Symbiosis-Specific Expression of Two *Medicago truncatula* Nodulin Genes, *MtN1* and *MtN13*, Encoding Products Homologous to Plant Defense Proteins

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Two *Medicago truncatula* nodulin genes putatively encoding proteins structurally related to two classes of proteins commonly associated with plant defense reactions have been characterized. *MtN1* is homologous to two small, cysteine-rich, pathogen-inducible proteins from pea (pI39 and pI230), whereas *MtN13* is closely related to the PR10 family of pathogenesis-related proteins. We show that neither *MtN1* nor *MtN13* is induced in leaves in response to pathogenic bacteria, and that both are exclusively expressed during nodulation. In situ hybridization experiments as well as Northern (RNA) studies of interactions between *M. truncatula* and either wild-type *Rhizobium meliloti* or mutants deficient in infection establish that *MtN1* is associated with the infection process, while *MtN13* represents the first specific marker described for the nodule outer cortex. Possible roles for *MtN1* and *MtN13* are discussed. We also present the identification of another member of the PR10 family, designated as *MtPR10-1*, whose regulation is strikingly different from that observed for *MtN13*, being constitutively expressed in roots and pathogen-inducible in leaves.

Additional keyword: gamma-thionin.

Endophytic symbioses are plant-microbe interactions in which the microorganism internally colonizes the host plant to the mutual benefit of both organisms. Rhizobia are soil bacteria that have the unique capacity to elicit on legumes the development of a novel organ, the nodule, in which they fix atmospheric dinitrogen.

Nodule organogenesis takes place through a series of developmental stages. The relationship between the symbionts starts with a molecular dialogue involving activation of rhizobial *nod* gene expression by plant-secreted inducers and resulting in the synthesis of specific lipo-chitooligosaccharides, the Nod factors (for recent reviews see Dénarié et al. 1996; Long 1996; Schultze and Kondorosi 1996; Spaink 1996). Nod factors are able to elicit most of the morphogenetic responses that are induced by the bacteria themselves (for reviews see Hirsch 1992; Kijne 1992).

In temperate legumes such as pea or alfalfa, the bacteria penetrate the root by means of an infection thread, a tubular structure that grows inside the root hair, and down toward the nodule primordium that forms in the inner cortex. Simultaneously, a second division center develops in the middle cortex to form the nodule meristem. In alfalfa, the meristem remains active for several weeks, resulting in the development of an indeterminate nodule through which a longitudinal section reveals the entire developmental pathway along the nodule axis. Histologically, a nodule comprises central and peripheral tissues. Central tissues are subdivided into zones, from the distal meristematic zone I and passing consecutively through the prefixing zone II (infection zone), the amyloplast-rich interzone II-III, the nitrogen-fixing zone III, and finally the proximal senescent zone IV (Vasse et al. 1990). Peripheral tissues include the nodule parenchyma, nodule vascular bundles, the nodule endodermis, and the nodule cortex (Vasse et al. 1990).

Nodulation involves specific expression of certain bacterial and plant genes, as well as the coordinated differentiation of the two symbionts. Plant genes that are preferentially expressed before the onset of nitrogen fixation are designated as early nodulin genes (Van Kammen 1984) and are postulated to play a role in morphogenetic and organogenetic plant responses. Whereas the exact functions of most remain elusive, early nodulin genes have proved to be useful as molecular markers to define cell- or tissue-specific responses (for review, see Pawlowski and Bisseling 1996). By differential screening of a cDNA library made from immature *Medicago truncatula* root nodules, we have recently identified 29 new *M. truncatula* nodulation-related genes, termed *MtN1* to *MtN29* (Gamas et al. 1996). In this paper, we have characterized two of these genes, *MtN1* and *MtN13*, that encode putative proteins structurally related to two classes of defense proteins. These genes were found to be differentially expressed during nodulation, with the expression of *MtN13* being associated with the differentiation of the nodule outer cortex, whereas *MtN1* expression appears to be related to infection. The possible roles of these two genes are discussed.

RESULTS

The *MtN1* putative protein is homologous to two pathogen-induced pea proteins.

Initial characterization of *MtN1* showed that its expression is induced approximately 48 h after *Rhizobium meliloti* in-

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Nucleotide and/or amino acid sequence data are to be found at the EMBL data bank as accession nos. Y10456 (*MtN1*), Y10455 (*MtN13*), and Y08641 (*MtPR10-1*).

oculation, and that its transcripts are abundant in *Rhizobium*-induced immature root nodules. Partial sequencing of the clone did not reveal any homologies with known proteins (Gamas et al. 1996).

