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The *Medicago truncatula N5* Gene Encoding a Root-Specific Lipid Transfer Protein Is Required for the Symbiotic Interaction with *Sinorhizobium meliloti*

Youry Pii,[1] Alessandra Astegno,[2] Elisa Peroni,[2] Massimo Zaccardelli,[3] Tiziana Pandolfini,[1] and Massimo Crimi[1]

[1]Dipartimento Scienze, Tecnologie e Mercati del Vino, University of Verona, San Floriano (VR); [2]Dipartimento Scientifico e Tecnologico, University of Verona, Verona, Italy; [3]CRA-Centro di Ricerca per l'Orticoltura, Pontecagnano-Battipaglia (SA), Italy

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The *Medicago truncatula N5* gene is induced in roots after *Sinorhizobium meliloti* infection and it codes for a putative lipid transfer protein (LTP), a family of plant small proteins capable of binding and transferring lipids between membranes in vitro. Various biological roles for plant LTP in vivo have been proposed, including defense against pathogens and modulation of plant development. The aim of this study was to shed light on the role of *MtN5* in the symbiotic interaction between *M. truncatula* and *S. meliloti*. MtN5 cDNA was cloned and the mature MtN5 protein expressed in *Escherichia coli.* The lipid binding capacity and antimicrobial activity of the recombinant MtN5 protein were tested in vitro. MtN5 showed the capacity to bind lysophospholipids and to inhibit *M. truncatula* pathogens and symbiont growth in vitro. Furthermore, MtN5 was upregulated in roots after infection with either the fungal pathogen *Fusarium semitectum* or the symbiont *S. meliloti.* Upon *S. meliloti* infection, MtN5 was induced starting from 1 day after inoculation (dpi). It reached the highest concentration at 3 dpi and it was localized in the mature nodules. *MtN5*-silenced roots were impaired in nodulation, showing a 50% of reduction in the number of nodules compared with control roots. On the other hand, transgenic roots overexpressing *MtN5* developed threefold more nodules with respect to control roots. Here, we demonstrate that MtN5 possesses biochemical features typical of LTP and that it is required for the successful symbiotic association between *M. truncatula* and *S. meliloti*.

Plant lipid transfer proteins (LTP) were isolated for the first time approximately 30 years ago and were characterized by their ability to facilitate the transfer of phospholipids between a donor and an acceptor membrane in vitro (Kader 1975). Plant LTP are small, soluble basic proteins with an isoelectric point (pI) ranging between 9 and 10 (Kader 1997). The primary sequence of LTP contains eight strictly conserved cysteine residues which are engaged in the formation of four intrachain disulfide bonds. The three-dimensional architecture of several LTP has been solved by nuclear magnetic resonance and X-ray crystallography, and a structure of four α-helices enclosing an internal hydrophobic cavity has been revealed (Lerche et al. 1997; Lee et al. 1998; Samuel et al. 2002; Pons et al. 2003; Hoh et al. 2005). Plant LTP are grouped into two main families (Boutrot et al. 2008). The first family, LTP1, comprises proteins which have a molecular mass of approximately 9 kDa. In addition to the eight perfectly conserved cysteine residues, type 1 LTP display other conserved characteristics, such as the presence of small hydrophobic amino acids throughout the whole sequence which participate in the definition of the binding tunnel hydrophobicity (Yeats and Rose 2008). This cavity runs through the molecule's axes and accommodates fatty acids or lysophospholipids (de Oliveira Carvalho and Moreira Gomes 2007).

The second family, LTP2, comprises proteins with an average molecular mass of 7 kDa. Compared with the LTP1 family, there are considerably fewer studies concerning the architecture of LTP belonging to family 2 (Samuel et al. 2002; Pons et al. 2003; Hoh et al. 2005). Site-directed mutagenesis studies have recently highlighted a crucial role for Leu8, Phe36, and Val49 in the maintenance of both structure and lipid-binding ability of rice LTP2 (Cheng et al. 2007). In other members of the LTP2 family, the last two amino acids can be replaced by residues with similar chemical characteristics (i.e., Leu and Ile, respectively).

All known plant LTP are synthesized as precursors with an N-terminal signal peptide which targets the mature protein toward the secretory pathway. Therefore, a role in the intracellular lipid trafficking has been ruled out for plant LTP (Kader 1997). The biological function of plant LTP is still not fully elucidated. Some experimental evidence suggests the involvement of LTP in different aspects of plant biology.

Many LTP belonging to the LTP1 family have been proven to participate in defense responses against bacterial and fungal pathogens. Several LTP1s from different plants exhibit antimicrobial activity against both pathogenic bacteria and fungi in vitro (Terras et al. 1992; Molina et al. 1993; Segura et al. 1993; Cammue et al. 1995; Ge et al. 2003) and the overexpression of these proteins in transgenic plants conferred enhanced resistance against a wide range of plant pathogens (Mourgues et al. 1998; Iwai et al. 2002; Jung et al. 2005; Roy-Barman et al. 2006; Patkar and Chatoo 2006; Yang et al. 2008). The defensive role of LTP has also been corroborated by the observation that many isoforms are upregulated during infection in plants (García-Olmedo et al. 1995; Jung et al. 2003), and this feature has resulted in the inclusion of LTP in the pathogenesis-related

Corresponding author: Tiziana Pandolfini; Telephone: +39-45-8027918; Fax: +39-45-8027929; E-mail: tiziana.pandolfini@univr.it

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(PR) protein family (Van Loon and Van Strien 1999). To our knowledge, there are no clear indications that LTP2s act as antimicrobial proteins.

LTP can participate in the defense reaction as components of the signaling pathway that lead to systemic acquired resistance (SAR). The evidence of this role was obtained with the *dir1-1* mutant of *Arabidopsis thaliana*, which was impaired in the SAR induction but not in the local response (Maldonado et al. 2002). DIR1 displays some structural and lipid-binding properties similar to plant LTP2s, except for the acidic pI. However, on the basis of both structural and functional specific features, it was proposed as a new type of LTP (Lascombe et al. 2008).

Evidence for LTP1s acting as systemic resistance inducers originated from the observation that a *Nicotiana tabacum* LTP1 (NtLTP1) has the capability to bind jasmonic acid (JA) (Buhot et al. 2004). The complex NtLTP1-JA is able to interact with the elicitin receptor on the plasma membrane, providing long-distance protection against *Phytophthora parasitica* infection in tobacco plants (Buhot et al. 2004).

Recently, LTP have also been implicated in plant developmental processes such as the induction of cell-wall loosening and the synthesis of the cuticle (Nieuwland et al. 2005; Cameron et al. 2006). A putative involvement of LTP in the symbiotic relationship has been hypothesized, based on the data obtained from a number of transcriptome analyses aimed at monitoring changes in gene expression elicited by rhizobia infection.

The transcriptome analysis of *Medicago truncatula* root nodules has led to the discovery of a gene family named nodule-specific cysteine-rich (NCR), which is composed of 300 members. Some of these NCR, shown to be expressed in different phases of nodule development and localized in different zones of the emerging organ, belong to the LTP family. This suggests that these genes could be involved in the formation of root nodules (Mergaert et al. 2003).

MtN5 is a gene from *M. truncatula,* which was identified by means of a differential screening aimed at isolating new genes induced during the early phases of the symbiosis between *M. truncatula* and *Sinorhizobium meliloti* (Gamas et al. 1996). Thus, it was recognized as an early marker of the symbiosis and, on the basis of the sequence homology, it was annotated as a putative LTP (El Yahyaoui et al. 2004).

In this study, we have demonstrated that MtN5 is able to bind lysophospholipids, possesses a weak antimicrobial activity *in vitro*, and is induced in *M. truncatula* roots upon *Fusarium* spp. infection. Furthermore, we have shown that MtN5 protein is specifically expressed during the early phases of the symbiosis and accumulates in the mature nodules. Using an RNA-interference (RNAi) approach to suppress MtN5 function, we proved that *MtN5* is required for the successful establishment of the symbiotic association between *M. truncatula* and *S. meliloti*. Accordingly, the overexpression of the MtN5 gene promoted nodulation.

