The MtMMPL1 Early Nodulin Is a Novel Member of the Matrix Metalloendoproteinase Family with a Role in Medicago truncatula Infection by Sinorhizobium meliloti$^1$[W][OA]

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We show here that MtMMPL1, a Medicago truncatula nodulin gene previously identified by transcriptomics, represents a novel and specific marker for root and nodule infection by Sinorhizobium meliloti. This was established by determining the spatial pattern of MtMMPL1 expression and evaluating gene activation in the context of various plant and bacterial symbiotic mutant interactions. The MtMMPL1 protein is the first nodulin shown to belong to the large matrix metalloendoproteinase (MMP) family. While plant MMPs are poorly documented, they are well characterized in animals as playing a key role in a number of normal and pathological processes involving the remodeling of the extracellular matrix. MtMMPL1 represents a novel MMP variant, with a substitution of a key amino acid residue within the predicted active site, found exclusively in expressed sequence tags corresponding to legume MMP homologs. An RNA interference approach revealed that decreasing MtMMPL1 expression leads to an accumulation of rhizobia within infection threads, whose diameter is often significantly enlarged. Conversely, MtMMPL1 ectopic overexpression under the control of a constitutive (35S) promoter led to numerous abortive infections and an overall decrease in the number of nodules. We discuss possible roles of MtMMPL1 during Rhizobium infection.

Rhizobium infection and nodulation are the two key developmental processes involved in establishing the nitrogen-fixing symbiosis between legume plants and the appropriate soil microsymbiont. Both of these processes require Nod factors, lipo-chito-oligosaccharide molecules that are synthesized by rhizobia in response to root-secreted flavonoids and play an essential role in the activation of symbiotic genetic programs in specific host plants (for review, see Oldroyd and Downie, 2004; Riely et al., 2004; Stacey et al., 2006). The plant responses to Nod factors and rhizobia integrate various internal cues, notably nutritional (particularly regarding carbon and nitrogen status), developmental (only specific root regions are responsive), and auxin signals. Indeed, both the location and the number of nodules and infections are tightly controlled by the plant at least two mechanisms, either ethylene dependent or independent, which lead to a superinfection/supernodulation phenotype when altered by mutations (skl, affected in ethylene perception [Penmetsa and Cook, 1997; Penmetsa et al., 2003]; har1 and sum, affected in a Leu-rich repeat receptor kinase gene [Krusell et al., 2002; Schnabel et al., 2005]).

In Medicago truncatula, as in many other temperate legumes, Rhizobium infection takes place via plant cell structures called infection threads (ITs), which form in curled, infected root hairs in the presence of Rhizobium, probably following Nod factor-dependent cytoskeleton reorganization (for review, see Brewin, 2004; Gage, 2004). The IT initially develops an invagination of the root hair wall, thereby producing an inwardly growing cylinder of wall material bounded by a membrane and containing the bacteria embedded in a matrix. In addition to bacteria and secreted bacterial products, such as exopolysaccharides (EPSs) and lipopolysaccharides (LPSs), the IT lumen contains many plant components in common with the extracellular matrix (ECM; Rae et al., 1992; Wisniewski et al., 2000; Rathbun et al., 2002), such as extensins and other...
In legumes with indeterminate nodules, like *M. truncatula*, ITs first grow inward as a branched network from the root hairs to the newly divided cortical cells of the nodule primordium, following the pathway created by preformed cytoplasmic columns (named pre-ITs) in aligned activated cells. Once the nodule meristem differentiates within this primordium (about 3–4 d post-inoculation [dpi] in *M. truncatula* A17) and the nodule starts to grow continuously out from the root, new IT branches grow outward and behind this apical meristem (Monahan-Giovanelli et al., 2006). The newly divided cells generated by the nodule meristem (zone I) are then infected within the adjacent so-called infection zone II, where bacteria are liberated from unwalled outgrowths of ITs termed infection droplets (Brewin, 2004). The mechanism for bacterial release within nodules might partly overlap with that proposed for IT initiation and progression, and depend on localized plant cell wall degradation and accumulation of osmotically active compounds leading to swelling of the IT droplet (for review, see Timmers et al., 2005). After release, rhizobia, now called bacteroids, remain surrounded by a plant membrane, the peribacteroid (or symbiosome) membrane, with features of the host plasma membrane and additional specific components. Nitrogen fixation takes place in the nodule zone III and requires the coordinated differentiation of both bacteroids and their host plant cells. A senescent zone IV, in which nodule cells and bacteroids undergo degradation, forms in older nodules proximally to zone III, usually about 4 weeks post-inoculation in *M. truncatula* A17.

A number of infection-defective mutants have been identified in the macro- and microsymbionts, showing that both partners are involved in this process. Plant mutants affected in early infection stages (IT initiation or progression) have been reported in pea (*Pisum sativum*; Tsyganov et al., 2002), *Lotus japonicus* (Bonfante et al., 2000; Tansengco et al., 2003; Yano et al., 2006), and *M. truncatula* (Catoira et al., 2000; Limpens et al., 2003; Kuppusamy et al., 2004). From these, only *lyk3* has been cloned, encoding a putative Nod factor receptor kinase (possibly a component of the so-called entry receptor; Limpens et al., 2003). Plant mutants affected in bacterial release have also been described in pea (Tsyganov et al., 1998; Morzhina et al., 2000), *L. japonicus* (Imaizumi-Anraku et al., 1997), and *M. truncatula* (Veereshlingam et al., 2004). Because most of the corresponding genes have not yet been cloned, important information about the way the plant controls ITs is still lacking. In addition, RNAi constructs knocking down the expression of a SymRK receptor-like kinase were also shown to alter the bacterial uptake process in *Sesbania rostrata* (Capoen et al., 2005) and *M. truncatula* (DM12; Limpens et al., 2005).