In this study we have completed the sequence determination of the *MtN1* cDNA (Fig. 1). The longest open reading frame (ORF) covers only 222 nucleotides of the 922 nucleotides. The Southern analysis shown in Figure 2 suggests that *MtN1* belongs to a small gene family, which could explain the existence of several cross-hybridizing mRNA species (Gamas et al. 1996). Data base interrogation (Table 1) revealed homologies between the MtN1 putative protein and two pea proteins, pI39 and pI230, whose corresponding mRNAs accumulate in response to the pathogen *Fusarium solani* (Chiang and Hadwiger 1991). A comparison of the noncoding regions did not show any similarity between the *MtN1* cDNA and the two pea sequences (data not shown). The MtN1, pI39, and pI230 proteins are very similar in size (73, 72, and 74 amino acids, respectively). A comparison of their primary sequences showed limited homologies between MtN1 and the pea proteins, as reflected by the modest statistical significance obtained with the BLASTX interrogation (Table 1). However, alignment of these proteins with programs allowing gaps to be introduced (such as gap or Multalin) revealed 67% similarity (39% identity) between MtN1 and pI230 (with three gaps), and 55% similarity (32% identity) between MtN1 and pI39 (with two gaps) (Fig. 3). Analysis of randomized alignments (randomizations = 100; gap program) indicated that these similarities are statistically significant (data not shown). The two pea proteins are related to the gamma-thionins (Kragh et al. 1995), a subfamily of low-molecular-weight, cysteine-rich proteins

(for a review, see Florack and Stiekema 1994). Weak homologies between MtN1 and wheat and barley gamma-thionins were detected by a BLASTX interrogation (Table 1). Alignments with gap and Multalin (Fig. 3, and data not shown) confirmed that MtN1 is more closely related to pI39 and pI230 than to sensu stricto gamma-thionins. The N terminus, which is strongly conserved in MtN1 and in the two pea proteins, has the features of a signal peptide, as commonly observed in thionins. The primary sequence of MtN1 downstream of the signal peptide is not very homologous to the two pea sequences, with the important exception that eight cysteine residues are found in the three proteins, at similar, but not strictly conserved, positions (Fig. 3). It is also striking that the hydrophobicity profiles are quite close for MtN1, pI39, and pI230 (Fig. 3). These observations suggest that the protein conformations are more conserved than the primary sequences. In terms of amino acid composition, it can be noted that MtN1 is more basic than pI230 and pI39 (isoelectric points of 7.79, 7.47, and 6.46, respectively, for mature proteins), and that cysteine, glycine, and basic residues are very abundant, as in pI230 and in the wheat and barley gamma-thionins.

MtN13 and MtPR10-1 are two members of the PR10 family.

Initial studies of *MtN* clones (Gamas et al. 1996) showed that the MtN13 putative protein was homologous to DRRG49-C, a pea protein whose gene expression is induced by the pathogen *F. solani* (Chiang and Hadwiger 1990), and more generally to the PR10 proteins, a large family of acidic, intracellular, pathogenesis-related proteins also including different allergens such as Bet v 1 (Van Loon et al. 1994). Since then,

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CGGCACGAGTTTTAGAAAGACAAAAAAGGTGATGGAGAGGAAACACTTGCTAGCTTGTG 60
G T S F R R Q K K V M E R K T L A S L C

CTTCTTTCTCATTGTTCTCTTAGCTGCTCAAGTAGTGGCGCAAATTGTGCCTTGTAAGAC 120
F F L I V L L A A Q V V A Q I V P C K T

ACGAAATCGTAACTTCAAGAGTGCATGTATTGCTGTATCTGGCGACAACGAAGAATGTGA 180
R N R N F K S A C I A V S G D N E E C D

CCATGATTGCAGGAGAGTAGGAGGCTGGTATGGTGGTTCTTGCAAAAATCAGAAATGTGT 240
H D C R R V G G W Y G G S C K N Q K C V

TTGTGACTGTTTGAACATCTCTACATAAAATAAATAATCTAGTTATAGTATGATTCATAGC 300
C D C *

TAGATTATGTTTTATGGAATAAATCATTGATCAATTCTCTTCCCTTGAGTGAGAAGAGAAT 360
TAATTAATATATAGAGTAATATGTGTGACAAGATAGCATGTTGTCTTATTAATAAACCG 420
AAATGATTATCATAGCAAGACATTGCATCGTGAGGATTTCCAGAATGATGTGGCAACATA 480
ATATCTTTTATTAAATTGGCTGAATAGTGTTCGCGGCCGTTTCACGATTTGAGGAATGCT 540
AGGGTCACACCCCTTAACCTCACCATTTTAACACACTCCAATAGGTCACCTCATCCAATTTT 600
TTATTTTCTTTTGGATTAAATTTGGGTGAAAAGCGACAATTCCTTCGGGACATACAAGAA 660
CTTGGTTTTTGGAGAAGGTCGAGCGAAGAAGATAGATATTAACTCCCAAAAATAAAAAAT 720
AAAATAAATGTTTCATAAAGTGAAACCGGTAGCTGTAAAGAAAATAAAAAAGTGGACTACA 780
TGGCTTATCTTGATTGGAGTGATTTAAAGGGTATGTTAGAAGGTGTGACCCTAGCATTC 840
CTCTTCATGATTTTTACCTTGGGATGTATAGCATCGCTGTTAAATAAAATATGTGTTAA 900
TTAAAAA 922

