A New Proline-Rich Early Nodulin from Medicago truncatula Is Highly Expressed in Nodule Meristematic Cells

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We cloned and characterized MtPRP4, a new member of the repetitive proline-rich protein gene family in Medicago truncatula. The sequence of MtPRP4 predicts a 62-kD protein consisting of a 22-amino acid N-terminal signal peptide and a 527-amino acid repetitive proline-rich domain composed of three repetitive pentapeptide motifs arranged into two decapeptide repeats: PPVEKPPVHK and PPVEKPPVYK. MtPRP4 is the largest PRP described to date and contains repeated motifs that have not previously been found together in a single polypeptide. RNA gel blot experiments detected MtPRP4 transcripts in symbiotic root nodules, but not in roots, hypocotyls, or leaves. Accumulation of MtPRP4 transcript was an early response to Rhizobium inoculation and did not depend on nodule infection. In situ hybridization experiments demonstrated that MtPRP4 was expressed early in the development of the nodule meristem and that expression was highest in the meristematic cells of mature indeterminate nodules. These data support the proposition that an important early response of legume host roots to Rhizobium involves remodeling the host extracellular matrix and that proline-rich wall proteins play an important role in this architectural modification.

INTRODUCTION

Plant cell wall architecture plays an important role in plant-microbe interactions and in plant growth and development. Wall structure is determined by both structural polysaccharides and structural (glyco)proteins, and recently a number of genes encoding extracellular structural proteins have been cloned and characterized (for recent review, see Showalter, 1993). Three distinct classes of wall structural proteins (proline-rich proteins or PRPs, hydroxyproline-rich glycoproteins or extensins, and glycine-rich proteins) are distinguished by characteristic amino acid compositions and repetitive peptide motifs and by unique patterns of gene expression within the plant body, presumably playing important roles in the differentiation and function of particular cell types (Ye et al., 1991). Expression of specific members of the gene families encoding these structural wall proteins correlates with important developmental processes, such as vascular differentiation, wound healing, and pathogen infection/resistance.

In legumes, several novel proline-rich gene products were cloned as early nodulin genes (e.g., ENOD2 and ENOD12) expressed early in the development of symbiotic nitrogen-fixing root nodules (Franssen et al., 1987; Scheres et al., 1990). Expression of these cell wall protein genes during nodule development correlates with the morphogenesis of this new plant organ or with the intracellular infection of this organ by symbiotic Rhizobium bacteria. Thus, nodule development provides an ideal system for investigating the structure, regulation, and function of cell wall PRPs in plant development and plant-microbe interactions.

Medicago truncatula is one model host plant for studies on Rhizobium-legume symbiosis (Barker et al., 1990). In this study, we report the cloning and characterization of a new member of the M. truncatula PRP gene family, MtPRP4. DNA sequence analysis of this clone predicts a 62-kD polypeptide (one of the largest plant PRPs identified to date) targeted to the secretory pathway in plant cells. The mature PRP4 protein is comprised of three distinct repetitive pentapeptides, PPVEK, PPVYK, and PPVHK, which have not been found previously in a single polypeptide. Expression analyses show that MtPRP4 expression is induced early in the development of symbiotic root nodules, where it may play a role in nodule morphogenesis.

RESULTS

Characterization of MtPRP4, a Novel PRP Gene

An M. truncatula genomic library was constructed and screened at low stringency with an internal coding region fragment of MsENOD2 from M. sativa (pA2ENOD2; Dickstein et al., 1988). This screen yielded six different genomic clones that hybridized to A2ENOD2 at low stringency but not at high

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stringency. These clones included both proline-rich and glycine-rich protein genes (J. Patterson, E.M. Maruoka, and J.B. Cooper, unpublished data) as observed in previous screens for proline-rich sequences (Keller et al., 1988; Showalter et al., 1991). The restriction map of a 9-kb Sall restriction fragment from one of these clones, λMM7-1, shown in Figure 1A, was determined and the region of ENOD2 homology was localized to two contiguous Spel fragments using DNA gel blotting experiments.