Other sources of information rely on the identification of plant genes whose expression is up-regulated during *Rhizobium* infection in roots or in the nodule infection zone, such as *MtLEC4* (Mitra and Long, 2004), *MtENOD11*, and *MtENOD12* (Pichon et al., 1992; Journet et al., 2001); *MtN1* and *MtN6* (Gamas et al., 1996, 1998); or *PsRNE1* (Rathbun et al., 2002). However, the lack of mutants (or knock-down constructs) in these genes has so far hampered direct investigations of their function. We show here that *MtMMPL1*, a *M. truncatula* gene identified by transcriptomics, also accompanies the *Rhizobium* infection process. Our attention was attracted to this gene because it encodes a putative protein belonging to the large matrix metalloendopeptidase (MMP) family, of particular interest in relation to developmental processes, notably invasion related.

MMPs are structurally related endopeptidases thought to play a key role in the breakdown of the ECM. Their importance is well documented in animals in the context of many normal biological processes involving remodeling of connective tissues (e.g. embryonic development, organ morphogenesis, angiogenesis, wound healing, and apoptosis), as well as pathological processes (notably arthritis, cancer metastasis, and inflammation; for review, see Nagase and Woessner, 1999). In animals, MMP expression is tightly regulated both at the transcript level (by growth factors, hormones, chemical agents, or physical stress) and protein level (by the control of their activation from precursor proteins and also by endogenous inhibitors; Nagase and Woessner, 1999).

Several MMPs have been described in plants, with different but still unclear functions and without determining their physiological substrates. The soybean (*Glycine max*) SMEP1 protein was the first plant MMP to be purified (Graham et al., 1991; McGeehan et al., 1992) and found to be transcribed only in mature leaves (Pak et al., 1997). Five MMP genes (*At1/5-MMP*) were then shown to be expressed with different patterns in Arabidopsis (*Arabidopsis thaliana*), with no hint of their possible function (Maidment et al., 1999). An insertion mutant in *At12-MMP* was later characterized and exhibited alterations in plant growth and development, notably with late flowering and early senescence (Golldack et al., 2002). Another MMP gene, *Cs-1MMP*, was identified in cucumber (*Cucumis sativus*) from a screen of genes up-regulated during programmed cell death in cotyledons (Delorme et al., 2000). Finally, a soybean gene, *GmMMP2*, was found to be activated during compatible and incompatible interactions with the oomycete pathogen *Phytophthora sojae* or the bacterial pathogen *Pseudomonas syringae pv glycinea* and suggested to be involved in a novel defense mechanism (Liu et al., 2001).

Considering the importance of ECM and tissue remodeling during nodulation and infection, we decided to further characterize *MtMMPL1* as an interesting example of a plant MMP and to make use of reverse genetics tools recently made available for *M. truncatula* to explore its possible function during symbiosis.
RESULTS

MtMMPL1: A Novel Variant of the MMP Family

MtMMPL1 is a *M. truncatula* nodulin gene that was first identified by a subtractive screen of a young nodule cDNA library (designated at that time MtIN9; Gamas et al., 1996). Subsequently, *MtMMPL1* was reidentified following large-scale EST sequencing approaches, as a cluster of nodule-specific ESTs (designated MtC40019 in the Medicago EST Navigation System [MENs] database and TC95584 in The Institute for Genomic Research [TIGR] MtGI database), and by macroarray/microarray analyses of nodule versus root gene expression (El Yahyaoui et al., 2004). BLASTN interrogation against analyses of nodule versus root gene expression ([TIGR] MtGI database), and by macroarray/microarray bases is Gm-MMP2 (62% conserved amino acids, protein. Its closest homolog in the SP/TrEMBL databases revealed sequences, with one Glu-to-Pro frequency among soybean ESTs, with one Glu-to-Pro

The Role of MtMMPL1 in Rhizobial Infection

Because the Glu-to-Gln variant had been confirmed in several *M. truncatula* MMP-like proteins, we then looked for similar variants in other species, using public nucleotide and protein databases. We first conducted a ScanProsite search on the SP and TrEMBL databases (release 50.2 and release 33.2, respectively) to identify proteins containing the following motif: [VAI]-A-[AMTLV]-H-Q-[FLIV]-G-H-[ALVIS]-L-G-[LM]-X-H-S. All four hits found (for an approximate no. of expected random matches of 3.6 e\(^{-88}\)) were from *M. truncatula*, and three as ESTs (MtC40019 = MtMMPL1, MtD00669, and MtD00924 clusters, containing ESTs from various cDNA libraries; see Supplemental Fig. S2). By comparison, when performing a search with the same sequence except with the Gln residue replaced by a Glu residue, 288 hits were obtained in a variety of plant and animal species.

To explore a larger range of species, we then carried out a TBLASTN search on dbEST (36,649,443 sequences), using a 31-amino acid sequence centered on the MMP active site (WDLETVMHQIQLLGDHSSDVEISMYPTI). A total of 502 hits were found in ESTs from 52 species (seven animals and 45 plants, including trees, monocots, and dicots), among which 101 corresponded to the variant (Gln) site. These Gln variants were found exclusively in legume species (one from *Trifolium pratense*, 14 from *M. truncatula*, and 86 from soybean). These came from 17 cDNA libraries (one from *T. pratense*, 10 from *M. truncatula*, and six from soybean) and often, but not always, corresponded to stress responses (e.g. response to salicylic acid, with 19 soybean ESTs). Legume MMP ESTs with a nonvariant (Glu) site were also found but overall with a lower frequency (one from *T. pratense*; six from *M. truncatula*, but not in the libraries where the Gln variants were found; 31 from soybean). In addition, two other active site variants were found at a very low frequency among soybean ESTs, with one Glu-to-Pro and one Glu-to-Asp substitution.
A phylogenetic tree analysis was carried out on all predicted plant MMP proteins or protein fragments containing at least the C switch and the catalytic domain (in total, 38 sequences from 18 plant species). In addition, this analysis included eight representatives of animal MMPs belonging to the same subfamily (M10B in PROSITE nomenclature). To compare fragments of similar lengths and therefore generate a more reliable tree, only sequences spanning the region from the Cys switch to the Met turn were considered. Figure 2 presents the resulting tree, showing clearly that the eight animal MMPs form a subgroup separated from the plant MMPs and that MtMMPL1 stands in a group of closely related proteins, which contains the six (Gln) legume MMP variants as well as GmMMP2. The fact that the soybean SMEP1 protein does not belong to this group indicates that this group does not simply correspond to legume sequences and suggests a very distinct function for SMEP1. None of the proteins of this group possesses the binding site for a calcium ion and a second Zn ion, known as structural Zn, found in many MMPs (Massova et al., 1998). The precise function of this site in plant MMPs is unclear, however, because the metalloproteinase activity of GmMMP2 was experimentally confirmed (Liu et al., 2001). All plant MMPs identified so far carry one conserved Glu residue located just upstream of the predicted catalytic site (Fig. 1), never found in animal MMPs (generally carrying a Phe or a Leu residue at this position). The fact that this residue seems to be conserved may indicate that it is part of the active site in plants.