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Fig. 1. cDNA and translated sequence of the *MtN1* clone. Start and stop codons, as well as putative polyadenylation sites, are underlined. Longest detected putative protein sequence is underlined, showing a signal peptide (dotted line) and eight cysteine residues (bold characters, doubly underlined) in the mature protein.

we have identified an *M. truncatula* root cDNA, picked up as a control clone not induced by *R. meliloti*, and also found to belong to the PR10 family (Table 1). This cDNA contained a 474-nucleotide ORF, encoding a putative protein almost identical to two *M. sativa* proteins, MsPR10-1 (99% amino acid identity; Breda et al. 1996) and Srg1 (98% amino acid identity; Truesdell and Dickman 1997). It was thus designated as *MtPR10-1*.

A comparison of the DNA sequences of *MtN13* and *MtPR10-1* showed that the conservation at the nucleotide level is very limited (data not shown), thus explaining the absence of cross-hybridization during the screening procedure. A Southern analysis of the *M. truncatula* genome with *MtN13* and *MtPR10-1* probes (Fig. 2) confirmed the absence of cross-hybridization and suggested that these two genes do not belong to large, multigene families.

MtN13 and *MtPR10-1* putative proteins were found to be similar in size (163 and 157 residues, respectively, with calculated molecular masses of 18.1 and 16.6 kDa) and charge (pI of 4.4). The PSORT program predicts that both proteins are cytoplasmic (certainty of 0.45). BLASTX interrogation clearly established that the *MtN13* and *MtPR10-1* putative proteins were homologous to the same set of PR10 proteins but with different levels of similarity (Table 1, and data not shown). A sequence alignment of *MtN13* and *MtPR10-1* with different members of the PR10 family showed that the overall amino-acid conservation was quite high (Fig. 4). This analysis also included two partial sequences of an 18-kDa ribonuclease purified from ginseng calluses, found to be homologous to

PR10 proteins (Moiseyev et al. 1994). It should be noted that these RNase fragments were more homologous to *MtN13* and *Betula verrucosa* Bet v 1J than to the other PR10 proteins analyzed in Figure 4. Interestingly, the Bet v 1 family is a group of highly homologous proteins, including pathogen-inducible proteins (Swoboda et al. 1995), which has recently been reported to exhibit RNase activity in vitro (Bufe et al. 1996; Swoboda et al. 1996).

Expression of *MtN1* and *MtN13* during nodule development.

In a previous study, we observed by Northern (RNA) analysis that *MtN13* mRNA increased in abundance approximately 2 days later than *MtN1* following inoculation by *R. meliloti* (Gamas et al. 1996). Further characterization of *MtN1* and *MtN13* established that transcripts for these two genes were not detectable by Northern analysis in stems, leaves, flowers, or roots of noninoculated *M. truncatula* plants (Fig. 5A). In contrast, *MtPR10-1* was found to be actively transcribed in roots and weakly in leaves and nodules.

To determine the tissue specificity of *MtN1* and *MtN13* expression during nodule development, we performed in situ hybridization on sections of developing nodules (6 days old) and nitrogen-fixing nodules (2 or 4 weeks old). The probes used were ³⁵S-radiolabeled, anti-sense *MtN1* and *MtN13* riboprobes. *MtN1* transcripts were detected uniquely in the region of the nodule where infection threads were observed (Fig. 6A–C). Thus, in developing nodules, a strong signal was seen in the central region that is almost entirely occupied, at this

Fig. 2. Southern analysis of *Medicago truncatula* genomic DNA with *MtN1*, *MtN13*, and *MtPR10-1* ³²P-labeled probes. Five micrograms of genomic DNA, hydrolyzed with the indicated restriction enzymes, was loaded per lane. Left lane shows a radiolabeled DNA size ladder (GIBCO-BRL, Life Technologies, Cergy Pontoise, France).

stage, by an abundant network of infection threads (Fig. 6A and B). In fully differentiated mature nodules, *MtN1* transcripts occurred exclusively in the distal part of prefixing zone II (Fig. 6C), a region adjacent to the meristem where infection threads develop and where rhizobia are released into the plant cytoplasm (Vasse et al. 1990). There was no evidence of a reduction in gene transcription in this zone during nodule maturation, as judged by the intensity of labeling in the young and mature nodules (compare Fig. 6A and C). No signal was observed in any of the other central or the peripheral nodule tissues (Fig. 6C).

MtN13 mRNA was specifically located in the nodule cortex, the outer tissue that surrounds the entire nodule and makes the interface with the rhizosphere (Vasse et al. 1990). Transcripts were observed at an early developmental stage, i.e., when the nodule was still enclosed in the root cortex (Fig. 6E) or had just emerged at the root surface (Fig. 6F). *MtN13* transcription continued as the nodule matured. In nitrogen-fixing nodules, the highest signal was seen in the cortical cells surrounding the apical meristem (Fig. 6G), while a significant decrease in labeling was observed in the cortex bordering the sides of the nodule (Fig. 6G). It is worth mentioning that neither *MtN1* nor *MtN13* transcripts were detected within axenically grown control roots, including sites of lateral root initiation (Fig. 6E and data not shown), and that the specificity of the hybridizations described above was confirmed with sense probes, which generated only low, even backgrounds for both genes (Fig. 6D and data not shown).