RESULTS

MtN5 is an LTP2-like protein.

The *MtN5* gene encodes for a 643-nucleotide-long messenger (m)RNA which produces a putative protein of 102 amino acids containing a 27-amino-acid-long N-terminal signal peptide for the secretory pathway. Recently, it has been experimentally demonstrated that MtN5 protein is localized in the apoplastic space (Kusumawati et al. 2008). The deduced sequence of the mature MtN5 protein was compared with the sequences of proteins belonging to the two different LTP families (type 1 and type 2) (Fig. 1A and B). The multiple sequence alignment shows that the homology with representative members of the LTP1 family is restricted to the eight-cysteine motif and a few other residues (14.5% identity, 34.2% similarity)

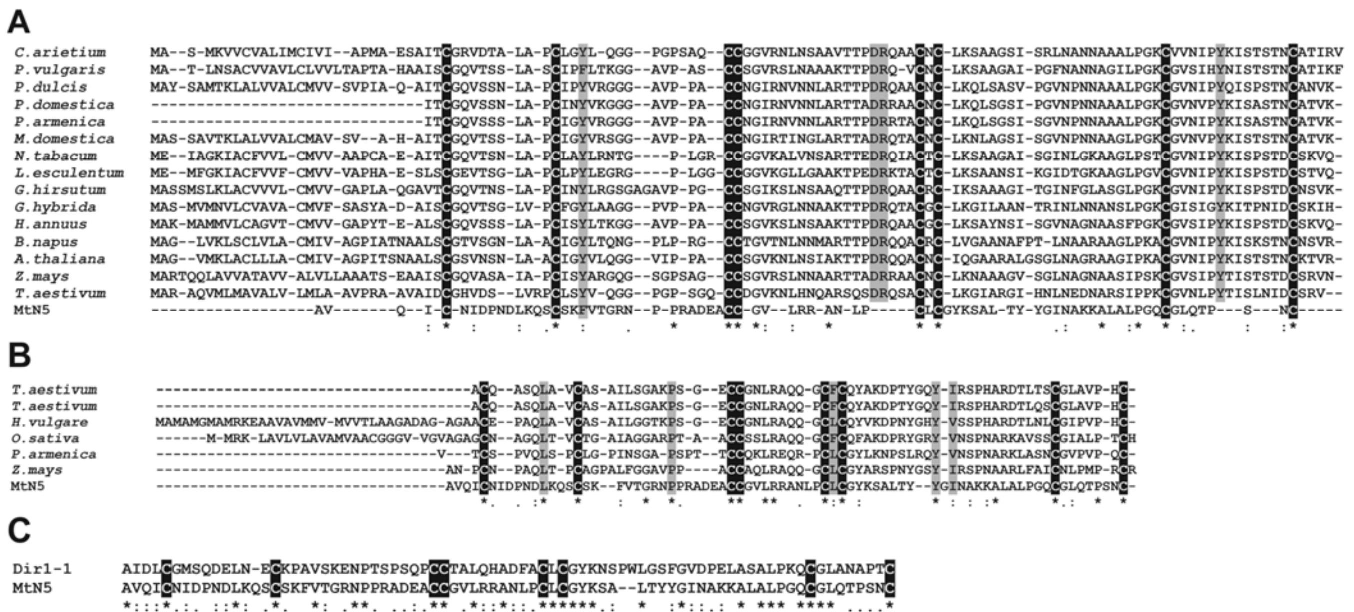

Fig. 1. Comparison of the amino acid sequences of *Medicago truncatula* N5 protein and representative members of lipid transfer protein (LTP)1 and LTP2 families. The sequence of the proteins were obtained from SWISS-PROT and aligned with Clustal W (Thompson et al. 1994). The black boxes indicate the eight-cysteine motif, whereas the residues that are fundamental for the folding and the lipid-binding activity are boxed in light gray. **A,** Multiple sequence alignment between members of the LTP1 family and MtN5. The data bank accession numbers of the proteins are the following: *Cicer arietinum* (O23758), *Phaseolus vulgaris* (O24440), *Prunus dulcis* (Q43017), *P. domestica* (P82534), *P. armeniaca* (P81651), *Malus domestica* (Q9M5X7), *Nicotiana tabacum* (Q42952), *Lycopersicon esculentum* (P93224), *Gossypium hirsutum* (Q9FVA5), *Gerbera hybrida* (Q39794), *Helianthus annuus* (Q39950), *Brassica napus* (Q42614), *Arabidopsis thaliana* (Q42589), *Zea mays* (P19656), and *Triticum aestivum* (Q8GZB0). **B,** Multiple sequence alignment between members of the LTP2 family and MtN5. The data bank accession numbers of the proteins are listed as follows: *T. aestivum* (P82900 and P82901), *Hordeum vulgare* (P20145), *Oryza sativa* (P83210), *P. armeniaca* (P82353), and *Z. mays* (P83506). **C,** Pairwise alignment between MtN5 and *A. thaliana* DIR1 (Q8W453); *, residues identical in all sequences; **:**, for conserved substitutions; **.**, for semiconserved substitutions.

(Fig. 1A) whereas the overall homology is higher with type 2 LTP (21% identity, 38% similarity) (Fig. 1B). In addition to the eight strictly conserved cysteines, MtN5 also displays several residues (i.e., L12, P25, L44, Y54, and I56), which were demonstrated to be crucial for the folding and the lipid-binding activity of LTP2 (Fig. 1B) (Cheng et al. 2007). Interestingly, the alignment between DIR1, which was recently defined as a new type of LTP (Lascombe et al. 2008), and MtN5 (Fig. 1C), highlighted a higher homology degree (37% identity, 83% similarity) with respect to the other type 2 LTP, even though the pI of DIR1 is acidic (pI 4.5) whereas MtN5 is a basic protein (pI 9.0).

Phylogenetic analysis was conducted to assess the relationships between MtN5 and several well characterized LTP from other plant species, also including *A. thaliana* DIR1 (Supplementary Fig. 1). The phylogenetic tree suggests that the proteins analyzed could be divided into three groups, two of which consist of family 1 and family 2 LTP. MtN5 and DIR1 grouped independently from the two major LTP families, despite the fact they display a higher degree of homology with family 2 LTP.

MtN5 expression and purification.

In order to further characterize MtN5, the nucleotide sequence encoding for the mature protein tagged with six His residues at the N-terminus was cloned in the pET12b vector and expressed in *E. coli* BL21 (DE3) pLysS. The $(His)_6$-tag enables affinity purification on the Ni–NTA affinity column. Recombinant MtN5 was purified from inclusion bodies using strong denaturing conditions and applying a refolding protocol in column, by removing the denaturing agent through a linear gradient (Fig. 2A). In addition to carrying out the in vitro tests, we also used the recombinant protein to produce polyclonal antibodies in rabbit. Such an antibody detects, in protein crude extracts of nodulated roots, a single principal band of approximately the same apparent molecular mass as the purified recombinant protein (Fig. 2B).

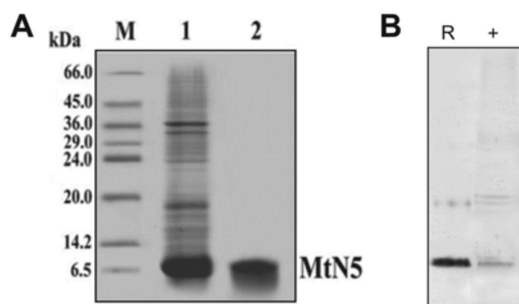

Fig. 2. *Medicago truncatula* N5 protein expression and purification. **A,** Expression and purification of recombinant MtN5 protein (Coomassie staining). Lane1: *Escherichia coli* BL21 (DE3) pLysS lysate. Lane 2: Affinity chromatography-purified recombinant MtN5. **B,** Western blot analysis of crude protein extract from *M. truncatula*-nodulated roots (R); **+**, purified recombinant MtN5 protein; M, molecular weight marker.