MtMMPL1 Transcription Is Specifically Associated with Sinorhizobium meliloti Infection

Using quantitative reverse transcription (qRT)-PCR analysis, we compared MtMMPL1 transcript levels in symbiotic and nonsymbiotic conditions. MtENOD11, a repetitive Pro-rich protein gene induced by purified Nod factors and by S. meliloti infection (Journet et al., 2001; Boisson-Dernier et al., 2005), was used as a positive control for all these qRT-PCR studies. Consistent with MtMMPL1 EST distribution, we could not detect significant MtMMPL1 transcripts in wild-type M. truncatula shoots, stems, flowers, or seed pods (data not shown), while we confirmed a strong MtMMPL1 induction in young nodules (Fig. 3A) using a sensitive method, MtMMPL1 expression could not be detected in Nod factor-treated root samples, in contrast to MtENOD11 (data not shown). MtMMPL1 transcripts were detected in wild-type M. truncatula roots at 3 dpi with S. meliloti, while practically undetectable
at 1 dpi and in noninoculated roots (Fig. 3B). *MtMMPL1* transcripts were about 5 to 14 times less abundant than *MtENOD11* mRNA in infected root and nodule samples. Figure 3B also shows that, at 3 dpi, *MtMMPL1* expression was much more strongly induced in the hypernodulating *M. truncatula* mutants *sunn* (TR122 allele; Sagan et al., 1995) and *skl* (Penmetsa and Cook, 1997) than in wild-type *M. truncatula*. However, *MtMMPL1* was about 3-fold less expressed in *skl* than in *sunn*, which contrasts with *MtENOD11*, which is transcribed at a similar level in both mutants. Another difference between these two genes is that *MtMMPL1* was not expressed during symbiotic interactions between *M. truncatula* and the arbuscular...
Comparison of MtMMPL1 and MtENOD11 expression in S. meliloti-induced nodules (A) and S. meliloti-inoculated roots (B). The level of expression was monitored by real-time PCR analysis using isolated wild-type M. truncatula nodules at 4, 10, and 14 dpi, compared to noninoculated nitrogen-starved root samples (time point 0; A). Gene expression in S. meliloti-inoculated roots (at 0, 1, and 3 dpi) is compared between wild-type M. truncatula and sunn and skl supernodulant mutants. All values were normalized using an EF-1α housekeeping gene as an internal control. The error bar depicts the variation between two biological repetitions and two technical repeats.

MtMMPL1

MtENOD11

Days post inoculation

Relative expression level

0.00 0.05 0.10 0.15 0.20 0.25

0.00 0.05 0.10 0.15 0.20 0.25

Figure 3. Comparison of MtMMPL1 and MtENOD11 expression in S. meliloti-induced nodules (A) and S. meliloti-inoculated roots (B). The level of expression was monitored by real-time PCR analysis using isolated wild-type M. truncatula nodules at 4, 10, and 14 dpi, compared to noninoculated nitrogen-starved root samples (time point 0; A). B, Gene expression in S. meliloti-inoculated roots (at 0, 1, and 3 dpi) is compared between wild-type M. truncatula and sunn and skl supernodulant mutants. All values were normalized using an EF-1α housekeeping gene as an internal control. The error bar depicts the variation between two biological repetitions and two technical repeats.

To determine more precisely the spatio-temporal pattern of MtMMPL1 expression, we then carried out in situ hybridizations (ISHs) with 35S-radiolabeled MtMMPL1 antisense riboprobes (Fig. 4). As expected, hybridization with the MtMMPL1 sense probe control did not give a detectable signal (data not shown). To study early infection stages, we carried out ISH on sections of roots that had been spot inoculated with S. meliloti and harvested at 2 dpi. At this stage, under our experimental conditions, ITs just begin to form in infected root hairs, concomitantly with cell divisions in the inner root cortex. Figure 4, A and B, shows an example of an epidermal cell bearing an IT at an early stage of development, to which MtMMPL1 transcripts were found to be specifically associated in several serial sections. No MtMMPL1 hybridization signal was observed in noninfected dividing cortical cells. Once the sequence of the MtMMPL1 genomic region became available, we could confirm this expression pattern in infected roots, using a promoter::GUS fusion generated with a 2.4-kb region upstream of the MtMMPL1 start codon and introduced in M. truncatula by Agrobacterium rhizogenes-mediated root transformation (Fig. 4C).

In young nodules, MtMMPL1 transcripts located to inner nodule tissues where ITs develop and ramify within newly divided cells (Fig. 4, D–F), as previously observed with several other early nodulin genes (de Carvalho Niebel et al., 1998; Gamas et al., 1998). In older, well-differentiated nodules, MtMMPL1 transcripts were only detected in the infection zone II where bacteroids are liberated from ITs and it could clearly be seen at high magnification that the hybridization signal was closely and exclusively associated with ITs (Fig. 4, G–I).

To confirm that MtMMPL1 expression is tightly correlated to S. meliloti infection, we then analyzed situations where infection was impaired due to various mutations in bacterial or plant symbiotic genes/locus. Thus, we used an exoA mutant of S. meliloti, defective for EPS production, which shows IT abortion in root hairs and elicits small empty nodules (Yang et al., 1994). MtMMPL1 induction was drastically reduced in roots at 3 dpi, as judged by qRT-PCR (Table I) or in isolated 10-d-old nodules (microarray analyses; S. Moreau and P. Gamas, unpublished data). We suggest that the low level of residual MtMMPL1 expression was associated with abortive infections. In contrast, almost normal MtMMPL1 transcript levels were found in nodules induced by bacA and fixL mutants of S. meliloti, which are defective in nitrogen fixation but not infection defective (S. Moreau and P. Gamas, unpublished data).