Table 1. Sequence homologies detected for *MtN1*, *MtN13*, and *MtPR10-1* clones

Protein	Four best homologies ^a	Probability ^b
MtN1	Pea disease resistance response protein 39 precursor (<i>Pisum sativum</i>) (Q01784)	2.8 10 ⁻⁴
	Pea disease resistance response protein 230 precursor (<i>Pisum sativum</i>) (Q01783)	1.9 10 ⁻³
	Gamma hordothionin (<i>Hordeum vulgare</i>) (P20230)	0.54
	Gamma-2 purothionin (<i>Triticum turgidum</i>) (P201159)	0.65
MtPR10-1	MsPR10-1 (<i>Medicago sativa</i>) (X98867)	2.9 10 ⁻¹⁰⁵
	Srg1 (<i>Medicago sativa</i>) (U42752)	1.7 10 ⁻¹⁰⁴
	Pea disease resistance response protein DRRG49-C (<i>Pisum sativum</i>) (P27047)	3.9 10 ⁻⁸⁷
	Pea disease resistance response protein pl49 (<i>Pisum sativum</i>) (P14710)	3.9 10 ⁻⁸⁷
MtN13	Major allergen Cor a1 (<i>Corylus avellana</i>) (Z72440)	7.6 10 ^{-44c}
	Pathogenesis-related protein (<i>Cicer arietinum</i>) (S47140)	2.6 10 ⁻⁴³
	DRRG49-C (<i>Pisum sativum</i>) (P27047)	2.6 10 ⁻⁴³
	Major pollen allergen Bet v 1 (<i>Betula pendula</i>) (P43183)	9.2 10 ⁻⁴³

^a Found with BLASTX interrogations (Altschul et al. 1990) of nonredundant data bases, using default parameter values.

^b Probability of finding the indicated homologies by chance.

^c For comparison, homologies between MtN13 and pea RH2, alfalfa MsPR10-1, and *M. truncatula* MtPR10-1 putative proteins were assigned BLASTX probabilities of 1.6×10^{-41} , 7.1×10^{-28} , and 6×10^{-27} , respectively. In addition to the proteins reported in this table, more than 100 proteins were found to have significant homologies with MtN13 and MtPR10-1 putative proteins.

Expression of *MtN1* and *MtN13* in *M. truncatula* inoculated with rhizobial mutant strains or treated with Nod factors.

Having determined the pattern of expression of *MtN1* and *MtN13* in the wild-type interaction, we used Northern analysis to further compare the regulation of these genes in *M. truncatula* roots either inoculated with *R. meliloti* mutant strains or treated with Nod factors, and in *M. sativa* NAR nodules that develop in the absence of rhizobia (Truchet et al. 1989). Four mutants were used whose modified symbiotic properties have been previously described on *M. sativa*. The first two mutants, *nodA* and *nodH*, neither infect nor induce any microscopically visible response (Debellé et al. 1986; Roche et al. 1991). The third strain, a double mutant *nodF/nodL*, does not infect or nodulate, but elicits a strong activation of the epidermal and cortical cells (Ardourel et al. 1994). The fourth was an *exoA* mutant, which induces the formation of small, bacteria-free nodules with infection threads being aborted in the root hairs (Yang et al. 1992). In the time scale considered in this study these mutant strains elicited responses in *M. truncatula* similar to those described in *M. sativa* (data not shown).

We found that neither *MtN1* nor *MtN13* was expressed in roots following inoculation by the noninfective and non-nodulating *nodA*, *nodH*, and *nodF/nodL* mutants (Fig. 5B). In contrast, in roots inoculated with the *exoA* mutant, expression of *MtN13* was clearly observed, while a very low signal was detected for *MtN1* on over-exposed autoradiographs (data not shown). These data are consistent with the hypothesis that expression of *MtN1* and *MtN13* is associated with, respectively, the infection process and the differentiation of the nodule. Moreover, these correlations were also supported by two other observations: (i) a homologue of *MtN13*, but not of *MtN1*, was expressed in *M. sativa* NAR nodules (Fig. 5B); and (ii) neither *MtN13* nor *MtN1* was induced by Nod factors in *M. truncatula*, following 6 h to 15 days of treatment (data not shown; see also Gamas et al. 1996). It should be mentioned that in *M. truncatula* Nod factors are unable to elicit nodule formation although they do activate epidermal and cortical cells (data not shown; see also Gamas et al. 1996).

MtN1, *MtN13*, and *MtPR10-1* display different patterns of expression in pathogenic interactions.