The nucleotide sequence of ~2.8 kb of DNA flanking the region of ENOD2 homology was determined, and a single long open reading frame (ORF) was identified in the sense strand (Figure 1B). This direction of transcription was confirmed using RNA gel blot experiments with strand-specific probes (data not shown). As shown in Figure 2, this DNA sequence encodes a long ORF predicting a 549-amino acid translation product with an extremely biased amino acid composition and an extremely repetitive sequence. Only six amino acids (Pro, Val, Lys, Tyr, Glu, and His) make up more than 90% of this polypeptide. Table 1 compares the amino acid composition of this M. truncatula ORF to the composition of other plant PRPs. Most obviously, all of the plant PRPs (as well as extensin) have a very high (hydroxy)proline content and high contents of both lysine and tyrosine. This new M. truncatula ORF also has a high content of valine, as do MtPRP1 and MtPRP2, a high content of glutamic acid, as do PRP2 and ENOD2, and a high content of histidine, as do ENOD2, ENOD12, and DcPRP1 from carrot. The predicted size of this ORF (~62 kD) is the largest yet among the known PRPs. We named this M. truncatula gene

![Figure 1. Structure of MtPRP4.](image)

(A) Restriction map of the 9-kb Sall fragment containing MtPRP4. The bold line represents the region of DNA sequenced in this analysis. The shaded box represents the region of homology to A2ENOD2. B, BamHII; H, HindIII; S, Sall; Sp, Spel.

(B) The arrow represents the long ORF and the direction of transcription identified by sequence and RNA gel blot analyses.

(C) Probes used for gene structure and gene expression analyses.

![Figure 2. Sequence of MtPRP4.](image)

(A) Deduced amino acid sequence of MtPRP4, arranged in repetitive decapptide motifs. The GenBank accession number is L23504. The arrow indicates the predicted signal peptide cleavage site.

(B) Schematic representation of the repetitive structure of MtPRP4.

product MtPRP4 based on (1) the similarity in amino acid composition with other PRPs (MtPRP1, MtPRP2, DcPRP1, and MtENOD12) and the distinct difference in composition from ENOD2 and extensins; (2) the presence of an N-terminal signal peptide and repetitive pentapeptide motifs characteristic of the PRP family (see below); and (3) the unusually large size of the MtPRP4 polypeptide.

Like the other legume PRPs, MtPRP4 contains two distinct domains: a 22-amino acid long N-terminal putative signal peptide and a mature protein domain consisting of proline-rich pentapeptide repeats, as shown in Figure 2A. Hydropathy plots of the putative PRP4 signal peptide show the typical hydrophobic pattern found in all signal peptides, consisting of a hydrophobic core flanked by charged residues (von Heijne, 1985). The amino acid triplet Gly-20 Leu-21 Ala-22 is a perfect candidate for the signal peptide cleavage recognition site
described by Perlman and Halvorson (1983). The presence of this N-terminal signal peptide indicates that MtPRP4, like other PRPs, is probably targeted to the secretory pathway in plant cells and thus localized in the extracellular matrix.

The mature MtPRP4 polypeptide is predicted to be 527 amino acids long and is composed almost entirely of three distinct consensus pentapeptide motifs, PWEK, PWHK, and PPWK, which are repeated 50, 28, and 21 times, respectively (Figure 2B). As shown in Table 2, all three of these motifs have been identified in the deduced amino acid sequences of other PRPs (e.g., both PPVEK and PPVYK in MtPRP1 and MtPRP2; PPVHK in both DcPRP1 and MtENOD12), but their coexistence in a single polypeptide is novel. Most of the pentapeptide motifs of PRP4 are themselves arranged into two distinct decapetide repeats, PPVEKPPVK and PPVEKPPVYK (Figure 2), as is the case with the PRP2 pentapeptide motifs. Overall, these characteristics distinguish MtPRP4 from all other previously described PRPs.