We also carried out qRT-PCR analysis of MtMMPL1 expression in three early symbiotic M. truncatula mutants altered in the infection process, namely, nsp1 (B85 allele), hcl (B56 allele), and lin (D8 allele). The nsp1 mutant is unable to trigger cortical cell division and is defective in root hair curling and IT formation in response to S. meliloti (ccd−, hac+, and inf phenotype, respectively), but exhibits normal root hair deformation and very limited MtENOD11 induction in response to Nod factors (Catoira et al., 2000). The hcl mutant is hac− ccd+ (Catoira et al., 2001) and the lin mutant is ccd+ hac−, but all infections are arrested within root hairs where ITs fail to elongate (Kuppusamy et al., 2004). We found that, following S. meliloti inoculation, MtENOD11 was induced in lin and hcl roots (Table I) but not in nsp1. Using the same samples, we were unable to detect MtMMPL1 expression in nsp1 and hcl, while in lin expression was around 10-fold lower than in wild-type M. truncatula and similar to the level obtained in wild-type M. truncatula in response to S. meliloti exoA (Table I).

Taken together, these results lead us to conclude that MtMMPL1 expression is specifically associated with S. meliloti infection and triggered at the onset of IT formation.

mycorrhizal fungus Glomus intraradices under conditions where MtENOD11 was up-regulated (Journet et al., 2001; data not shown).

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RNA Interference and Ectopic Overexpression of MtMMPL1

We further investigated the functional role of MtMMPL1 by an RNAi approach. An RNAi construct, covering most of the MtMMPL1 translated and 3′ untranslated regions, was cloned into pRedRoot II. This vector, derived from pRedRoot (Limpens et al., 2004), allows transformed roots to be selected both by their resistance to kanamycin and their expression of the fluorescent DsRED1 protein. This RNAi construct led to an approximately 4-fold reduction in MtMMPL1 transcript levels by comparison with roots transformed with the empty vector roots (at 6 dpi with S. meliloti), as estimated by qRT-PCR analysis of pooled transformed roots (Fig. 5A). MtMMPL1 belongs to a multigene family, but, as judged by ESTs, no other family member is expressed at a comparable level in young nodules or nodulated roots. The phenotype conferred by the RNAi construct is thus likely to result from the alteration of MtMMPL1 expression.

The symbiotic competence of MtMMPL1 RNAi roots was compared with that of control roots (empty vector transformed). Similar kinetics of nodulation and nodule numbers were observed (Fig. 6A). MtMMPL1 RNAi nodules were clearly functional because plants
Table I. Real-time PCR analysis of MtMMPL1 and MtENOD11 expression in roots of wild-type M. truncatula, lin (D8 allele), and hcl (B56 allele) mutants, inoculated by wild-type or exoA S. meliloti

|                | A17 S. meliloti Wild Type  | A17 S. meliloti exoA  |
|----------------|---------------------------|-----------------------|
|                | T0 1 dpi 3 dpi            | T0 1 dpi 3 dpi        |
| MtMMPL1        | ND 1.3 × 10^-4 ± 4.4 × 10^-5  | ND 1.3 × 10^-4 ± 4.4 × 10^-5 |
| MtENOD11       | 5.5 × 10^-4 ± 2.5 × 10^-4  | 5.1 × 10^-3 ± 1.7 × 10^-3  |
| lin (D8)       | ND 1.3 × 10^-4 ± 4.4 × 10^-5  | ND 1.3 × 10^-4 ± 4.4 × 10^-5  |
| MtENOD11       | 5.6 × 10^-3 ± 7.8 × 10^-3  | 7.0 × 10^-2 ± 6.9 × 10^-2  |
| hcl (B56) S. meliloti Wild Type | ND 5.2 × 10^-3 ± 3.5 × 10^-3  | ND 1.1 × 10^-3 ± 9.0 × 10^-4  |

Table II. Number of S. meliloti colony-forming units recovered from MtMMPL1 RNAi-transformed nodules versus control (empty vector-transformed) nodules

| Experiment No. 1 | Experiment No. 2 | Experiment No. 3 |
|------------------|------------------|------------------|
| (n = 10) 21 dpi  | (n = 15) 21 dpi  | (n = 11) 44 dpi  |
| MtMMPL1 RNAi nodules (×10^3) | 42.8 ± 7.6    | 134 ± 24         | 355 ± 85        |
| Control nodules (×10^3) | 1.70 ± 0.6    | 2.6 ± 2.5        | 28 ± 1.0        |
| Ratio RNAi/control | 25.2          | 50.9             | 12.7             |

grew vigorously for several weeks in a medium without combined nitrogen. In fact, these nodules were slightly larger (about 1.3-fold) than control nodules in three out of the four experiments performed. However, while the overall structure of nodules appeared normal, vibratome (50 or 70 μm) and semi-thin (4 μm) nodule sections revealed that, within the infection zone, ITs were on average significantly larger in diameter in comparison to those found in control nodules (Fig. 7, A and B). These enlarged ITs were not accompanied by obvious signs of plant defense reactions such as autofluorescence and thickening of IT walls. Electron microscopic observations of ultra-thin sections (Fig. 7, C and D) confirmed the presence of very abundant (but otherwise apparently normal) bacteria filling these enlarged ITs. Bacteroid differentiation and bacterial release appeared normal in the nodule zones II and III, associated with type I to type 4 bacteroids (Vasse et al., 1990).

To explore whether IT enlargement was accompanied by a difference in bacterial viability, we counted the bacteria recovered from crushed nodules (following surface sterilization). Using nodules harvested at 21 or 44 dpi, we found a substantial increase in the number of bacterial colonies recovered from MtMMPL1 RNAi nodules in comparison to control nodules (Table II).

