided was used for the amplification step, according to the manufacturer’s instructions. RACE products were cloned in the pGEM-T vector (Promega) and sequenced. The full-length sequence was designated *Srprx1*, reamplified with primers sh27 (5'-ATGGCCCTCAAAGCGGTATC-TCTCTG-3') and sh28 (5'-CAATAATCTAATTTGCTTTCAAATT-3') with Vent polymerase (New England Biolabs), and cloned in the pGEM-T vector as pGEMTc2.2f14.

For *SrRNE1*, plasmids (3 x 10⁵) of a ZAP cDNA library of developing nodules (Goormachtig et al., 1995) were screened with a 32P-labeled *rlld*-2 fragment, an extensin-like partial cDNA isolated by differential display (Goormachtig et al., 1995). Plasmages from single positive plaques were transferred to their corresponding plasmid form, according to the manufacturer’s protocol (Stratagene). The plasmid with the largest insert was sequenced and designated pSrExT1.

### MATERIALS AND METHODS

#### Biological Material

*Sesbania rostrata* ‘Brem’ seeds were surface sterilized, grown, and inoculated as described (Goormachtig et al., 1995; Fernández-López et al., 1998). For root assays, plants were grown either in tubes with sterile nitrogen-free liquid Noris medium, at pH 7.0 (Vincent, 1970), or in Leonard jars with vermiculite, root assays, plants were grown either in tubes with sterile nitrogen-free liquid Noris medium, at pH 7.0 (Vincent, 1970), or in Leonard jars with vermiculite, covered with perlite. For H₂O₂ treatment, a 30% (w/w) aqueous solution of butyl-methyl-embedded (5 m) or butyl-methyl-embedded (8 m) was used. As controls, a ubiquitin (Corich et al., 1998) and a corresponding tissue samples on a 1% (w/v) agarose gel containing 2% (w/v) formaldehyde, dried in a gel air dryer (Bio-Rad).

#### RNA Analysis

RNA of roots was prepared according to Kiefer et al. (2000) and template cDNA was synthesized from 2 or 5 µg of total RNA with the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). For the specific amplification of a 300-bp fragment of the 5’ end of *Srprx1*, primer sSr38 was used. PCR products were detected radioactively with probes generated from the *SrRNE1* fragment with primers Extl (5’-CCACCTCCATTTCCCATATCCCATCTC-3’) and Ext2 (5’-CCCATTTGATTACACACACAC-3’). The program comprised 20 cycles of amplification for 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. PCR products were radioactively labeled with probes generated from the cDNA fragment *Srdd15*, *Srnt1* (Corich et al., 1998), *Srglu2* (Lievens et al., 2004), and *Srext1* by means of the Rediprime II random prime labeling system (GE Healthcare). Membranes were analyzed with a PhosphorImager (GE Healthcare). RT-PCR analysis was repeated at least twice with independent material.

RNA blot was performed by separation of 10 µg RNA from the different tissue samples on a 1% (v/v) agarose gel containing 2% (v/v) formaldehyde, transfer to Hybond-N filters (GE Healthcare), and hybridization with the corresponding *adi*-2 fragment. As a control for equal loading, filters were stained with methylene blue (Sambrook et al., 1989).

#### In Situ Hybridization

Sections of paraffin-embedded (10 µm) or butyl-methyl-embedded (8 µm) root primordia and developing adventitious root nodules were hybridized in situ as described by Goormachtig et al. (1997). The plasmid pGEMTc2.2f14 was digested with SacII and SacI to yield templates for the 32P-labeled antisense and

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sense probe production with SP6 and T7 RNA polymerase (GE Healthcare), respectively. For SrNRE1, sense and antisense probes were generated by digestion of pSrExt1 with XhoI or EcoRI and transcription with T7 and T3 RNA polymerase, respectively (Goormachtig et al., 1995). Hybridizations with the sense probe did not yield signal above background (data not shown).

Phylogenetic Analysis

All potential peroxidases were collected by running reciprocal best hits iteratively with BLASTP over different proteomes, namely, poplar (Populus trichocarpa; Joint Genome Institute), Medicago (International Medicago Genome Annotation Group), and Arabidopsis (Arabidopsis thaliana; The Arabidopsis Information Resource) starting with the S. rostrata gene. From the nonredundant set of 275 proteins collected over the three genomes (137, 64, and 57 proteins, respectively), a guide tree was made on the most conserved regions in the alignment. Based on this cladogram, a proper phylogenetic tree was built for a subset of proteins with the Tree-puzzle program (Schmidt et al., 2002) to calculate a neighbor-joining tree with puzzling steps.

Sequence Analysis

DNA sequencing was carried out with universal SP6 and T7 primers. DNA sequence data were assembled and analyzed with the GCG package (Accelrys). Percentage of identity and similarity between sequences was determined with the GAP program and aligned with the PileUp program. The SrPrx1 protein sequence was deduced with the Translate program and further mined with the MOTIFS, PeptideSort, and SPScan programs. Nucleic Acids Res 25: 433–439. The genome Annotation Group), and Arabidopsis (Arabidopsis thaliana; The Arabidopsis Information Resource) starting with the S. rostrata gene. From the nonredundant set of 275 proteins collected over the three genomes (137, 64, and 57 proteins, respectively), a guide tree was made on the most conserved regions in the alignment. Based on this cladogram, a proper phylogenetic tree was built for a subset of proteins with the Tree-puzzle program (Schmidt et al., 2002) to calculate a neighbor-joining tree with puzzling steps.

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