MtPRP4 Is a Member of a Small Gene Family Not Closely Related to ENOD2

Genomic DNA gel blotting experiments were used to investigate the organization of PRP4-related sequences in the M. truncatula genome. Figure 3A shows strong hybridization to three HindIII fragments of 8.4, 6.5, and 3.6 kb observed when genomic DNA gel blots were probed with the coding sequences of MtPRP4. Identical DNA gel blots probed with a 3' nontranslated region probe (see Figure 1) showed strong hybridization to an 8.4-kb HindIII fragment and much weaker hybridization to a 6.5-kb HindIII fragment (Figure 3B). Only the 8.4-kb fragment contains the 2.1-kb BamHI-HindIII fragment of hMM7-1 (data not shown), indicating that the 6.5-kb HindIII fragment represents either a related gene or a different allele of MtPRP4. At high stringency, a coding region probe from MtPRP2, the M. truncatula homolog of soybean SbPRP2 (GenBank accession number L25799; Wilson and Cooper, 1994), hybridized strongly with 3.8-, 6.5-, and 8.4-kb HindIII fragments, indicating that MtPRP4 is closely related to the PRP gene family in M. truncatula (Figure 3C). This interpretation is corroborated by the identity of repetitive sequence motifs between MtPRP4 and other legume PRPs (SbPRP1 and SbPRP2, Hong et al., 1990; MtPRP1 and MtPRP2, Wilson and Cooper, 1994). In contrast, the alfalfa A2ENOD2 probe hybridized at high stringency with only a 14-kb HindIII fragment in M. truncatula genomic DNA, but showed no hybridization to 8.4-, 6.5-, or 3.6-kb fragments (data not shown). Thus, despite the fact that MtPRP4 was isolated by (low-stringency) hybridization to A2ENOD2, these data indicate that ENOD2 is not closely related to other members of the legume PRP gene family.

MtPRP4 Is a New Nodulin

In soybean, PRP genes are expressed specifically in different plant organs at different stages of development (Datta et al., 1989; Hong et al., 1989). We analyzed the expression of MtPRP4 in different plant organs using RNA gel blot experiments. Figure 4A demonstrates that a coding region probe for MtPRP4 hybridized to two transcripts in hypocotyls and three transcripts of 1.05, 1.35, and 1.65 kb observed when genomic DNA gel blots were probed with the coding sequences of MtPRP4. Identical DNA gel blots probed with a 3' nontranslated region probe (see Figure 1) showed strong hybridization to an 8.4-kb HindIII fragment and much weaker hybridization to a 6.5-kb HindIII fragment (Figure 3B). Only the 8.4-kb fragment contains the 2.1-kb BamHI-HindIII fragment of 1.1M7-1 (data not shown), indicating that the 6.5-kb HindIII fragment represents either a related gene or a different allele of MtPRP4. At high stringency, a coding region probe from MtPRP2, the M. truncatula homolog of soybean SbPRP2 (GenBank accession number L25799; Wilson and Cooper, 1994), hybridized strongly with 3.8-, 6.5-, and 8.4-kb HindIII fragments, indicating that MtPRP4 is closely related to the PRP gene family in M. truncatula (Figure 3C). This interpretation is corroborated by the identity of repetitive sequence motifs between MtPRP4 and other legume PRPs (SbPRP1 and SbPRP2, Hong et al., 1990; MtPRP1 and MtPRP2, Wilson and Cooper, 1994). In contrast, the alfalfa A2ENOD2 probe hybridized at high stringency with only a 14-kb HindIII fragment in M. truncatula genomic DNA, but showed no hybridization to 8.4-, 6.5-, or 3.6-kb fragments (data not shown). Thus, despite the fact that MtPRP4 was isolated by (low-stringency) hybridization to A2ENOD2, these data indicate that ENOD2 is not closely related to other members of the legume PRP gene family.

Table 1. PRP Amino Acid Compositions Deduced from DNA Sequence Dataa

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<tr>
<th>Amino Acid</th>
<th>MtPRP4 (62.2 kDa)</th>
<th>MtPRP1 (23.3 kDa)</th>
<th>MtPRP2 (42.0 kDa)</th>
<th>DcPRP1 (25.4 kDa)</th>
<th>SbENOD2 (35.9 kDa)</th>
<th>MtENOD12 (12.5 kDa)</th>
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<tr>
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<td>12.7</td>
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Data are presented as mole percent.