To determine whether these genes could be transcribed in response to plant pathogens, we studied their expression in leaves of *M. truncatula* infiltrated with *Pseudomonas syringae* pv. *pisi* or with *Xanthomonas campestris* pv. *alfalfae*, two pathogens that have been reported to induce, respectively, a hypersensitive reaction or a compatible interaction in alfalfa (Esnault et al. 1993). We first verified that *M. truncatula* responded to these pathogens in a manner similar to that of *M. sativa*. Visible leaf lesions appeared approximately 6 and 20 h after *P. syringae* and *X. campestris* infection, respectively, leading to localized necrosis with *P. syringae* and diseaselike symptoms with *X. campestris* (data not shown). A molecular marker of pathogenic interactions, the chalcone synthase *CHS1* gene (Breda et al. 1996; Esnault et al. 1993), was found to be induced in *M. truncatula* in a manner similar to that in alfalfa (Fig. 5C and data not shown).

Leaf RNA analysis from two independent experiments with different inoculums revealed that *MtPR10-1* was strongly induced by both pathogens, while neither *MtN1* nor *MtN13* ap-

peared to be induced (Fig. 5C and data not shown). It is worth mentioning that (i) the infiltration process in itself transiently induced *MtPR10-1* expression, as suggested by the analysis of water-treated leaves (Fig. 5C and data not shown), and (ii) a moderate *MtPR10-1* induction was also detected in leaves after wounding (data not shown). Neither *MtN1* nor *MtN13* expression was observed in any of these conditions (data not shown).

DISCUSSION

In this paper, we report an analysis of three *M. truncatula* cDNA clones, *MtN1*, *MtN13*, and *MtPR10-1*, that putatively encode proteins showing homologies with proteins commonly associated with defense responses. Two of them, *MtN1* and *MtN13*, are expressed specifically during nodulation, while the third, *MtPR10-1*, is expressed in roots and in pathogenic interactions in leaves. We have been particularly interested in

studying the two former genes, which encode two novel types of nodulins.

Homologies between *MtN1* and *MtN13* and defense-related proteins.

Data base interrogation revealed that *MtN13* and *MtN1* have homologies to two distinct classes of defense-related proteins in plants. The strongest homologies were found between the deduced amino-acid sequence of *MtN13* and numerous members of the PR10 family, a large and ubiquitous family comprising many pathogen-induced proteins, such as MsPR10-1 in alfalfa (Breda et al. 1996) and DRRG49-C in pea (Chiang and Hadwiger 1990). The pattern of expression of *MtN13* appears to be quite novel for this family since *MtN13* transcripts were not detected in roots or in pathogen-treated leaves, and were found to be strictly correlated with nodule development. In contrast, *MtPR10-1* displayed an expression pattern similar to that observed for other PR10 genes, being

Fig. 3. Structure of *MtN1* and related proteins. Top panel: sequence alignments between deduced sequence of *MtN1* and of the two pathogen-induced preproteins p1230 and p139 from pea, and the protein sequence of two purified, mature gamma-thionins (barley gamma-hordothionin and wheat gamma-2 purothionin). This alignment was generated with the Multalin program with default parameter values. Dark shading indicates homologies with the two gamma-thionins; light shading corresponds to homologies restricted to the pea proteins. Homologies are defined here as identities and similarities with a score equal or superior to +2 in the Multalin alignment matrix (blosom 62). Bottom panel: hydrophobicity profiles of *MtN1*, p1230, and p139 preproteins, obtained with the GCG pepplot program, using the Kyte and Doolittle (1982) measure and a window of nine residues. An overall view of the primary structure of the three proteins is also presented, with an emphasis on the spacing of cysteine residues.

pathogen-inducible in leaves and expressed in roots. Sequence alignment showed that MtPR10-1 and, to a lesser extent, MtN13 are closely related to *M. sativa* MsPR10-1 and to *P. sativum* DRRG49-C proteins. These data strongly suggest that *M. truncatula* MtN13 and MtPR10-1 genes are related. However, it should be emphasized that these two genes have diverged, at the DNA level, to such an extent that no cross-hybridization could be detected by Northern or Southern blots. The divergence between the two genes is consistent with a scenario in which a gene duplication event has allowed independent evolution and regulation of the two sequences, one being specifically recruited for nodule development. This would be reminiscent of data showing that two non-cross-hybridizing sets of hemoglobin genes exist in soybean, one encoding the nodular leghemoglobin, and the other a hemoglobin expressed in non-symbiotic tissues (Andersson et al. 1996).

The MtN1 putative protein does not exhibit as strong a homology with known defense-related proteins as MtN13. Nevertheless, it shares several structural features with two pea proteins, pI39 and pI230, that accumulate in response to compatible and incompatible races of the fungal pathogen *F. solani*. All three proteins are predicted to have a similar molecular weight, a signal peptide, eight cysteine residues with similar spacings, and a very similar hydrophobicity profile. Such similarities raise the possibility that MtN1 and the pea proteins pI39 and pI230 share common functional properties, although additional studies are required to assess the importance of nonconservation of many of the amino acid residues.

MtN1 and MtN13 are nodulation-related genes.