We also examined the phenotype of transgenic roots expressing MtMMPL1 constitutively under the control of the 35S promoter, which resulted in a more than a 1,000-fold increase in MtMMPL1 transcript accumulation in noninoculated roots (Fig. 5B). Under these conditions, the number of S. meliloti-induced nodules was decreased by about 60% (Fig. 6B). These nodules were fully functional and indistinguishable from control nodules in terms of structure or number of viable rhizobia. However, small regions of the root displayed a large number of infections aborting in the epidermis, as shown in Figure 7E, which was never seen in control roots (Fig. 7F). It thus seems that expressing MtMMPL1 before the initiation of S. meliloti infection leads to a decrease in the number of productive infections.

DISCUSSION

In this study, we investigated the possible role in the nitrogen-fixing symbiosis of a nodulin gene, MtMMPL1, which encodes a member of a family of proteins frequently involved in ECM and tissue remodeling.

MtMMPL1, a Novel and Specific Marker for S. meliloti Infection

The combined use of ISH, a promoter::GUS fusion, and infection-defective mutants allowed us to establish that MtMMPL1 transcription accompanies IT development from root hairs to the nodule primordium and then subsequently in the nodule infection zone. MtMMPL1 was not found to be induced by purified nod factors, and during the preinfection stage, unlike MtENOD11, a well-characterized early nodulin gene also activated by rhizobial infection. MtENOD11 is activated before the formation of ITs (Boisson-Dernier et al., 2005), as shown with a nodF nodL S. meliloti mutant (Ardourel et al., 1994; Catoira et al., 2000), which elicits root hair curling but no IT initiation. In contrast, we have no indication that MtMMPL1 is induced before IT formation because MtMMPL1 was not induced by the nodF nodL mutant in a S. meliloti sunn background (TR122 allele), which amplifies many nodulin gene responses (data not shown). Finally, MtMMPL1 was substantially less induced in a skl
than in a sunn mutant background, which is not the case for MtENOD11.

MtMMPL1 thus shows both common and distinct features in comparison to MtENOD11 and is therefore quite complementary, e.g. for characterizing the phenotype of early symbiotic plant mutants or for identifying cis- and trans-regulatory elements controlling infection-induced genes.

MtMMPL1 Has an Impact on IT Structure and Rhizobium Multiplication

Nodules with enlarged ITs and more viable rhizobia were observed when MtMMPL1 expression was decreased by RNAi. The bacteria recovered on plates from crushed nodules probably corresponded mostly to those present in ITs because differentiated bacteroids found in indeterminate nodules are unable to divide when cultured (Mergaert et al., 2006).

There are several reports of enlarged ITs, either due to Rhizobium or plant host mutations. Thus, alterations in the EPSs or in the LPSs of various rhizobia (R. leguminosarum bv. viciae, S. meliloti, Azorhizobium caulinodans) lead to the formation of enlarged ITs with thickened cell walls, often associated with plant defense reactions, and to the production of ineffective nodules in their plant host (pea, M. truncatula, and S. rostrata, respectively; Niehaus et al., 1993, 1998; Perotto et al., 1994; Mathis et al., 2005). Rhizobium surface polysaccharides are thought to play an important role in plant-bacteria communication, for the invasion process via IT (EPSs) and after the bacterial release stage.
(LPSs), possibly to evade plant immune responses (for review, see D’Haeze and Holsters, 2004). To our knowledge, viability of nodule bacteria was determined only in the study of an LPS- and EPS-defective mutant of \textit{A. caulinodans}, where about 100-fold fewer viable bacteria were recovered from \textit{S. rostrata} 2-month-old nodules than with wild-type \textit{A. caulinodans} (Mathis et al., 2005). Altered IT structure has also been reported for several plant mutants. Thick abortive ITs were observed in root hairs of infection-defective mutants in \textit{L. japonicus} (Yano et al., 2006), pea (Sagan et al., 1994), and \textit{M. truncatula} (TE7 mutant: Benaben et al., 1995; \textit{rpg} mutant: J.-F. Arrighi and C. Gough, personal communication). Enlarged ITs were also found in nodules exhibiting bacterial release defects, as with \textit{Mt} sym6 and \textit{nip} mutants or DMI2 RNAi lines in \textit{M. truncatula} (Tirichine et al., 2000; Veereshlingam et al., 2004; Limpens et al., 2005), or premature degradation of nodule tissues, as with \textit{Pss} sym33, \textit{Pss}ym40, or \textit{Ris} fix pea mutants (Novak et al., 1995; Tsyganov et al., 1998).

Enlarged ITs due to \textit{Mt}MMPL1 RNAi differed strikingly from these cases because they were found in fully functional nodules with an increased \textit{Rhizobium} accumulation and without any sign of plant defense responses. It could be argued that the \textit{Mt}MMPL1 RNAi used here corresponded to a weak mutant allele, for which an altered IT structure was perhaps the most easily observed phenotype. Bacterial accumulation in ITs could thus result from nonoptimal bacterial release but without problems of viability because these bacteria were wild type. Bacterial surface components are

\begin{figure}
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\caption{Modifications of \textit{S. meliloti} infections in \textit{M. truncatula} \textit{Mt}MMPL1 RNAi nodules (B and D) or in 35S::\textit{Mt}MMPL1 roots (E). A, C, and F, Control samples, transformed with an empty cloning vector. A and B, Bright-field microscopy of 4-\textmu m sections. C and D, Electronic microscopy of a section made in the nodule infection zone. E and F, Bright-field microscopy of \textit{S. meliloti} hemA::\textit{lacZ}-inoculated roots at 7 dpi after \textbeta-galactosidase activity detection (note the numerous abortive infections in E, in comparison to F). Transverse and longitudinal sections of ITs are shown by pink and black arrows, respectively (note the enlarged ITs in B and D); green arrowheads point to nucleoli. Bars = 50 \textmu m, except in C and D (5 \textmu m).}
\end{figure}
indeed likely to be important for *Rhizobium* protection (D’Haeze and Holsters, 2004) against the harsh conditions encountered in ITs, notably with abundant reactive oxygen species (Pauly et al., 2006) and possibly also defensin-like and thionin-like proteins (Gamers et al., 1998; Mergaert et al., 2003; Silverstein et al., 2005; see below). Alternatively, it may be that rhizobial accumulation in *MtMMPL1* RNAi nodules was due to a change in IT structure or internal composition, making it more hospitable for bacteria.