Carrot, Chen and Varner (1985a) and Ebensr et al. (1993).
Soybean, Franssen et al. (1987).
Carrot, Chen and Varner (1985b).
Table 2. Number of Repetitive Pentapeptide Motifs Found in Different PRPs

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<th>Consensus Motif</th>
<th>MtPRP4</th>
<th>MtPRP1</th>
<th>MtPRP2</th>
<th>DcPRP1</th>
<th>SbENOD2</th>
<th>PsENOD12</th>
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<tr>
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<td>-</td>
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<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>13 x</td>
<td>-</td>
</tr>
</tbody>
</table>

a —, motif not found in this PRP.

Figure 3. Organization of PRP Gene Sequences in the M. truncatula Genome.

Genomic gel blots of 10 μg of DNA restricted with EcoRI or HindIII are shown. (A) Genomic DNA probed with MtPRP4 coding region DNA showing cross-hybridization with the PRP family. (B) Genomic DNA probed with the MtPRP4 3' nontranslated region probe. (C) Genomic DNA probed with an MtPRP2 coding region probe. Lengths of hybridizing restriction fragments are shown at left (for EcoRI) and right (for HindIII).

MtPRP4 Is an Early Nodulin Expressed in Invasion-Deficient Nodules

Nodulin genes have been divided into two groups, early and late, based on the timing of gene activation during nodule development (see Gloudemans and Bisseling, 1989). Early nodulins are expressed before the onset of nitrogen fixation.
Figure 4. Organ-Specific Expression of MtPRP4 and Related Sequences.

(A) and (B) RNA gel blots containing 5 µg of total RNA isolated from hypocotyls (hypo), roots, and mature root nodules (nod) probed with MtPRP4 coding sequences or MtPRP4 3’ nontranslated sequences, respectively. The additional lane to the right in (A) represents a longer exposure of the adjacent lane containing nodule RNA.

(C) and (D) Blots were reprobed with 18S rDNA sequences to normalize loading. Transcript lengths are provided between (A) and (B).

and are therefore likely to be involved in the morphogenesis and infection of symbiotic nodules. To investigate the temporal regulation of PRP4 expression during nodule development, we used alfalfa root nodules produced by spot inoculation with R. meliloti 1021 (Dudley et al., 1987). Alfalfa was used for these experiments because M. truncatula roots were not readily amenable for spot inoculation experiments. RNA was extracted from spot-inoculated alfalfa nodules harvested 2, 4, and 6 days after inoculation, and RNA gel blots were hybridized with the MtPRP4 3’ nontranslated region probe. Figure 5A demonstrates that expression of the PRP4 transcript was detected as early as 2 days after inoculation and reached a maximum level within 4 days. In contrast, ENOD2 was not expressed until day 5 to 6 (data not shown; Pichon et al., 1992). Because nitrogen fixation commences on day 6 to 7 of nodule development in Medicago (Gallusc et al., 1991), these data indicate that MtPRP4 encodes a new early nodulin.

Because of the early induction of MtPRP4 expression by R. meliloti and the sequence homology with the MtENOD12 promoter sequences (see Discussion), we were interested in determining whether MtPRP4 gene expression requires

Figure 5. Regulation of MtPRP4 Expression during Nodule Development.

(A) RNA gel blot containing 5 µg of total RNA from spot-inoculated alfalfa nodules 2, 4, and 6 days postinoculation and from mature nodules (more than 21 days) produced by flood inoculation with R. meliloti probed with MtPRP4 3’ nontranslated sequences.

(B) RNA gel blot containing 5 µg of total RNA from nodules formed by inoculation with wild type (wt, R. meliloti 1021), ndvB− (R. meliloti TY7), and exoF− (R. meliloti 7055) probed with MtPRP4 3’ nontranslated sequences.

(C) and (D) Blots were reprobed with 18S rDNA sequences to normalize loading.
successful symbiotic infection. Figure 5B shows a comparison of MtPRP4 expression levels in nitrogen-fixing nodules formed by wild-type *R. meliloti* (strain 1021) and in ineffective nodules formed by two mutant strains reported to be defective in nodule invasion (Finan et al., 1985; Dylan et al., 1986; Finan, 1988). Nodules formed by the nodule development mutant ndvB::Tn5 (*R. meliloti* TY7) expressed MtPRP4 at high levels (Figure 5B, second lane), indicating that MtPRP4 expression is not dependent upon successful symbiotic infection. In contrast, ineffective nodules formed by the exopolysaccharide mutant exoF::Tn5 (*R. meliloti* 7055), which are also devoid of intracellular nitrogen-fixing bacteroids, showed markedly reduced MtPRP4 expression. Equal loading of RNA was verified by reprobing these blots with an 18S rDNA clone (Figures 5C and 5D).