The results presented in this paper strongly suggest that the expression of *MtN1* and *MtN13* is strictly restricted to developmental events specific to root nodulation. Therefore, these genes can be considered nodulin genes (Van Kammen 1984).

MtN1, which is induced significantly earlier than *MtN13*

(about 24 h after infection versus approximately 3 days for *MtN13*; Gamas et al. 1996), seems to be related to the infection process. Thus, mRNA is not detected in conditions in which infection does not occur (axenically grown plants, plants either inoculated with noninvasive *Rhizobium* mutants or treated with Nod factors, *M. sativa* NAR nodules), while a very low expression is detected in plants inoculated with the poorly invasive *exoA* mutant. Moreover, in the wild-type association, in situ hybridization has shown that this gene is specifically expressed in the infection part of prefixing zone II. The observation that the intensity of the signal in this zone did not vary significantly in nodules collected at different stages of development indicates that the signal decrease noticed by Northern analysis as the nodule ages (Gamas et al. 1996) is likely due to a dilution effect.

Previous studies have already resulted in the cloning and characterization of pea and alfalfa early nodulin genes that are expressed in the infection zone of the nodule, such as *ENOD12* (Allison et al. 1993; Pichon et al. 1992; Scheres et al. 1990), *ENOD5* (Scheres et al. 1990), and *ENOD10* (Löbner and Hirsch 1993). The proteins encoded by these genes are (hydroxy)proline-rich proteins (PRPs) with an N-terminal signal peptide sequence and they are suspected to be localized in the cell wall. Our sequence data show that MtN1 is an infection-related protein structurally unrelated to this PRP family.

Several early nodulin genes have already been shown to be expressed in noninvaded peripheral or central nodule tissues, such as *ENOD2* in the nodule parenchyma (Van de Wiel et al. 1990) or *MtPRP4* in the nodule meristem (Wilson et al. 1994). To our knowledge, *MtN13* is the first nodulin found to be specifically expressed in the outer cortex, another noninvaded tissue that surrounds developing and mature nodules (Vasse et al. 1990). *MtN13* activation is observed only in situations in which nodules develop, i.e., in wild-type nodules or in nodules elicited by a noninfective *exoA* mutant of *R. meliloti*.

Fig. 4. Sequence alignment between MtPR10-1, MtN13, different members of PR10 family, and the partial sequence of a ginseng ribonuclease (RNase), generated with the Multalin program, using default parameter values. Dark shading indicates strong homologies, defined as identities and similarities with a score equal or superior to +2 in the Multalin alignment matrix (blossom 62). Light shading corresponds to weaker homologies, defined here as similarities with a score of +1 in the alignment matrix. Homologies specifically observed with the ginseng RNase (Moiseyev et al., 1994) are underlined.

Fig. 5. Northern (RNA) analysis of *Medicago truncatula* total RNA. Corresponding ^{32}P -radiolabeled probes are indicated on the left. Two transcripts are detected with the *MtN1* probe. Approximately 6 μg of RNA was loaded per lane. Bottom lane shows the ethidium bromide-stained 28S rRNA band, after transfer of total RNA onto the blotting membrane. **A**, RNA extracted from different *M. truncatula* organs. **B**, RNA extracted from *Rhizobium*-treated *M. truncatula* roots, and from isolated *M. sativa* spontaneous (NAR) nodules. *M. truncatula* RNA was isolated from whole root systems, harvested 6 days after inoculation with wild-type (w.t.) or mutant strains of *R. meliloti*. At this time point, small, empty nodules were visible after treatment with the *exoA* mutant. The NAR lane corresponds to RNA extracted from isolated *M. sativa* nodules, harvested after 3 weeks of combined nitrogen starvation. When the *exoA* mutant and the NAR lanes are compared, it is apparent that RNA prepared from isolated nodules gives a more intense signal than RNA extracted from whole root systems. **C**, RNA extracted from leaves infiltrated with *Xanthomonas campestris* pv. *alfalfae* (*X. c.*), *Pseudomonas syringae* pv. *pisi* (*P. s.*), and water. Blots were hybridized with probes corresponding to *MtN1*, *MtN13*, *MtPR10-1*, and a chalcone synthase gene (*CHS1*) from *M. sativa* (Esnault et al. 1993). Samples were harvested at indicated time points. A previous experiment with a more detailed kinetic analysis showed that cytological symptoms and gene activation were more rapidly induced by *P. syringae* than by *X. campestris* (data not shown), in agreement with results by Esnault et al. (1993) on *M. sativa*. The t₀ lane corresponds to a leaf sample harvested just before the beginning of the infiltration procedure. Lane C shows positive controls corresponding to nodule RNA for the *MtN1* and *MtN13* lanes, or to root RNA for the *MtPR10-1* and *CHS1* lanes.