Biochemical characterization of MtN5.

Lipid binding by plant LTP can be assessed by means of equilibrium titration experiments using lipids as substrates (Gomar et al. 1996; Lascombe et al. 2008). The binding of lysolipids induces the enhancement of intrinsic fluorescence emitted by aromatic side chains which are affected by the hydrophobic environment (Crimi et al. 2006). To probe the lipid-binding capacity of MtN5 protein, lyso-phosphatidylcholines (LPC) with various fatty acids chain lengths (LPC-C12, LPC-C16, and LPC-C18) were used. MtN5 presents, in its amino acid sequence, three tyrosine residues (Y47, Y53, and Y54) and displays a maximum of fluorescence emission at 305 nm in an aqueous environment. Upon the binding of LPC-C12 and LPC-C16, the MtN5 maximum of fluorescence emission at 305 nm remained unchanged; however, the relative fluorescence intensity increased as a function of lipid concentration up to 12 μM (Supplementary Fig. 2). The addition of Tween 20 to MtN5 in solution did not affect the fluorescence emission spectrum (data not shown), thus demonstrating that the changes in fluorescence intensity observed after the addition of lyso-phosphatidylcholines were due to a specific lipid–protein interaction. The fitting of the titration points (i.e., relative fluorescence intensity at 305 nm versus lipid/protein ratio) using a noncooperative binding model revealed that the saturation of the lipid-binding sites was reached when the molar ratio between MtN5 and the ligand was approximately 1:1 for both LPC-C12 and LPC-C16 (Fig. 3A and B). In the binding assay carried out with LPC-C18, MtN5 did not display the increase in the intrinsic fluorescence emission observed when the ligands were LPC-C12 and LPC-C16 (data not shown). These findings indicate that MtN5 protein possesses a lipid-binding activity, as observed in several other members of the plant LTP superfamily.

MtN5 exhibits antimicrobial activity in vitro.

To examine whether the recombinant MtN5 exerts antimicrobial activity, we tested its action on *Xanthomonas campestris* pv. *alfalfa*, a bacterial pathogen that infects the aerial parts

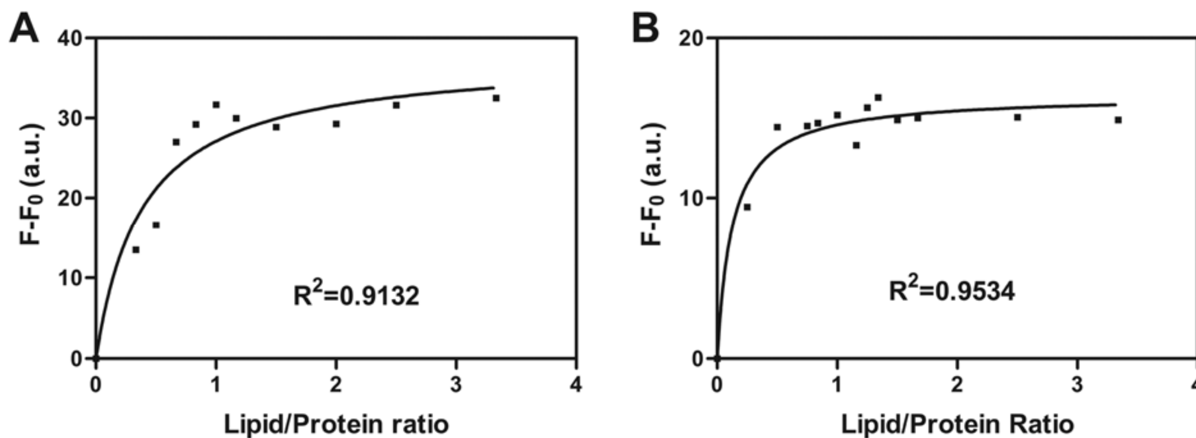

Fig. 3. Lipid-binding assays. **A,** Titration points, increasing in fluorescence intensity (F to F_0) at 305 nm versus LPC-C12/protein molar ratio and **B,** LPC-C16/protein molar ratio. The fitting curves of the titration points were obtained with a noncooperative binding model. F: fluorescence emission of the *Medicago truncatula* N5 protein in the presence of lipids; F_0: fluorescence emission of MtN5.

of *Medicago* spp.; on *Fusarium semitectum*, a soilborne fungus which attacks the root apparatus of *Medicago* plants; and on *S. meliloti*, the natural host of *M. truncatula*. The action of MtN5 on the growth of the microorganisms tested was compared with that exerted by the same amounts of bovine serum albumin (BSA). The growth of *F. semitectum* in the presence of MtN5 was slower than untreated culture, already at low concentrations (starting from 0.5 µM), and the effect was dependent on the amount of protein added to the medium up to a concentration of 10 µM (Fig. 4A). MtN5 also affects *X. campestris* growth; the inhibition of bacterial growth displays a dose-dependent trend and the effects were detectable starting from 0.4 µM (Fig. 4B). Furthermore, MtN5 showed an antibiotic activity also against *S. meliloti*, which is the natural symbiont of *Medicago* plants (Fig. 4C). Interestingly, the inhibitory effect against *Rhizobium* spp. was observed at concentrations higher (>1 µM) than those necessary to inhibit the growth of the two pathogens.

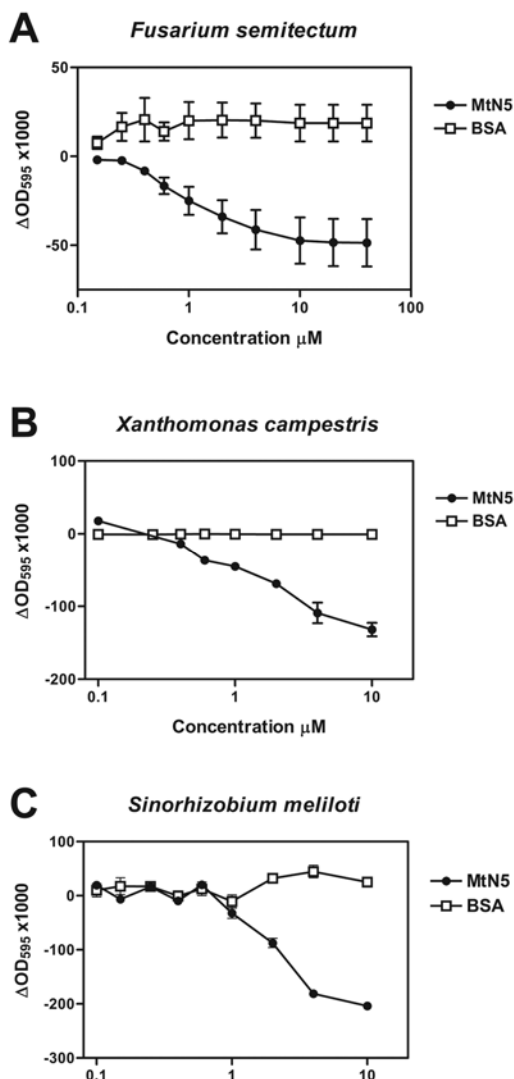

Fig. 4. In vitro antimicrobial assays. The microbial growth was determined by measuring the absorbance at 595 nm of the microorganism suspensions after 72 h of incubation in microplates with different concentrations of the *Medicago truncatula* N5 protein. **A,** Effect of MtN5 on *Fusarium semitectum* growth. **B,** Effect of MtN5 on *Xanthomonas campestris* growth. **C,** Effect of MtN5 on *Sinorhizobium meliloti* growth. ΔOD_{595} represents the difference in the optical density between the untreated and the treated samples. The effect of MtN5 on microorganism growth was compared with that of bovine serum albumin (BSA).

Root MtN5 expression is induced after *F. semitectum* infection.