**Possible Roles of the *MtMMPL1* MMP-Like Protein during Rhizobial Infection**

Considering the preproprotein structure of *MtMMPL1* and the localization of the corresponding transcript, *MtMMPL1* is probably exported into the IT cell wall or lumen. The lumen matrix shares many components with the ECM (Rae et al., 1992; Rathbun et al., 2002), and therefore it is interesting to have identified a MMP-like nodulin that can influence IT structure. Because *MtMMPL1* is expressed as soon as ITs are formed, it is likely to play a role in IT growth or to be induced by this process. In the previous section, we proposed two alternative ways in which an MMP could influence rhizobial infection.

First, *MtMMPL1* could play a positive role in infection, contributing to the formation of the IT cell wall or ECM. Decreasing *MtMMPL1* expression could lead to a modified IT structure with an indirect effect on bacterial accumulation. Major proteins in the IT ECM are extensin-like Hyp-rich glycoproteins (HRGPs), hypothesized to play a role both in polar growth of the IT and in the control of bacterial divisions, which takes place only at the IT tip where the ECM is not rigidified by HRGP cross-linking (Wisniewski et al., 2000). The fact that *MtMMPL1* production needs to be tightly coordinated with other IT components could explain why overexpressing *MtMMPL1* under the control of the 35S promoter led to frequent infection defects.

Second, the primary role of *MtMMPL1* could be to control the number of infecting bacteria, for two reasons: first, no obvious defects in IT growth or bacterial release were observed in *MtMMPL1* RNAi roots, and, second, the phylogenetic tree analysis showed a higher level of homology with an MMP associated with a stress/defense reaction (Gm-MMP2) than with other plant MMPs involved in developmental processes (e.g. At2-MMP). In this respect, we note that *MtMMPL1*-like ESTs come from legume stress-response libraries (such as the response to salicylic acid in soybean). The legume host may control not only the number of productive *S. meliloti* infections (via ethylene-dependent and -independent pathways) but also the number of infecting bacteria within ITs. As mentioned above, various plant proteins induced during nodulation could have a defense-related function and be involved in this kind of control, notably extensin-like proteins and antimicrobial peptides (defensins) that could inhibit cytokinesis and induce bacteroid differentiation (Mergaert et al., 2006). In this scenario, *MtMMPL1* would be one (among several) elements involved in the fine balance between promoting and restricting rhizobial infection. Expressing *MtMMPL1* before infection has started, as in the 35S::*MtMMPL1* transgenic roots, could lead to frequent abortions and a decrease in nodule number (indirectly leading to abortive hyperinfections). Residual nodules, however, would be normal because the 35S promoter is certainly not as strong as the *MtMMPL1* promoter in the nodule infection zone.

**The Mechanism of *MtMMPL1* Activity Raises Intriguing Questions**

The key amino acid residue (Glu) known to be involved in the metalloproteinase activity of MMPs is replaced by a Gln residue in *MtMMPL1*. This Glu-to-Gln substitution prevents protease activity in human MMPs (Crabbe et al., 1994; Rowsell et al., 2002). We found this mutation not only in a cluster of *MtMMPL1*-like genes, but also within ESTs from two other legume species (soybean and *T. pratense*). This mutation appeared recently during evolution, because it seems to be legumep-specific, but it is not possible to deduce with the current data available whether the *MtMMPL1* paralogs were generated before or after *M. truncatula* speciation.

One hypothesis is that there is a compensatory mutation elsewhere in *MtMMPL1*-like genes enabling some protease activity to be restored. We could not find a collagenase activity in an *MtMMPL1* extract synthesized in vitro (data not shown), but this does not, of course, rule out possible protease activity with natural plant substrates and under the particular conditions existing in ITs. Indeed, Dow et al. (1998) showed that a *Xanthomonas campestris* metalloprotease was enzymatically active on defense-related ECM HRGPs but not on model substrates such as casein. Another possibility is that a functional *MtMMPL1* does not need an active protease site and that its substrate(s) may be bound without degradation, resulting in substrate protection from proteases of bacterial or plant origin. This would be somewhat reminiscent of Srch24, a chitinase homolog induced during nodule development in *S. rostrata* and lacking a Glu residue essential for hydrolytic activity, hypothesized to trap Nod factors to protect them or to facilitate interactions with a receptor protein (Goormachtig et al., 2001). To push speculation even further, it can be imagined that *Rhizobium*, as for *X. campestris*, produces proteases that can degrade ECM HRGPs and that *MtMMPL1* provides a way to control them. To clarify these points, it will be necessary to establish the precise subcellular localization of *MtMMPL1*, to identify protein(s) interacting with *MtMMPL1*, and to test them as possible substrates.

**CONCLUSION**

This study highlights *MtMMPL1* as a particularly useful gene for studying various aspects of the infection...
process, including IT structure. Furthermore, MtMMPL1 represents a novel variant of the MMP family. Even though its precise role remains to be elucidated, this is the first member of this biologically important protein family with a clear function in plant-microbe symbiotic associations.

MATERIALS AND METHODS

Biological Material

Sinorhizobium meliloti RCR2011 pXLGD4 (GMI 6526) and S. meliloti RCR2011 exaaA pXLGD4 (GMI 3072) were grown at 30°C in tryptone yeast medium supplemented with 6 mM calcium chloride and 10 μg mL^-1 tetracycline.