### MtPRP4 Expression Is Highest in Nodule Meristem Cells

The tissue specificity of MtPRP4 expression in developing root nodules was investigated using in situ hybridization experiments. Sense and antisense probes consisting of 3′ nontranslated sequences of MtPRP4 were hybridized to sections of *M. truncatula* root nodules produced by inoculation with wild-type *R. meliloti*. As shown in Figure 6A, the PRP4 antisense probe hybridized at high stringency to transcripts expressed in cells throughout the interior regions of a 4-day-old emerging nodule, including both the undifferentiated primordium cells (arising from root cortical cell mitoses) and the newly forming distal meristem. In mature elongated nodules, the highest levels of PRP4 transcripts were observed in the nodule meristem (Figures 6C, 6D, and 6G). Control experiments using sense strand probes showed very low levels of background hybridization and/or alkaline phosphatase activity (Figures 6B and 6E).

Lower levels of MtPRP4 expression were consistently observed in the early symbiotic zone (or invasion zone) and in cells associated with the peripheral nodule vascular system (arrows in Figures 6C and 6D) of mature elongated nodules, and peripheral PRP4 expression superficially resembled the peripheral expression pattern reported for *ENOD2* (Van de Wiel et al., 1990; Allen et al., 1991). To directly compare the expression of these two proline-rich early nodulins, antisense probes from the 3′ nontranslated regions of *MtENOD2* and *MtPRP4* were hybridized to adjacent sections of mature nodules. As shown in Figures 6F and 6H, *ENOD2* was expressed only in the parenchyma cells surrounding the nodule vascular system. *MtPRP4* was also expressed (at low levels) in the nodule parenchyma (Figure 6G), but PRP4 expression was much higher in the vascular tissues differentiating inside this parenchyma cell layer (Figures 6G and 6I). Significant hybridization of the MtPRP4 probe was also consistently observed throughout the vascular system of roots attached to developing nodules (small arrowheads in Figures 6A, 6C, 6D, and 6G).

### DISCUSSION

We isolated and characterized a genomic clone encoding a novel member of the repetitive PRP family in *M. truncatula*. Similar to other plant cell wall PRPs, PRP4 consists of a conserved N-terminal signal peptide and a repetitive pentapeptide domain rich in proline, lysine, and tyrosine. As shown in Figure 7, signal peptide sequences are remarkably conserved among members of the legume PRP gene family and are distinct from signal peptide sequences of other hydroxy-proline-containing wall proteins (e.g., extensins). Based on this unprecedented high level of signal peptide sequence homology among the legume PRPs, we speculate that members of the PRP family are processed through a distinct secretory pathway involving specific and distinctive post-translational modifications. PRP4 is unique among the characterized legume PRPs, containing all three of the repeating consensus amino acid motifs PPVEK, PPVYK, and PPVHK. Most of these repetitive pentapeptides (98 of 99) are themselves arranged into the larger decapeptide repeats PPVEKPPVHK and PPVEKPFVYK. Protein analyses of other plant PRPs (SbPRP1, SbPRP2, and DcPRP1) have shown that 50% of the prolines are hydroxylated and that hydroxylation occurs specifically at the second proline residue in the repeating tetrameric motifs (Averyhart-Fullard et al., 1988; Kleis-San Francisco and Tierney, 1990; Lindstrom and Vodkin, 1991; Bradley et al., 1992; Millar et al., 1992). We predict that a similar hydroxylation pattern is likely to be found in the MtPRP4 protein.