In order to test whether MtN5, like other plant LTP, is upregulated in infected tissues, the expression pattern of MtN5 protein was investigated in plants inoculated with either *F. semitectum* ISCF20a (Zaccardelli et al. 2006) or *X. campestris* pv. *alfalfae*. *F. semitectum* is often isolated from plants with complex diseases: in legume crops *F. semitectum* may cause seed rot, collar rot and root rot (Zaccardelli et al. 2006). The roots were inoculated with two different concentrations of the pathogenic fungus (10 and 50%, vol/vol) and collected 48 h after the infection. In nonnodulated plants, the expression of MtN5 in the root apparatus was elicited by *F. semitectum* at both concentrations (Fig. 5). However, in plants treated with *F. semitectum* at 50%, the MtN5 level that resulted was lower than that obtained in the plants infected with the fungus at 10%. In nodulated roots, we observed a similar increase in the amount of protein upon treatment with 10 and 50% of fungus compared with nodulated roots of untreated plants. In addition, the increase in MtN5 level was restricted to root apparatus, because the protein was not detected in the shoots of infected plants (data not shown).

In *M. truncatula* plants inoculated with the bacterial pathogen *X. campestris,* MtN5 protein was detected in neither the infected shoot tissue nor the root tissue of both nonnodulated plants and nodulated plants (data not shown). In addition, MtN5 protein was not elicited in the root tissue even by wounding (data not shown).

Considering these findings, we can infer that MtN5 is a root-specific protein which is upregulated in response to microorganisms that infect the root apparatuses.

MtN5 is specifically expressed in root nodules during rhizobial symbiosis.

The expression pattern of *MtN5* was evaluated in 40-day-old *M. truncatula* plants by means of quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). In nodulated plants, the *MtN5* gene was highly expressed in the root nodules, where the level of expression was sevenfold higher with respect to the transcript level detected in either nonnodulated

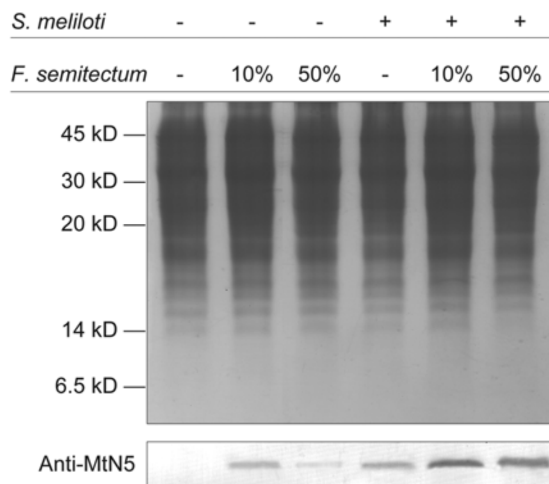

Fig. 5. *Medicago truncatula* N5 protein expression in root apparatuses of plants infected with *Fusarium semitectum*. *M. truncatula* plants (40 days old), either nodulated or nonnodulated, were treated with two different concentrations (10 and 50%, vol/vol) of *F. semitectum*. Total proteins were extracted from root apparatuses of both infected and noninfected plants and analyzed by Western blot. An equivalent amount of proteins was loaded in each lane, as shown by the Coomassie staining (upper panel).

roots or in nodulated roots deprived of nodules (Fig. 6A). The aerial part of both nonnodulated and nodulated plants displayed an extremely low level of *MtN5* mRNA with respect to root tissue (2 and 18% in nonnodulated plants and nodulated plants, respectively, compared with nonnodulated roots).

We also looked for the presence of MtN5 protein in 40-day-old *M. truncatula* plants nodulated by *S. meliloti* (Fig. 6B): MtN5 protein was detectable only in root nodules, suggesting that the nodules represent the organs where the protein is preferentially expressed and accumulated.

MtN5 was classified as an early nodulin, whose mRNA is induced well before the onset of nitrogen fixation, maintained in mature nodules (14 days after inoculation), and also expressed in N fixation-defective Nar nodules (Gamas et al. 1996). On the basis of these findings, it has been proposed that the protein encoded by *MtN5* could play a role in root nodule organogenesis rather than in nitrogen fixation (Gamas et al. 1996). We studied the induction kinetic of MtN5 protein during different phases of rhizobia infection (Fig. 6C). Seedlings (7 days old) were microflood inoculated with *S. meliloti* and the roots were collected 0, 1, 3, 4, 5 and 14 days post inoculation (dpi). MtN5 was detected starting from 1 dpi and it reached the highest concentration at 3 dpi. These data suggest that *MtN5* induction is an early event that might occur before invasion of the root by rhizobia. The MtN5 protein content decreased after 3 dpi, and remained stable between 7 and 14 dpi (Fig. 6C).

Overall, these results confirm that *MtN5* expression is induced during symbiotic interaction and demonstrate that MtN5 protein is produced in the early phases of *S. meliloti* infection and is localized in the mature nodules.

Silencing and overexpression of MtN5.

The functional study of MtN5 during the establishment of the symbiosis was carried out by both silencing and overexpression approaches. A hairpin (*hp*) gene construct designed to silence the endogenous *MtN5* was transferred to *M. truncatula* roots by *Agrobacterium rhizogenes*-mediated transformation. As negative control, *M. truncatula* plants inoculated with *A. rhizogenes* harboring the empty pBIN19 binary vector were used. *A. rhizogenes* generates adventitious, genetically transformed roots at the site of inoculation. The transformed roots were selected by growing them on kanamycin, and the transgenic state of adventitious roots was checked by PCR analysis (discussed below) because other laboratories had previously observed that not all *M. truncatula* hairy roots selected on antibiotic-containing medium were transformed (Limpens et al. 2004; Huo et al. 2006). In addition, the effective expression of the *hp* construct was tested by means of RT-PCR (Fig. 7A).

The expression of the *hp* construct induced the silencing of the *MtN5* gene in the roots (Fig. 7B). The steady-state level of *MtN5* mRNA in silenced hairy roots was reduced, on average, by approximately 70 to 75% when compared with either control noninoculated roots or control nodulated roots deprived of nodules (Fig. 7B). In addition, the *MtN5* expression was also evaluated in the nodules, generated on both control and *MtN5*-silenced roots. In silenced nodules, the relative amount of *MtN5* mRNA turned out to be reduced by 98% when compared with control nodules (Fig. 7C). The *MtN5*-silenced roots were impaired in nodulation, because they developed 50% fewer nodules with respect to the inoculated control roots (Fig. 7D).

We observed that the presence of the antibiotic kanamycin used as selective agent in the medium somewhat affects the

