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 in both 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, Srprx1 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 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; Gucciaro 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 to 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.
RESULTS

Srprx1 Encodes a Functional Peroxidase

Differential display was used to compare gene expression in nonnodulated roots and in inoculated adventitious root primordia of *S. rostrata* at different time points (Goormachtig et al., 1995; Liewens 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 *H*$_2$*O*$_2$, 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 kDa 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 observed. 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 *H*$_2$*O*$_2$. 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 *H*$_2$*O*$_2$-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 upon inoculation 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).
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 (harp) 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 pathogenosis (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 isofrom 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, ITs were formed that guide the bacteria to the nodule primordium and Srprx1 expression was very prominent in the cells that were flanking these ITs (Fig. 5, C and F). At 4 dpi, ITs reached the nodule primordium and traversed the newly formed cells. Interestingly, Srprx1 expression stopped abruptly once the ITs 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 butyl-methyl-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 H2O2

To analyze whether Srprx1 transcript accumulation depends on NFs, inoculations with bacterial mutants were carried out. The A. caulinodans strain ORS571-X15 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’Haese 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’Haese 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 H$_2$O$_2$ 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 H$_2$O$_2$. 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 H$_2$O$_2$ (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., 2003), Srprx1 expression was induced by ORS571-X15, but not triggered by ORS571-V44 (Fig. 6A).

Figure 3. Expression analysis of Srprx1 during nodule development. A, Semiquantitative RT-PCR performed on RNA from uninoculated root primordia (−) and primordia harvested at 4, 8, and 12 h and 1, 2, 3, 4, 5, 7, 12, and 20 dpi with A. caulinodans ORS571. B, RT-PCR on hydroponic (LRB) and aeroponic (RHC) roots after bacterial inoculation. Primers and probes specific for Srprx1 (top) or the ubiquitin gene Srubi1 (bottom), as a constitutive control, were used. C, Protein gel-blot of stem protein extracts before or every day after inoculation until 8 dpi. Visualization was done with primary antibody Pep2#17_63d and an anti-rabbit-IgG-HRP secondary antibody, and subsequent chemiluminescent detection. Arrow indicates Srprx1.

Figure 4. Srprx1 expression in response to wounding and pathogen infection. Expression levels determined by semiquantitative RT-PCR with primers and probes specific for Srprx1, Srglu2, a β-1,3-glucanase gene as positive control, and Srubi1 as constitutive control. A, Transcripts in leaves before (−) and 1, 2, 4, and 8 h after inoculum and reached a maximum after 24 h, whereas thereafter it decreased again to initial levels, indicating that the expression level could be modulated by H$_2$O$_2$ (Fig. 6C).
1988; VandenBosch et al., 1989), 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–I) 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; 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 H₂O₂ (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 H₂O₂ 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 H₂O₂. 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 10⁻³ μM *A. caulinodans* NFs to hydroponic roots. Samples were taken from untreated roots (−) and from roots treated for 30 min (30'), 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 μM H₂O₂, 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 μm.
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’Haeze 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; Gucchiardo et al., 2005). The extensin motif with contiguous Hyp residues, such as SPPPP, 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 RNEs 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 nodule 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’Haese 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 exopolysaccharide 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 components of the NF signal perception system in the cortex.

**MATERIALS AND METHODS**

**Biological Material**

*Sebania 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 Norris 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 (Sigma-Aldrich) was used. Purified NFs were obtained as described (Mergaert et al., 1997) and added to a final concentration of 10^{-5} M (5 \times 10^{-5} M of each fraction pl and pII). Agrobacterium rhizogenes-mediated transformation of S. rostrata was done as described (Van de Velde et al., 2003).

*Azoishizobium caulinodans* ORS571, ORS571-X15 (Goethals et al., 1994), and ORS571-V44 (Van den Eede et al., 1996) were cultivated and used for inoculation as previously described (Goormachtig et al., 1995). Infections with *Ralstonia solanacearum* and *Botrytis cinerea* were done as described by Lievens et al. (2004).