As would be expected for a multigene family, hybridization probes that include the coding region of MtPRP4 hybridized to several genomic DNA fragments and several PRP transcripts (expressed in hypocotyls, roots, and symbiotic root nodules) under high-stringency wash conditions. The use of probes corresponding to the 3′ nontranslated region of MtPRP4 and high-stringency hybridization conditions minimized cross-hybridization to other genomic fragments (strong hybridization to the 8.4-kb HindIII fragment of genomic DNA containing the sequenced MtPRP4 gene and weak hybridization to an uncharacterized 6.5-kb HindIII fragment that probably represents a related PRP gene; Figure 3) and allowed hybridization only to a 2.35-kb transcript expressed specifically in root nodules (Figure 4). Expression of this nodule-specific transcript was detected very early in nodule development and was independent of nodule invasion (Figure 5 and data not shown). As shown in Figure 8, the MtPRP4 5′ promoter sequences show about 70% homology with the upstream promoter sequences of MtENOD12, a different proline-rich early nodulin expressed within 2 days of inoculation (Pichon et al., 1992). Both of these early nodulin promoters contained sequences identical to the Goldberg-Hogness TATAA element (Shenk, 1981), and MtPRP4 also contained the CCAAT element (Espositi et al., 1980) found in many eukaryotic genes (underlined in Figure 8). These regulatory elements were 60 and 110 bp upstream of the AUG.
Figure 6. Localization of MtPRP4 Transcripts in Developing Root Nodules.

Root nodules were sectioned and hybridized with digoxigenin-labeled single-stranded RNA probes and washed at high stringency. Probes were localized by immunocytochemistry using alkaline phosphatase–conjugated digoxigenin-binding Fab fragments.

(A) and (B) Bright-field photomicrographs of adjacent longitudinal sections of a 4-day-old M. truncatula root nodule hybridized with antisense (A) and sense (B) PRP4 probes showing PRP4 expression in both the differentiating nodule meristem and throughout the primordial cells of the emerging nodule.

(C) to (E) Bright-field photomicrographs of mature, elongated M. truncatula root nodules hybridized at high stringency with antisense (C) and (D) and sense (E) PRP4 probes showing the highest levels of PRP4 expression in the distal nodule meristem.

(F) to (I) Bright-field photomicrographs of adjacent sections of mature, elongated M. truncatula root nodules hybridized at high stringency with antisense MtENOD2 probes (F) and (H) and MtPRP4 probes (G) and (I). Details in (G) and (I) indicate that ENOD2 expression was limited to the parenchyma cells surrounding the differentiating vascular system, whereas PRP4 expression included both the parenchyma cells and the differentiating vascular tissues inside this parenchyma cell layer.

Bars represent 0.4 mm for all micrographs. np, nodule primordium; m, nodule meristem; nc, nodule cortex; es, early symbiotic zone; sz, bacteroid-filled cells in the symbiotic zone; vb, vascular bundle; p, nodule parenchyma. Arrows in (C) and (D) indicate PRP4 expression associated with the peripheral vascular system of elongated nodules. Small arrowheads in (A), (C), (D), and (G) indicate expression associated with the vascular system of attached roots.
initiation codon, respectively. The functional significance of these sequence elements remains to be tested.

In situ hybridization experiments using high-stringency conditions indicated that MtPRP4 was expressed throughout the undifferentiated inner regions of young emerging root nodules and in the small dividing cells of the differentiating nodule meristem. In mature nodules, the highest levels of PRP4 expression were in the distal nodule meristematic region. RNA gel blots showed that maximal levels of MtPRP4 expression were obtained after 4 days, correlating with the initial organization of the nodule meristem. These spatial and temporal patterns of gene expression are consistent with a role for PRP4 in the establishment of the indeterminate nodule meristem. Also consistent with this hypothesis are the lower levels of MtPRP4 expression observed in nodules formed by exopolysaccharide mutants (Figure 5) that are reported to lack the PRP4 expression observed in nodules formed by exopolysaccharide mutants. In situ hybridization and washing conditions (Meyerowitz, 1987; Smith et al., 1987) indicated that PRP4 transcripts were primarily localized in the nodule cortex (data not shown). The unique hybridization pattern shown in Figure 6 was consistently observed (29 elongated nodules) using high-stringency wash conditions designed to minimize cross-hybridization. Because a cortical expression pattern was never observed using high-stringency conditions, we speculated that the standard in situ conditions allowed cross-hybridization of this PRP4 probe to distantly related transcripts. In this context, we note that the 3' nontranslated region of MtPRP4 is highly homologous with the 3' regions of both MtPRP1 and MtPRP2 (69 and 75% identity, respectively) (Wilson and Cooper, 1994) and that the MtPRP4 nontranslated region probe hybridized to at least five additional transcripts in an RNA gel blot experiment performed using the low-stringency conditions normally used for in situ hybridization experiments (final wash was 0.12 M Na\(^+\) at room temperature; R.C. Wilson, unpublished data).