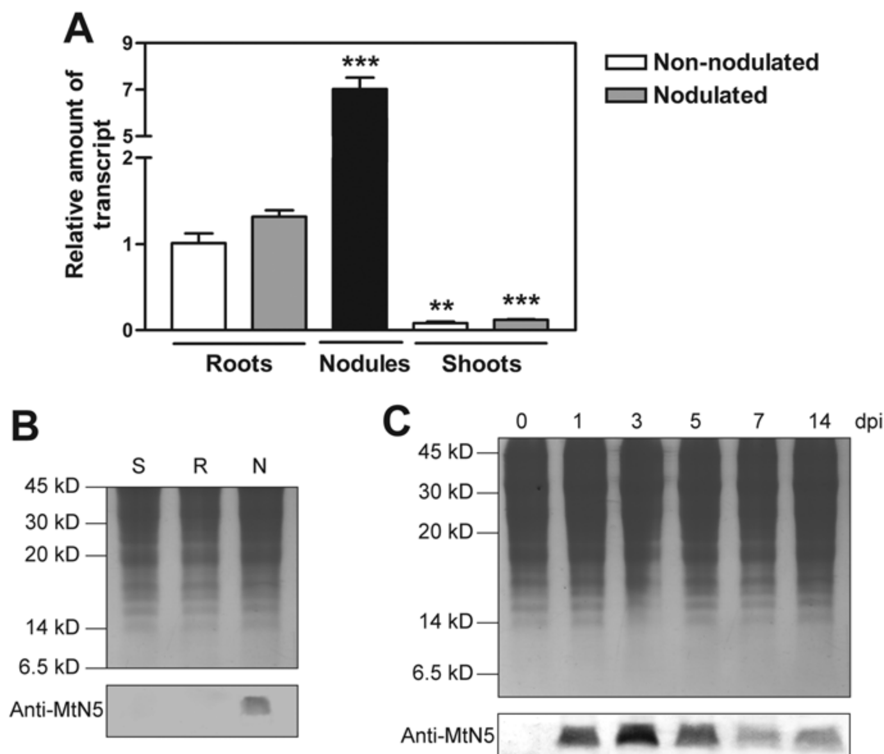

Fig. 6. Expression pattern in *Medicago truncatula* plants. **A,** Expression of the *M. truncatula N5* gene in roots and shoots of both nonnodulated and nodulated plants and in root nodules was evaluated by quantitative reverse-transcriptase polymerase chain reaction. Total messenger RNA was extracted from 40-day-old plants. The expression levels were normalized using actin as endogenous control gene and the relative expression ratios were calculated using nonnodulated roots as calibrator sample. The values reported are means ± standard error ($n = 3$; **, $P < 0.01$; ***, $P < 0.001$). **B,** Western blot analysis of proteins extracted from shoots (S), roots deprived of nodules (R), and nodules (N) of 40-day-old *M. truncatula* plants. An equivalent amount of proteins was loaded in each lane, as shown by the Coomassie staining (upper panel). **C,** Western blot analysis of proteins extracted from root apparatuses after microflood inoculation with *Sinorhizobium meliloti*. Roots were collected 1, 3, 5, 7, and 14 days post inoculation (dpi). An equivalent amount of proteins was loaded in each lane as shown by the Coomassie staining (upper panel).

general status of the plant and might also impair nodulation. Therefore, we used a different selection approach based on the use of a fluorescent selection marker (Limpens et al. 2004) to confirm the results of MtN5 silencing.

In an independent test, a pBINPLUS (van Engelen et al. 1995) derivative binary vector, named pRedRoot (Limpens et al. 2004), was chosen to harbor the *hp* gene construct with the aim of silencing *MtN5*. Approximately 50% of the roots displayed the red fluorescence due to DsRED1 expression, confirming the transformation efficiency already reported by other groups using the same transformation protocol (Fig. 8A and B) (Limpens et al. 2004; Huo et al. 2006). Only the nodules present on roots expressing the fluorescent reporter were considered for the analyses (Fig. 8A through F). The number of nodules developed by the silenced roots were reduced by approximately 80% when compared with control adventitious roots, generated by transformation with the empty pRedRoot vector (Fig. 8A, B, E, F, and G).

On the other hand, when roots were transformed with the *35S::MtN5* construct, the *MtN5* transcript level of transgenic adventitious roots was approximately sevenfold higher than control adventitious roots (Fig. 8H) and they developed approximately 300% more nodules with respect to control roots (Fig. 8C, D, E, F, and G).

Nodules developed by *MtN5hp* and *35S::MtN5* transgenic roots appeared, at maturation, to be morphologically similar and of approximately the same size; in addition, nodules were all apparently functional because they exhibited the typical red color due to the presence of leghemoglobin.

DISCUSSION

Plants belonging to the *Leguminoseae* family are able to establish a symbiotic association with soilborne bacteria, called rhizobia. This mutualistic relationship leads to the development of a new organ, the root nodule. The symbiosis starts with an exchange of signals between the host plants and the symbiont. The flavonoids exuded from the roots act on rhizobia, inducing the synthesis of Nod factors, whose perception results in the activation of a signaling cascade and the induction of symbiosis-specific host plant genes. This class of genes, named nodulins, is subdivided on the basis of the timing of their expression into early and late nodulins. The early nodulins are expressed before the onset of nitrogen fixation and are considered to be involved in both the infection process and the organogenetic plant response. The late nodulins are induced in mature nodules and are related to the nitrogen-fixing metabolism and root nodule physiology. In recent years, many nodulin genes have been isolated from different leguminous plants. Mergaert and co-workers (2003) identified a gene family (NCR) composed of 300 members encoding for small (60 to 90 residues), cysteine-rich proteins, which are upregulated during rhizobial infection. On the basis of their specific temporal and spatial expression profile, it has been proposed that they participate in nodule development (Mergaert et al. 2003).

MtN5 codes for a cysteine-rich protein, which has been classified as early nodulin because it is induced during the early phases of the symbiotic association between *M. truncatula* and

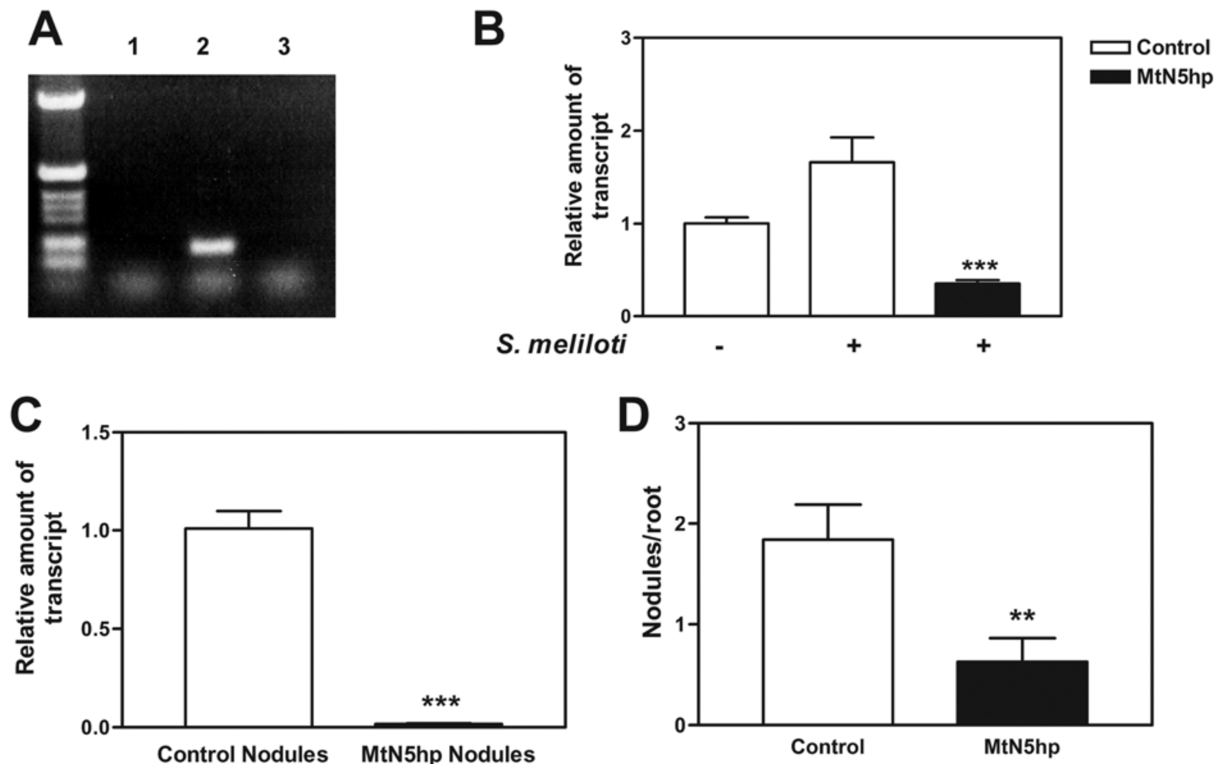

Fig. 7. *Medicago truncatula N5* gene silencing in transgenic *M. truncatula* roots. **A,** Expression of the *MtN5* hairpin (*hp*) gene construct in hairy roots. Agarose gel electrophoresis of reverse-transcriptase polymerase chain reaction (RT-PCR) product obtained from total RNA extracted from control roots (lane 1) and from *MtN5*-silenced roots (lane 2). Lane 3: no-template control. **B,** Expression of *MtN5* in hairy roots. The expression level was evaluated by means of quantitative RT-PCR in *MtN5hp* roots inoculated with rhizobia and in control roots, both noninoculated (–) and inoculated (+). The expression levels were normalized using actin as endogenous control gene. The relative expression ratios were calculated using noninoculated control root as calibrator sample. The values reported are means ± standard error (SE) ($n = 3$; ***, $P < 0.001$). **C,** Expression of *MtN5* in root nodules generated on control and *N5hp* hairy roots. The expression levels were normalized using actin as endogenous control gene. The relative expression ratios were calculated using control nodule as calibrator sample. The values reported are means ± SE ($n = 3$; ***, $P < 0.001$). **D,** Number of nodules per root. The values reported are means ± SE ($n = 18$; **, $P < 0.01$). Control: hairy roots generated by *Agrobacterium rhizogenes* carrying an empty pBIN19 vector; *MtN5hp*: hairy roots generated by *A. rhizogenes* carrying the recombinant pBIN19 vector containing the *hp* construct.