MtN13 transcripts are not detected in situations in which symbiotic plant responses are not elicited (inoculation with *nodA* and *nodH* mutants), or in which they are limited to root-hair or cortical-cell activation (inoculation with the *nodF/nodL* dou-

ble mutant or treatment with wild-type Nod factors) (Ardourel et al. 1994; Gamas et al. 1996; P. Gamas, *unpublished data*). That a close homologue of *MtN13* is expressed in spontaneous *M. sativa* NAR nodules (Gamas et al. 1996; this study) also

supports the conclusion that bacterial infection is not required for *MtN13* induction. Moreover, our data show that *MtN13* is not expressed in root tissues, including the root cortex, but requires the formation of an active nodule meristem, able to sustain an organogenetic process and tissue differentiation. This, and the fact that *MtN13* is strongly expressed in the cortex of mature nodules, indicates that the nodule cortex is a genuine tissue, originating from the nodule meristem and displaying properties different from epidermal and cortical root tissues.

Possible roles for MtN1 and MtN13.

Two alternative hypotheses can be considered for the roles of MtN1 and MtN13 in the symbiosis: either they are defense proteins or they are involved in developmental processes unrelated to defense reactions.

The possibility that MtN1 and MtN13 are involved in defense mechanisms is suggested by the sequence homologies discussed above. To explain the fact that neither *MtN1* nor *MtN13* appear to be induced by the two bacterial pathogens tested in this study, it can be proposed that these two genes belong to a class of nodulation-specific defense genes, whose expression would be specifically triggered by *Rhizobium* in the same way as, for example, nodulation-specific copies of leghemoglobin genes are activated during symbiosis. Following on from this idea, it could be proposed that MtN13 is a symbiotic homologue of MtPR10-1, with both proteins having a similar function, one in response to *Rhizobium* and the other in response to pathogens.

If such specific defense proteins are expressed during nodulation, it is interesting to envisage their possible functions. One possibility is that they could play a protective role to prevent pathogenic infections of the root nodule, an organ representing a rich carbon source for the soil micro-flora. MtN13 could constitute a protection against pathogens present in the external medium, whereas MtN1 might be part of a mechanism preventing pathogens from taking advantage of the infection threads to invade the plant. This idea is supported by the fact that MtN13 is closely related to RH2, a pea protein specifically expressed in root epidermal cells and proposed to contribute to a constitutive defense mechanism (Mylona et al. 1994). It is also worth noting that, despite the fact that the nodular outer cortex is a loose peripheral tissue (Vasse et al. 1990), pathogenic contamination has never been described to occur in nodules developing in soil. Finally, that MtN13 exhibits substantial sequence similarities with two proteins showing a ribonuclease activity (ginseng RNase: Moiseyev et al. 1994; Bet v 1: Bufe et al. 1996; Swoboda et al. 1996) opens the possibility that MtN13 has such an RNase activity, possibly involved in mediating cytotoxic effects.

Alternatively to the hypothesis of a protective role against pathogens, it can be imagined that MtN1 represents a defense

protein turned on by the infection process and possibly directed against *Rhizobium* itself. This speculation would be contrary to the hypothesis that efficient nodulation involves the suppression of plant defense mechanisms (see reviews by Baron and Zambriski 1995; McKhann and Hirsch 1994). Indeed, a clear activation of defense reactions in the symbiosis has so far been observed only in the case of late, unsuccessful infections occurring in wild-type association (Vasse et al. 1993), or in noneffective associations involving plant or *Rhizobium* mutants (Benaben et al. 1995; Niehaus et al. 1993; Noel et al. 1986). However, it cannot be excluded that partial defense reactions are turned on by successful wild-type *Rhizobium* infections, which might not have been detected so far due to the lack of appropriate markers.

As mentioned earlier, MtN1 and MtN13 may alternatively play a developmental role not directly related to a defense mechanism, despite the sequence homologies found with defense proteins. Indeed, in the absence of detailed structure-function analysis, it can be misleading to extrapolate functional similarities from structural homologies, which might simply reflect common evolutionary origins. Moreover, even if MtN1 and MtN13 are functional homologues of pathogenesis-related (PR) proteins, it should be emphasized that the actual functions of these PR proteins, during pathogenic interactions, are not known. For example, thionins, although inhibiting microbial growth, have also been proposed to be involved in signal transduction or modification of the cell wall upon infection (reviewed in Florack and Stiekema 1994). The expression of several PR proteins has already been observed in the absence of pathogens, especially during developmental processes such as flower development (Cutt and Klessig 1992; Gu et al. 1992; Lotan et al. 1989; Milligan and Gasser 1995; Neale et al. 1990). It could thus be argued that morphogenetic processes such as nodulation or flower development might require proteins also implicated in defense mechanisms. It should also be noted that several common markers of defense reactions, such as chitinases or peroxidases, are already suspected to play various roles during nodulation other than defense responses (Cook et al. 1995; De Jong et al. 1992; Staehelin et al. 1994).

It is intriguing to have identified nodulins that present homologies to defense proteins and that are expressed during early nodulation steps. To evaluate whether or not *MtN1* and *MtN13* are indeed involved in defense-reactions, we now plan to analyze the behavior of transgenic plants in which the expression of these genes is altered.