The RNA gel blot data shown in Figure 4 also indicated that expression of different members of the legume PRP gene family was coordinately regulated during nodule development. Specifically, expression of the two abundant root PRP transcripts was down-regulated in mature nodules, while expression of the nodule-specific PRP4 gene was induced. Constitutive PRPs are rapidly cross-linked during pathogen attack and are thus thought to play a role in plant defense (Bradley et al., 1992). Our data support the hypothesis that the successful infection of developing nodules by *Rhizobium* may depend on an attenuated or modified defense response as has been proposed

Figure 7. Sequence Comparison of PRP Signal Peptides.

N-terminal amino acid sequences from several cell wall structural PRPs, proline-rich early nodulins, and cell wall extensins are compared. a, Han et al. (1990); b, Scheres et al. (1990); c, Pichon et al. (1992); d, Franssen et al. (1987); e, Chen and Varner (1985b); f, Evans et al. (1990); g, Corbin et al. (1987).

**Figure 8.** Comparison of MtPRP4 and Mienod2 Promoter Sequences.

The proximal 5' upstream promoter sequence of MtPRP4 is aligned with the analogous *Mienod2* promoter sequence from Pichon et al. (1992). The boldface lettering indicates the protein coding region. The TATAA and CCAAT boxes are underlined. Vertical lines indicate sequence identity.
previously (Vance, 1983; Werner et al., 1985; Estabrook and Sengupta-Gopalan, 1991; Niehaus et al., 1993; Perotto et al., 1994).

We suggest that the changing pattern of cell wall gene expression induced by Rhizobium reflects a significant reorganization of the host extracellular matrix architecture in early nodule development. Despite the lower expression levels of *MtPRPl* and *MtPRP2* during nodule development, these two (root) genes were still expressed at higher levels than *MtPRP4* in mature nodules. Thus, the extracellular modifications taking place as quiescent root cortical cells dedifferentiate and reorganize to form the nodule primordium, and as *Rhizobium* cells invade host tissues, may be subtle. PRPs are thought to form covalently cross-linked networks, but the structural properties and the functional significance of such networks are not yet known. Perhaps low levels of nodule-specific PRPs function to regulate the molecular architecture of PRP networks in a manner that allows both new developmental fates for root cells and infection by *Rhizobium*. Further investigation of the structure and regulation of legume PRPs will provide a more complete picture of the involvement of PRP genes in developing symbiotic root nodules and in plant development in general.

**METHODS**

**Biological Materials**

Seeds of *Medicago truncatula* cv Jemalong (Purkiss Seeds, Newcastle, Australia) and *M. sativa* cv GT13 (Ferry Morse Seed Co., Mountain View, CA) were surface sterilized (35 min in 70% EtOH followed by 35 min in 5.25% hypochlorite), rinsed thoroughly with sterile H2O, and imbied overnight. Imbied seeds were germinated for 22 to 24 hr on inverted 0.8% agar plates and planted on Petri plates containing NO3, a buffered nodulation medium (2 mM CaSO4, 1 mM MgSO4, 0.5 mM KH2PO4, 1 mM 2-[N-morpholino]ethanesulfonic acid, pH 6.5, and trace elements of Murashige-Skoog medium salts lacking KI). Alternatively, seeds were primed (Berger and Kimmel, 1987). Ligation products were transformed into *E. coli* XL-1Blue competent cells. Nested deletions were generated using exonuclease III and S1 nuclease according to Henikoff (1987), and nucleotide sequencing was performed using dideoxy chain termination sequencing kits (United States Biochemical Corp.). Complete sequence data were obtained from both DNA strands and analyzed using University of Wisconsin Genetic Computer Group (Madison, WI) software run on a Silicon Graphics workstation.