S. meliloti (Gamas et al. 1996; El Yahyaoui et al. 2004). MtN5 was annotated as a putative plant LTP because of the presence of the typical eight-cysteine motif in its amino acid sequence (El Yahyaoui et al. 2004). Plant LTP are grouped in two classes, LTP1 and LTP2, on the basis of their molecular mass, which is approximately 9 and 7 kDa for LTP1 and LTP2, respectively (Yaets and Rose 2008). MtN5 protein has a molecular mass of approximately 8 kDa and displays a higher sequence similarity with members of the LTP2 family than with members of the LTP1 family. Site-directed mutagenesis studies carried out on LTP2 from *Oryza sativa* individuated a set of amino acids that are fundamental for the tertiary structure and the lipid-binding activity of this protein (Cheng et al. 2007). These amino acids are conserved in MtN5 sequence, strengthening its resemblance with type 2 LTP. Notably, MtN5 shows a higher sequence homology with *Arabidopsis thaliana* DIR1 (37% identity, 83% similarity), which was recently proposed as a peculiar LTP2 (Lascombe et al. 2008). Despite being closer to LTP2, the phylogenetic analysis shows that MtN5 and DIR1 group independently from the two major LTP families.

Although plant LTP have not yet been provided with a clear biological role, their characteristic biochemical properties, such as the ability to bind lipids in vitro (Kader 1996) and to inhibit microbial growth in vitro, have been associated with a possible defensive role of these proteins in vivo (Van Loon and Van Strien 1999).

The data presented here demonstrate that, in addition to sharing sequence similarities with LTP, MtN5 exhibits biochemical features typical of this protein family because it is able to bind lysophospholipids; possesses a slight antimicrobial activity in vitro against *X. campestris, F. semitectum*, and *S. meliloti*; and is upregulated upon microorganism infection.

The specific role of these cysteine-rich proteins in the rhizobial infection and nodule development is not fully elucidated. The upregulation of these cysteine-rich proteins has been explained as either a response that limits rhizobia invasion and spreading or a mechanism that protects root nodules from nonsymbiotic microorganisms (Gamas et al. 1998; Mergaert et al. 2003; El Yahyaoui et al. 2004; Chou et al. 2006). It was shown that, during the early stages of nodule formation, the Nod factors can elicit reactions that are similar to those induced by the match with pathogens (e.g., production of reactive oxygen species, phytoalexins, and PR proteins) (Santos et al. 2001; Mithöfer, 2002; El Yahyaoui et al. 2004; Mitra and Long 2004). However, it was proposed that these responses most likely are attenuated by bacterial effectors (e.g., lipopolysaccharides and exopolisaccharides) (Mithöfer 2002; Scheidle et al. 2005; Tellstrom et al. 2007). Furthermore, it was observed that the induction of plant defense responses is involved in autoinhibitory control of the nodule number (van Brussel et al. 2002).

MtN5 induction is an early event of the rhizobial infection and was shown to be dependent on Nod factor signaling; it has been observed that, in *M. truncatula* plants grown in aeroponic conditions, a 24-h treatment with Nod factors induces *MtN5* gene expression (Gamas et al. 1996). However, it has been reported that *MtN5* is not expressed in the *M. truncatula nfp* mutant impaired in Nod factor perception (El Yahyaoui et al 2004) and that the inoculation of roots with the *S. meliloti nodA*$^-$ mutant, defective in Nod factor synthesis, causes only a transient induction of *MtN5* expression between 3 and 24 h (Gamas et al. 1996; El Yahyaoui et al 2004). Our findings confirm the rapid accumulation of MtN5 protein in the root tissue during the early stages of rhizobial invasion. Furthermore, we have shown that, in the late phases of the symbiosis, MtN5 is predominantly localized within the nitrogen-fixing nodules. This expression pattern suggests that the role of MtN5 in the symbiosis might be either to protect mature nodules against