MATERIAL AND METHODS

Bacterial strains.

Xanthomonas campestris pv. *alfalfae*, strain #3837, was provided by the French Collection of Phytopathogenic Bacteria

Fig. 6. In situ hybridization of *Medicago truncatula* nodule sections with *MtN1* (A–C) and *MtN13* (E–H) ³⁵S-labeled anti-sense probes, and with *MtN1* ³⁵S-labeled sense probe (D). A, B, E, and F, 6-day-old nodules. C, D, and G, 4-week-old nodules. Dark field microscopy. A, Longitudinal section. A strong signal is seen in submeristematic region that corresponds to growing part of infection network. Bar = 100 µm. B, Transverse section through hybridizing region. A uniform pattern of hybridization is seen in the central tissue. Bar = 100 µm. C, Longitudinal section. Hybridization signal (asterisk) is observed exclusively at distal end of the prefixing zone II. No signal is seen in the proximal part of this zone (star). Bar = 100 µm. D, *MtN1* sense probe. Labeling is absent. Bar = 100 µm. E and F, Longitudinal sections. A uniform pattern of hybridization is observed within the nodule cortex. Nodule meristem is indicated by asterisks. No signal is observed in the root tissues. Bars = 100 µm. G, Longitudinal section. A significant decrease of the hybridization signal within the cortex (black arrows) is observed from top to sides of nodule. White arrows point to nodule endodermis. Bar = 100 µm.

(INRA, Station de Phythobactériologie, 49070, Beaucauzé, France). *Pseudomonas syringae* pv. *pisi* was obtained from D. Roby (our laboratory). *Rhizobium meliloti* 1021 *exoA32::Tn5* (pXLGD4) was provided by J. Leigh (Washington University, Seattle). The other *Rhizobium* strains were constructed by J. Dénarié's group: wild-type *R. meliloti* RCR2011 pXLGD4 (GMI 6526; Ardourel et al. 1994), *R. meliloti* RCR2011 *nodA::Tn5* #2208 (GMI 5382; Debelle et al. 1986), *R. meliloti* RCR2011 *nodH::Tn5* #2313 pXLGD4 (GMI 6527; Faucher et al. 1989), *R. meliloti* RCR2011 $\Delta(nodF)13$ *nodL::Tn5* pXLGD4 (GMI 6630), *R. meliloti* RCR2011 $\Delta(nodF)13$ *nodL::Tn5* pMH682 (GMI 6629; Ardourel et al. 1994). *X. campestris* was grown in Kado medium (Kado and Heskett 1970), *P. pisi* in King's B medium (King et al. 1954), and *Rhizobium* in TY medium (Sambrook et al. 1989) supplemented with 6 mM calcium chloride and appropriate selective antibiotics.

Plant growth and treatment conditions.

Plants of *M. truncatula* cv. Jemalong genotype J5 (Barker et al. 1990) were grown in growth pouches, in the nitrogen-free growth medium described by Gallusci et al. (1991). Rhizobial inoculation was performed on 8-day-old plants, with exponentially growing bacteria resuspended in the plant growth medium (10^7 CFU/ml, 1 ml per pouch). For treatments with pathogenic bacteria, plants were grown in the same medium supplemented with 5 mM ammonium nitrate. Leaf infiltration by *P. syringae* pv. *pisi* or by *X. campestris* pv. *alfalfae* was carried out on 2-week-old plants with bacteria from overnight cultures, pelleted by centrifugation, washed in sterile water, and resuspended in water (10^9 and 10^8 CFU/ml).

RNA and DNA analysis.

Total RNA was extracted from whole root systems following the procedure of Jackson and Larkins (1976). For RNA extraction from isolated nodules, flowers, stems, and leaves, plants were grown in aeroponic conditions, as in Gamas et al. (1996). Northern and Southern analyses were conducted with Nytran Plus membranes according to the manufacturer's instructions (Schleicher & Schuell, Dassel, Germany). Varying the wash stringency did not qualitatively affect the hybridization signals.

DNA sequencing was carried out on both strands, manually as in Gamas et al. (1996), or automatically with a Perkin-Elmer/ABI 373A sequencer (Foster City, CA).

In situ hybridization.

In situ hybridizations were conducted on 7- μ m sections, following the procedure described in de Billy et al. (1991), except that slides were exposed for 3 weeks, with Amersham LM1 emulsion (Amersham France, Les Ulis). Hybridized and exposed sections were stained with 0.02% toluidine blue for 20 s, and finally observed by bright- or dark-field microscopy with an Olympus Vanox light microscope.

Computer analysis.

Sequence data were analyzed with the University of Wisconsin Genetic Computer Group (GCG) software (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, Madison, WI). The following GCG programs were used: bestfit, fasta, gap, pepplot, peptidesort, prime. Predictions for protein localization were obtained with the PSORT program (Nakai and Kanehisa 1992). Homology searches in data bases

by the BLASTX program were carried out through the NCBI BLAST E-mail Server. Sequence alignments were realized with the Multalin program (Corpet 1988).

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