**Expression Analyses**

*M. truncatula* total RNA was prepared from leaves, hypocotyls, uninoculated roots, and mature nodules using a protocol modified from Maniatis et al. (1989). Plant tissue was harvested, frozen on dry ice, and stored at −80°C until use. Frozen tissue was ground to a powder under liquid nitrogen, warmed to −20°C for at least 15 min, and dispersed into 4 M guanidinium thiocyanate/1% β-mercaptoethanol using a polytron. Poly(C)* RNA was prepared from total RNA using magnetic oligo(dT) beads (Dynal A.S., Oslo, Norway). Riboprobes for RNA gel blot analyses were prepared using an in vitro transcription kit (Stratagene). Five micrograms of total RNA from each organ was subjected to electrophoresis on 1.2% formaldehyde-Hepes agarose gels (Maniatis et al., 1989). An RNA ladder (0.24 to 95 kb; Bethesda Research Laboratories-GIBCO) was used for size markers. Fractionated RNA was transferred to a GeneScreen hybridization transfer membrane, UV cross-linked for 15 sec, and baked at 80°C for 30 to 60 min in vacuo. Filters were stained with methylene blue to evaluate equivalent loading, and hybridizations were performed in the presence of 50% formamide and 6 x SSPE at 60°C for at least 24 hr. Blots were washed at 68°C with 0.2 x SSPE, 0.1% SDS, and exposed to x-ray film at −80°C with an intensifying screen.

**In Situ Hybridization Experiments**

In situ hybridization experiments were performed essentially as described by Meyerowitz (1987) and Smith et al. (1987). Tissues were fixed in 20 mM KPi, 20 mM KCl containing 1% glutaraldehyde at 0°C for 4 hr, dehydrated through an EtOH series, and embedded in Paraplast (Oxford Labware, St. Louis, MO). Tan-micron sections taken with a sliding microtome (American Optical, New York, NY) were dried onto Vectabond coated slides (Vector Laboratories, Burlingame, CA) at 55°C for at least 1 hr. To prepare for hybridization, slides were deparaffinized.
with toluene, rehydrated through an ETOH series, rinsed with 1 x PBS (Maniatis et al., 1989), treated with proteinase K (1 mg/mL in 20 mM Tris, 2 mM CaCl₂, pH 7.5) for 20 min at 37°C, rinsed in PBS, dehydrated through an ETOH series, and air dried. Sections were hybridized overnight at 55°C with digoxigenin-labeled RNA probes in 50% formamide, 100 mg/mL dextran sulfate, 0.6 M NaCl, 20 mM Tris, pH 7.6, 1 mM EDTA, 1 x Denhardt's solution (0.02% Ficoll, 0.02% PVP, 0.02% BSA), 100 mM DTT, 0.5 mg/mL poly(A) RNA, and 0.5 mg/mL yeast tRNA. Probes were produced by in vitro transcription of linearized plasmid DNA with T3 or T7 polymerase, using the Stratagene kit. Following hybridization, slides were washed with 2 x SSPE, treated with RNase A (50 µg/mL in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA) for 30 min at 37°C, washed with RNase buffer (30 min, 37°C), 2 x SSPE (30 min, room temperature), 1 x SSPE (30 min, room temperature), 0.1 x SSPE (30 min, 55°C), and rinsed in TBS (100 mM Tris, 150 mM NaCl, pH 7.5) for 5 min at room temperature. Sections were blocked with 2% sheep serum (Sigma) 0.3% Tween 20 in TBS, and incubated overnight at 4°C with digoxigenin-binding Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1:1000 in TBS containing 1% sheep serum and 0.1% Tween 20. Alkaline phosphatase activity was visualized using 5-bromo-4-chloro-3-indolyl phosphate (525 µg/mL) and 4-nitro blue tetrazolium chloride (675 µg/mL) in 50 mM MgCl₂, 150 mM NaCl, 100 mM Tris, pH 9.5, containing 10% polyvinyl alcohol (Sigma), as suggested by De Block and Debrouwer (1993), and photographed using a Zeiss Axiolab microscope (Cari Zeiss, Thornwood, NY).

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