Medicago truncatula seeds were surface sterilized and germinated on inverted agar plates in the dark for 3 d at 8°C and 1 d at 20°C. Plants used for qRT-PCR were grown in aeroponic cassettes. Plant growth chamber conditions were the following: temperature, 22°C; 75% humidity; light intensity, 200 μE m^-2 s^-1; light-dark photoperiod, 16 h/8 h. Control root (T0) and nodule samples from wild-type Medicago truncatula Gaertn ‘Jemalong’ genotype A17 were prepared as described by El Yahyaoui et al. (2014). For analysis of inoculated roots (1 and 3 dpi) of M. truncatula A17, supermutagenic mutant sumo (TR122 allele; Sagan et al., 1995; Penmetsa et al., 2003) and skl (Penmetsa and Cook, 1997) A17 mutants (D8 allele; C. Gough and J.F. Arrighi, unpublished data), bol (B58 allele; Catoira et al., 2001), and np1 (B85 allele; Catoira et al., 2000), chamber and harvesting conditions were the same, but roots were grown directly on a nitrogen-free medium. In the case of Nod factor experiments, M. truncatula A17 plants were treated by 10^-4 M Nod factor after 4 d of nitrogen starvation, and whole root systems were harvested before and after 1, 3, 6, 24, and 48 h after treatment and then frozen in liquid nitrogen.

Analysis of Gene Expression by qRT-PCR

RNA was extracted using the SV total RNA extraction kit (Promega) according to the manufacturer’s recommendations. RNA quality was checked using a Bioanalyser (Agilent Technologies), and the absence of DNA contamination was verified by a PCR reaction with EF1-α primers. RT-PCR was performed using the SuperScript reverse transcriptase II (Invitrogen) according to the manufacturer’s recommendations. The primer RNase quality. qPCR was conducted on a Roche Lightcycler system (Roche Diagnostics) according to the procedure described by Boisson-Dernier et al. (2005). This method was modified by transferring the transformed plants (checked by red fluorescence at 3 weeks post transformation) to growth pouches watered with 6 mL of the mineral plant growth medium used for aeroponic cultures (Journet et al., 2001). Plants were inoculated 1 week after transfer, with 1 mL of S. meliloti suspension at an OD600 = 0.05. Pouches were watered every week alternatively with water and growth medium.

Histochemical Staining and Microscopy Studies

GUS staining (using only 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid UA) and double staining for both GUS and β-galactosidase activities after inoculation with a S. meliloti strain carrying a constitutive henh-lacZ fusion (Arseneul et al., 1994) were performed as described by Boisson-Dernier et al. (2005). For simple β-galactosidase activity, we used 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (MBI) Biomedicals instead of Magenta-Gal. Roots and nodule sections (50 μm thick) were done in 4% agarose with a vibrating microtome (Leica VT 1000S), and stained samples were observed with a Zeiss Axioskop light microscope (Carl Zeiss). Expression of IFS was repeated in three independent experiments, including one with double blind scoring of RNAi and control (empty vector-transformed) roots.

Histology of the nodule was performed after fixation in 2.5% glutaraldehyde buffered in 0.1 M sodium phosphate buffer, pH 7.2, dehydrated in an alcoholic series and embedded in Technovit 7100 resin (Heraeus Kulzer). Sections (4 μm thick) were observed after counterstaining in a 0.2% aqueous toluidine blue solution. For electron microscopy studies, following the glutaraldehyde fixation step, nodules were postfixed in 1% osmium tetroxide buffer solution; we then proceeded to epoxy embedding steps as previously described (Vasse et al., 1993). Grids were examined using a Hitachi H600 electron microscope, and images were recorded on Kodak film 4489 Estar thick base.

ISHs were carried out as described by de Billy et al. (2001).

Counting Bacteria Recovered from Nodules

Nodules were cut from roots at the indicated times. They were surface sterilized using the following procedure: immersion for 30 s in 70% ethanol, wash with distilled water (five times), immersion for 30 s in 25% (v/v) commercial bleach, and final wash with water (six times). Sterilized nodules were ground with a glass tube in 100 μL of water. Aliquots of serial dilutions (10^-5 to 10^-9 fold) were spread on tryptone yeast plates supplemented with 6 mM calcium chloride.

Phylogenetic Tree Analysis

Protein segments of similar lengths were manually defined, as indicated. Amino acid sequences were then aligned using ClustalW (http://clustalw.genome.ad.jp/) and analyzed with the PHYLIP software package (http://www.csc.fi/mobio/progs/phylip/doc/main.html). Bootstrap values were obtained from 1000 replicates. The branch lengths indicate the frequency of the corresponding clade in the set of bootstrap trees.

Sequence data from this article can be found in the GenBank/EMBL database under accession number Y18249.
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Multiple alignment analysis of putative proteins encoded by MtMMPL1 and the cluster of MtMMPL1-like genes located on the bacterial artificial chromosome clone CR962135.

Supplemental Figure S2. Electronic northern of MtMMPL1 EST cluster (Mic40019) and of two closely related EST clusters.

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LITERATURE CITED

Ardourel M, Demont N, Debeille F, Maillet F, de Billy F, Prome JC, Denarie J, Truchet G (1994) Rhizobium meliloti lipopolysaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell Environ 17: 1357–1374

Benaben V, Duc G, Lefebvre V, Huguet T (1995) TET, an inefficient symbiotic mutant of Medicago truncatula Gaertn. cv Jemalong. Plant Physiol 107: 53–62

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340: 783–795

Boisson-Dernier A, Andriankaja A, Chabaud M, Niebel A, Journet EP, El-Gachtouli N, Vernoud V, de Billy F, Pichon M, Dedieu A, Cullimore JV, Gamas P, de Billy F, Truchet G (2005) MtENOD11 gene activation during rhizobial infection and mycorrhizal arbuscule development requires a common AT-rich-containing regulatory sequence. Mol Plant Microbe Interact 18: 1269–1276

Bonfante P, Genre A, Faccio A, Martini I, Schauer L, Stougaard J, Webb J, Parniske M (2000) The Lotus japonicus LSF4m6 gene is required for the successful symbiotic infection of root epidermal cells. Mol Plant Microbe Interact 13: 1109–1120

Brewin NJ (2004) Plant cell-wall remodeling in the Rhizobium-legume symbiosis. CRC Crit Rev Plant Sci 23: 293–316

Capoen W, Goormachtig S, De Rycke R, Schroeyes K, Holsters M (2005) SrSymRK, a plant receptor essential for symbiosome formation. Proc Natl Acad Sci USA 102: 10369–10374