**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 Srd21. cDNA was synthesized from RNA extracted from root primordia harvested at 2 dpi with *A. caulinodans* ORS571. Antiserum primer sh18 (5’-CTCTGCAGCTCAACCGGTATCTTCTCAGTG-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 sh22 (5’-TGGGCTTTACACCATCGGATTCTCTCGTGTGCT-3’) and sh28 (5’-CAATAATCTTAAATGCTTTACAAAT-3’) with Vent polymerase (New England Biolabs), and cloned in the pGEM-T vector as pGEMTc2.184.

For *SrRNE1*, plagues (3 \times 10^{3}) of a ZAP cDNA library of developing nodules (Goormachtig et al., 1995) were screened with a 3P-labeled ddi-2 fragment, an extension-like partial cDNA isolated by differential display (Goormachtig et al., 1995). Plages from single positive plagues 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.

**Protein Analysis and Activity Assay**

A polyclonal antibody was raised by several rabbit injections of a 12-mer peptide sequence (LVKYQSSYPEAF) 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 Immune X Active Immunoconjugation 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 volume 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 [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 for 4°C for 30 min. Protein concentration of the supernatant was determined with the D2 Protein assay (Bio-Rad) and 20 g of each sample were used for SDS-PAGE. Immunoblot was performed by blocking the membrane in 5% (w/v) skim milk in Tris-buffered saline-Tween and overnight incubation with the primary antibody (Pep217, 63d) at 4°C (1/1,000). 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 SrRNE1 occurred with 1% (v/v) of MAC265 hybridoma culture supernatant (Bradley et al., 1988) and anti-rat-IgG-HRP (1/1,000) 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).

**RNA Analysis**

RNA of roots was prepared according to Kiefer et al. (2000) and template cDNA was synthesized from 2 or 5 g of total RNA with the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). For the specific amplification of a 390-bp fragment of the 3’ end of Srprx1, primers SH8 (5’-TCTGCGAG-GACACACGATG-3’) and sh17 (5’-TAGTAGTGCTACCTTCGAGCCTG-3’) were used. As controls, a ubiquitin (Corich et al., 1998) and a b-1,3-glucanase (Lievens et al., 2004) fragment were amplified. Amplification of the SrRNE1 fragment occurred with primers Ext1 (5’-CACACTCTACCTCCCCATATCC-3’) and Ext2 (5’-CAGCTCATTTAAACCTTCTC-3’) of the ZAP cDNA library of developing nodules (Goethals et al., 1994). 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 detected radioactively with probes generated from the cDNA fragment Srd15, Srd1b1 (Corich et al., 1998), Srgu2a (Lievens et al., 2004), and Srext1 by means of the Rediprime II random prime labeling system (GE Healthcare). Membranes were hybridized with a Phosphorimager (GE Healthcare). RT-PCR analysis was repeated at least twice with independent material.

RNA blot was performed by separation of 10 g RNA from the different tissue samples on a 1% (w/v) agarose gel containing 2% (w/v) formaldehyde, transfer to Hybond-N filters (GE Healthcare), and hybridization with the corresponding ddi-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 mm) or butyl-methyl-embedded (8 mm) root primordia and developing adventitious root nodules were hybridized in situ as described by Goormachtig et al. (1997). The plasmid pGEMTc2.184 was digested with Sall and Sall to yield templates for 35S-labeled antisense and...
sense probe production with SP6 and T7 RNA polymerase (GE Healthcare), respectively. For SrN1E7, sense and antisense probes were generated by digestion of pSrEx1 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., 1999) and 57 proteins, respectively), a guide tree was made on the most conserved nonredundant set of 275 proteins collected over the three genomes (137, 64, 726 Plant Physiol. Vol. 144, 2007

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 Srprrx1 protein sequence was deduced with the Translate program and further mined with the GAP program and aligned with the PileUp program. The gene. From the Medicago truncatula; Plant Cell 7: 43–55


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LITERATURE CITED