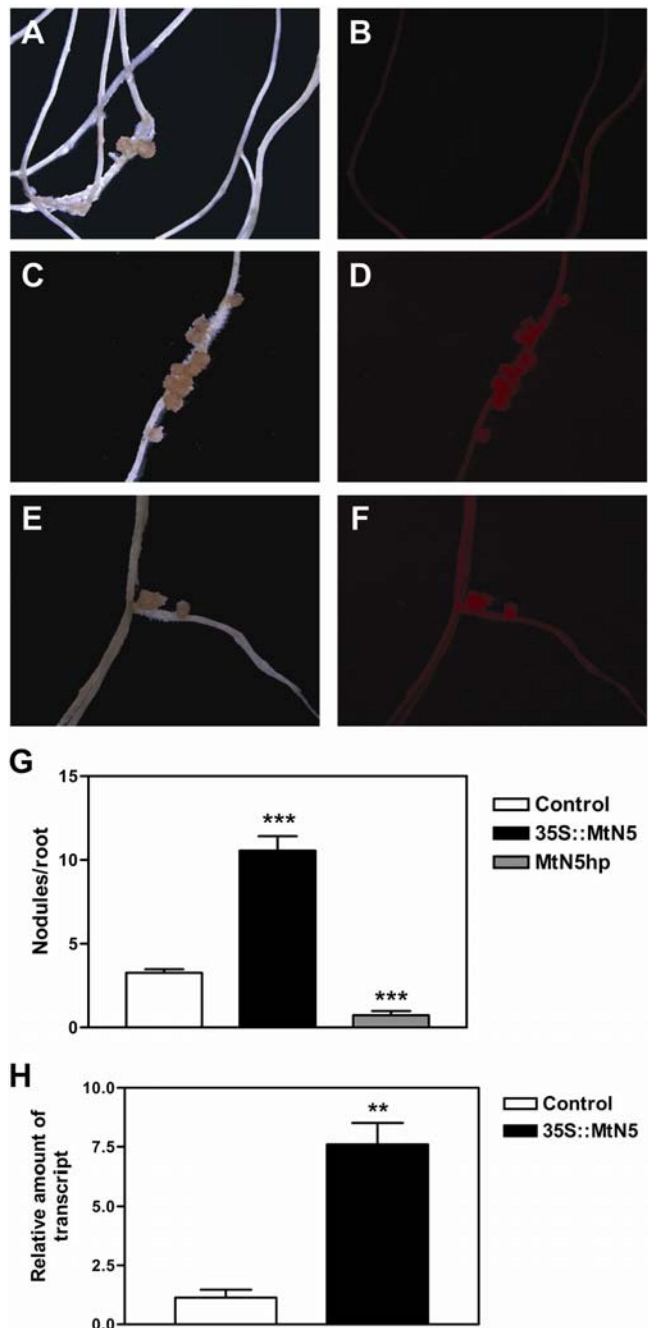

Fig. 8. Effects of silencing and overexpression of the endogenous *Medicago truncatula N5* gene. *M. truncatula* hairy roots transformed with *MtN5hp* harbored in the pRedRoot binary vector **A,** Bright-field picture. **B,** *DsRED1* fluorescence (red fluorescence). *M. truncatula* hairy roots transformed with *35S::MtN5* gene construct harbored in the pRedRoot binary vector. **C,** Bright-field picture. **D,** *DsRED1* fluorescence (red fluorescence). *M. truncatula* hairy roots produced by *Agrobacterium rhizogenes* harboring the empty pRedRoot binary vector. **E,** Bright field. **F,** *DsRED1* fluorescence (red fluorescence). **G,** Number of nodules per root. The values reported are means ± standard error (SE); ***, $P < 0.001$. **H,** Expression of *MtN5* in hairy roots transformed with *35S::MtN5* gene. The expression level was evaluated by means of quantitative reverse-transcriptase polymerase chain reaction in both *MtN5*-overexpressing and control roots inoculated with rhizobia and deprived of nodules. The expression levels were normalized using actin as endogenous control gene. The relative expression ratios were calculated using inoculated control root as calibrator sample. The values reported are means ± SE ($n = 3$; **, $P < 0.01$). Control: hairy roots generated by *A. rhizogenes* infection, carrying an empty pRedRoot vector. 35S::MtN5: hairy roots generated by *A. rhizogenes* infection, carrying the recombinant pRedRoot vector containing the gene construct for the overexpression. MtN5hp: hairy roots generated by *A. rhizogenes* infection, carrying the recombinant pRedRoot vector containing the *hp* gene construct.

pathogens or to promote nodule development, rather than to limit rhizobia infection. Indeed, the suppression of *MtN5* results in a reduced nodulation whereas its overexpression increases the number of nodules produced by *S. meliloti*. Although MtN5 exhibits an inhibitory effect on *S. melilot*i growth in vitro, the results of MtN5 silencing and overexpression rule out a putative role of MtN5 in the limitation of rhizobia infection and spreading. This is the first demonstration that a gene coding for a lipid transfer protein is required for the successful invasion of host roots by rhizobia. Our results also concur with the fact that *MtN5* does not belong to those genes that are downregulated in the supernodulating *M. truncatula* mutant TIR122 (El Yahyaoui et al. 2004).

The timing of expression of MtN5 might indicate a putative role of this protein in the early phases of symbiotic interaction. Interestingly, phospholipids also seem to act as signal molecules in the establishment of rhizobial symbiotic association (Charron et al. 2004). More recently, it has been shown that lyso-phosphatidylcholine is involved in the signaling pathway of the arbuscular mycorrhizal symbiosis (Drissner et al. 2007). We have demonstrated here that MtN5 is able to bind lysolipids and, thus, might be involved in lipid-mediated signaling processes during symbiosis. The apoplastic localization of MtN5 is in accordance with a possible role in the exchange of signals between the host plant and the *Rhizobium* sp. It is remarkable that MtN5 shares a high degree of homology with DIR1 an LTP that takes part in the signaling cascade activated by pathogen infection in *A. thaliana*. However, considering that MtN5 is also present in mature nodules, it cannot be excluded that it might have a role in later phases of nodule development too.

There is experimental evidence suggesting that LTP could also function as lipid carriers from the intracellular compartment toward the apoplast; for instance, during the synthesis of the cuticle (Cameron et al. 2006). Following from this idea, we can speculate that MtN5 might also be involved in the targeting of new lipidic materials during the stages of both infection thread and symbiosome development that require reorganization and de novo formation of membranes.

The results herein presented also demonstrate that the accumulation of MtN5 protein is rapidly induced by the infection of a fungal root pathogen. The induction of MtN5 protein upon *F. semitectum* infection might suggest that MtN5 can exert a defensive role against fungi that attack the root apparatus. It was shown that plant LTP can exert their antimicrobial activity either via permeabilization of a pathogen's plasma membrane (Regente et al. 2005; Diz et al. 2006) or as lipid sensors in defense signal pathways, as is the case with *A. thaliana* DIR1 (Maldonado et al. 2002). Lipid molecules, such as oxolipins (JA), phosphatidic acid, and N-acylethanolammines, are produced by plants as a consequence of pathogen attacks and act as second messengers in plant defense signaling (Wasternack and Parthier 1997; Chapman 2000; Munnik 2001). Because MtN5 is not induced by either *X. campestris* infection in vivo or wounding (data not shown), our findings also suggest that the expression of MtN5 protein is not a generic response to biotic stresses but is restricted to those microorganisms that interact with plants at root level. The persistence of MtN5 in mature nodules could represent a form of rapid protection of nodules against fungal attacks.

Further studies are needed to get a clear picture of the role played by this gene during symbiotic interaction between *M. truncatula* and *S. meliloti*. In particular, it would be interesting to study the localization of MtN5 both within the root nodule and in the root tissue during the different stages of rhizobia infection, and to identify which phases of the nodulation process MtN5 takes part in.

In conclusion, our results indicate that MtN5 exhibits biochemical features typical of plant LTP and displays similarity to type 2 LTP. Furthermore, we have demonstrated that MtN5 is specifically expressed in root nodules and required for the successful establishment of the symbiotic interaction between *M. truncatula* and *S. meliloti*.

Based on the observation that MtN5 is also induced by a pathogenic microorganism, we can speculate that this protein might have a dual function, participating in both the defense of roots and nodules against pathogens and in the establishment of a symbiotic interaction with rhizobia.

MATERIALS AND METHODS

Bacterial strains.

S. meliloti 1021 (Meade et al. 1982) was grown at 28°C in LBMC medium (tryptone at 10 g/liter, yeast extract at 5 g/liter, NaCl at 10 g/liter, 2.6 mM $MgSO_4$, and 2.6 mM $CaCl_2$) supplemented with streptomycin at 200 µg/ml. *Agrobacterium rhizogenes* ARqua1 (Quandt et al. 1993) was grown at 28°C in TY medium (tryptone at 5 g/liter, yeast extract at 3 g/liter, and 6 mM $CaCl_2$, pH 7.2) supplemented with streptomycin at 100 µg/ml. *F. semitectum* ISCF20a is a wild-type field isolate (Zaccardelli et al. 2006) and it was grown on solid-medium potato dextrose agar (PDA) (Sigma, St. Louis). *X. campestris* pv. *alfalfae* was grown on YDA medium (glucose at 10 g/liter, yeast extract at 5 g/liter, peptone at 5 g/liter).

Construction of recombinant plasmid and protein expression.

MtN5 coding sequence (accession no. Y15371) was amplified using, as template, cDNA obtained by reverse transcribing mRNA extracted from *M. truncatula* roots. The upstream primer was 5′-CATATG*CATCATCATCATCATCAC*GTTCAAA TATGTAACATAGACCCAAATGAT-3′ (*Nde*I site is underlined and His-tag is in italics) and the downstream primer was 5′-GGATCCTTAACAGTTGGAAGGTGTTTG-3′ (*Bam*HI site is underlined). The PCR product corresponding to the coding sequence of mature MtN5 was double digested with *Nde*I and *Bam*HI, cloned into pET12b (Novagen, Madison, WI, U.S.A.), and checked by sequencing. The recombinant vector pET12-N5 was mobilized into the host strain *E. coli* BL21 DE3 pLysS.

MtN5 protein was purified from inclusion bodies using strong denaturing conditions (20 mM Tris HCl, pH 8.0; 0.5 M NaCl; 5 mM imidazole; and 6 M guanidine hydrochloride) and loaded on a Hi-trap column. MtN5 was refolded, applying a linear gradient from 6 to 0 M guanidine hydrochloride. The recombinant protein was used to produce polyclonal antibodies in rabbit (PRIMM s.r.l.; Milano, Italy).

RNAi plasmid construction.

The RNAi *hp* construct was built following the construct design previously described (Pandolfini et al. 2003). The arms of the *hp* construct consist of two 200-bp-long fragments homologous to the 5′ end of the *MtN5* coding sequence from base +93 to +292 of the mRNA sequence (Y15371) placed in inverted orientations. The chimeric gene was cloned into both the pBIN19 and pRedRoot (Limpens et al. 2004) binary vectors and the recombinant plasmids were mobilized into *A. rhizogenes* ARqua1.

In order to build the gene construct for *MtN5* overexpression, the coding sequence corresponding to the mature protein was PCR amplified (primers used: 5′-GGTACCATGGCACAT TCTCAGGGCAA-3′ and 5′-GGATCCTTAACAGTTGGAAG GTGTTTG-3′), subcloned in pGemT (Promega Corp., Madison, WI, U.S.A.), and checked by sequencing. The *MtN5* coding region was *Kpn*I-*Bam*HI cloned in pRedRoot in between the

constitutive promoter *35S* from *Cauliflower mosaic virus* and the nopoline synthase terminator sequence. The recombinant vector was mobilized into *A. rhizogenes* ARqua1.

Plant growth and rhizobia inoculation.

M. truncatula cv. Jemalong seeds were scarified and sterilized in 5% commercial bleach for 3 min. Seeds were germinated on 0.8% agar plates in a growth chamber at 25°C for 7 days. Inoculation experiments were carried out using plants grown in pots as previously described (Pii et al. 2007). For the time course assay, germinated seedlings of *M. truncatula* were placed in square petri plates containing slanted BMN agar medium (Engstrom et al. 2002) supplemented with 0.1 µM L-α-2-aminoethoxyvinyl glycine (AVG). The plates were kept vertically in a growth chamber at 25°C and a regimen of 10 h of light and 14 h of darkness. Seven days after germination, seedlings were microflood inoculated. Bacteria were grown overnight in liquid LBMC medium, collected by centrifugation, and suspended in 10 mM $MgSO_4$ to an optical density at 600 nm of 0.2. Microflood inoculation was performed by placing five drops (0.5 µl) of bacterial suspension on the surface of the root. Root apparatuses were collected 1, 3, 5, 7, and 14 dpi.

Plant infection.

F. semitectum strain ISCF 20 (Zaccardelli et al. 2006) was grown on solid-medium PDA (Sigma) for a week. Ten plugs (6 mm in diameter) excised from the margin of the fungus colony grown in the petri plate were transferred to an Erlenmeyer flask containing 270 g of sterile sand, 30 g of corn meal, and 60 ml of water. After incubation for 3 weeks at 25°C, the fungal suspension was diluted at 10 and 50% (vol/vol) with double-sterilized soil. At 30 days after rhizobia inoculation, the plants, grown on sand and perlite as previously described (Pii et al. 2007), were transferred to pots containing double-sterilized soil at the bottom; the root apparatuses were covered with the diluted fungal suspension. Plants were collected 48 h after infection for further analysis.

Plant transformation.

Transformation with *A. rhizogenes* ARqua1 was performed as previously described (Boisson-Darnier et al. 2001). Plants infected with ARqua1 were kept in square petri dishes containing Fåhraeus Modified Medium (1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 0.5 mM NH_4NO_3, 50 µM FeNaEDTA, $MnSO_4$, $CuSO_4$, $ZnSO_4$, H_3BO_3, and Na_2MoO_4 each at 0.1 mg/liter) for approximately 3 weeks. When the binary vector employed in the transformation was pBIN19, FMM was supplemented with kanamycin at 50 µg/ml for transformants selection. Well-developed hairy roots were analyzed by PCR for the presence of the *hp* gene construct. Approximately 95% of the roots tested were positive for the gene construct insertion. The expression of the *hp* construct was checked by RT-PCR. Total RNA was isolated by using RNeasy plant mini kit (Qiagen, Basel, Switzerland). RNA (0.5 µg) extracted from hairy roots was treated with 0.5 units of RQ1 DNase (Promega Corp.) and then used as a template for a reverse-transcriptase (Superscript II; Invitrogen, Carlsbad, CA, U.S.A.) reaction primed with the oligo-dT. The complementary DNA was amplified with the forward primer 5′-CTGCG GTTACAAGTCCCTAAC-3′, annealing on the second arm of the hp construct, and with the primer 5′-GCCAAGCTTGCGC GATGAAATCAAGTATCCAGT-3′, complementary to the 5′ region of the termination sequence. In the pRedRoot, the antibiotic resistance-encoding gene was replaced with the gene encoding for the fluorescent protein DsRED1, which provides a nondestructive selectable marker that allows the discrimination of transgenic roots from nontransformed ones, thus avoiding the use of antibiotics for transgenic root selection (Limpens et al. 2004). Transformed roots were checked using the Leica MZ16F fluorescence microscope using an appropriate filter setting for DsRED1 detection.

For nodulation experiments, plants were nitrogen starved on BMN medium supplemented with 0.1 µM AVG for 7 days and microflood inoculated as described above.

qRT-PCR.

The qRT-PCR analyses were carried out as already described (Pii et al. 2007). The nucleotide sequences of the primers used for the qRT-PCR are the following: *MtN5*, forward primer 5′-A TGGCACATTCTCAGGGCAA-3′ and reverse primer 5′-GGT TTCTACCGGTAACGAATT-3′; actin, forward primer 5′-AGA TGCTGAGGATATTCAAC-3′ and reverse primer 5′-GTATGA CGAGGTCGGCCAAC-3′. The couple of primers used to analyze the expression of *MtN5* in hairy roots was specifically chosen at the 3′ end of the transcript to avoid the amplification of sequences derived from the hp construct itself. The nucleotide sequences of the primers used are the following: forward primer 5′-CTGCGGTTACAAGTCTGCCCTAAC-3′ and reverse primer 5′-GCGGATCCTTAACAGTTGGAAGGTGTTT G-3′.

Western blot analysis.

Total proteins were extracted by grinding frozen tissues in homogenizing buffer (30 mM Tris-HCl, pH 8.2; 50 mM KCl; 0.5% Tween 20; 0.1% polyvinyl-polypyrrolidone; and 1 mM EDTA) supplemented with 0.04% 2-mecaptoethanol and 0.1% plant protease inhibitor cocktail (Sigma). The soluble fraction was obtained by centrifugation at 12,000 × *g* for 20 min and the protein concentration was determined by the Bradford method (Bradford 1976). Proteins were acetone precipitated, separated by a 15% Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electroblotted to polyvinylidene diflouride membrane (Amersham, Tokyo). The membrane was probed with the primary polyclonal antibody against MtN5 and, afterward, treated with the alkaline phosphatase-conjugated secondary antibody. The immunoblot was placed in the alkaline phosphatase reaction buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; and 50 mM $MgCl_2$) containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as color-developing reagent.

Lipid-binding assay.

Lipid-binding capacity of MtN5 was assayed by monitoring tyrosine fluorescence. MtN5 protein was dissolved at a suitable concentration in 20 mM TrisHCl, pH 7.8, and 50 mM NaCl. Changes in fluorescence intensity were measured at 25°C with a Jasco FP-777 spectrofluorimeter using an excitation wavelength at 275 nm and recording emission between 280 and 340 nm. Lyso-phosphatidilcholine dissolved in ethanol was added in a stepwise manner and fluorescence changes were measured after 2 min of equilibration.

Antimicrobial activity.

Antimicrobial activity of the MtN5 protein was assayed by microspectrophotometry on liquid cultures grown in microtiter plates as described previously (Broekaert et al. 1990). Briefly, in a well of a 96-well plate, 50 µl of microorganism (*X. campestris*, *F. semitectum*, and *S. meliloti*) cultures at a concentration of 10^4 CFU/ml was mixed with MtN5 recombinant protein at different concentrations. The plates were incubated for 72 h at a suitable temperature according to the type of microorganism. The antimicrobial effect was estimated as the difference between the optical density, measured at 595 nm, of the treated sample and that of the untreated sample.

Statistical analysis.

The mean values ± standard error are reported in the figures. Statistical analyses were conducted using a Student's *t* test.

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