Catoira R, Galera C, de Billy F, Pennetsa RV, Journet EP, Maillet F, Rosenberg C, Cook D, Gough C, Denarie J (2000) Four genes of Medicago truncatula controlling components of a nod factor transduction pathway. Plant Cell 12: 1647–1666

Catoira R, Timmers AC, Maillet F, Galera C, Pennetsa RV, Cook D, Denarie J, Gough C (2001) The HCL gene of Medicago truncatula controls Rhizobium-induced root hair curling. Development 128: 1507–1518

Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernie T, Ott T, Gamas P, Crespi M, et al (2006) MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in Medicago truncatula. Genes Dev 20: 3084–3088

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16: 10881–10890

Crabbe T, Zucker S, Cockett M, Willenbrock F, Tickle S, O’Connell JP, Scouthern JM, Murphy G, Docherty AJ (1994) Mutation of the active site glutamic acid of human gelatinase A: effects on latency, catalysis, and the binding of tissue inhibitor of metalloproteases-1. Biochemistry 33: 6684–6690

de Billy F, Grosjean C, May S, Bennett M, Cullimore JV (2001) Expression studies on AUX1-like genes in Medicago truncatula suggest that auxin is required at two steps in early nodule development. Mol Plant Microbe Interact 14: 267–277

de Carvalho Niebel F, Lescure N, Cullimore JV, Gamas P (1998) The Medicago truncatula MtAN1 gene encoding an annexin is induced by Nod factors and during the symbiotic interaction with Rhizobium meliloti. Mol Plant Microbe Interact 11: 504–513

Delorme VG, McCabe PF, Kim DJ, Leaver CJ (2000) A matrix-metalloproteinase gene is expressed at the boundary of senescence and programmed cell death in cucumber. Plant Physiol 123: 917–927

D’Haese W, Holsters M (2004) Surface polysaccharides enable bacteria to evade plant immunity. Trends Microbiol 12: 555–561

Dow JM, Davies HA, Daniels MJ (1998) A metalloprotease from Xanthomonas campestris that specifically degrades proline/hydroxyproline-rich glycoproteins of the plant extracellular matrix. Mol Plant Microbe Interact 11: 1085–1093

El Yahyaoui E, Kuster H, Ben Amor B, Hohncje N, Puhler A, Becker A, Gouzy J, Vernie T, Gough C, Niebel A, et al (2004) Expression profiling in Medicago truncatula identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol 136: 3159–3176

Gage DJ (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol Mol Biol Rev 68: 280–300

Gamas P, de Billy F, Truchet G (1998) Symbiosis-specific expression of two Medicago truncatula nodulin genes, MINI1 and MINI3, encoding products homologous to plant defense proteins. Mol Plant Microbe Interact 11: 393–403

Gamas P, de Carvalho Niebel F, Lescure N, Cullimore J (1996) Use of a subtractive hybridization approach to identify new Medicago truncatula genes induced during root nodule development. Mol Plant Microbe Interact 9: 233–242

Goldack D, Popova OV, Dietz KJ (2002) Mutation of the matrix metalloproteinase cAt-MMP inhibits growth and causes late flowering and early senescence in Arabidopsis. J Biol Chem 277: 5541–5547

Goormachtig S, Van de Velde W, Lievens S, Verplancke C, Herman S, De Keyser A, Holsters M (2001) SrcH24, a chitinase homolog lacking an essential glutamic acid residue for hydrolytic activity, is induced during nodulation development on Sesbania rostrata. Plant Physiol 127: 78–89

Graham IA, Xiong J, Gillikin JW (1991) Purification and developmental analysis of a metalloproteinase from the leaves of Glycine max. Plant Physiol 97: 786–792

Imaiuzumi-Anraku H, Kawaguchi M, Koiwa H, Akao S, Syono K (1997) Two ineffective-nodulating mutants of Lotus japonicus LjSym4 suggest that auxin is required at two steps in early nodule development. Mol Plant Microbe Interact 11: 1451–1459

Inoue JT, Endre G, Prabhu R, Penmetsa RV, Veereshlingam H, Kuppusamy KT, Cook DR, Dickstein R, Vandenbosch KA (1998) The Medicago truncatula gene encoding a annexin is induced by Nod factors and during the symbiotic interaction with Rhizobium meliloti. Mol Plant Microbe Interact 11: 504–513

Journet EP, El-Gachtouli N, Vernoud V, de Billy F, Pichon M, Dedieu A, Arnould C, Morandi D, Barker DG, Gianinazzi-Pearson V (2001) Medicago truncatula ENOD11: a novel RRPP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol Plant Microbe Interact 14: 737–748

Kussell I, Madsen LH, Sato S, Aurbert G, Genua A, Szczylowski K, Duc G, Kaneko T, Tabata S, de Bruijn E, et al (2002) Shoot control of root development andnodulation is mediated by a receptor-like kinase. Nature 420: 422–426

Kuppusamy KT, Endre G, Prabhu R, Pennetsa RV, Veereshlingam H, Cook DR, Dickstein R, Vandenbosch KA (2004) LIN, a Medicago truncatula gene required for nodule differentiation and persistence of rhizobial infections. Plant Physiol 136: 3682–3691

Limpens E, Franken C, Smit P, Willemsen J, Bisseling T, Geurts R (2003) LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. Science 302: 630–633

Limpens E, Mirabella R, Fedorova E, Franken C, Fransen H, Bisseling T, Geurts R (2005) Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by DM12. Proc Natl Acad Sci USA 102: 10375–10380

Limpens E, Ramos J, Franken C, Raz V, Compaan B, Fransen H, Bisseling T, Geurts R (2004) RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula. J Exp Bot 55: 983–992

Liu Y, Dammann C, Bhattacharyya MK (2001) The matrix metalloproteinase gene GmMMP2 is activated in response to pathogenic infections in soybean. Plant Physiol 127: 1786–1797
Niehaus K, Kotra L P, Friman R, Mobasher S (1998) Matrix metalloproteinases: structures, evolution, and diversification. FASEB J 12: 1075–1095

Mathis R, van Gijsegem F, De Rycke R, D'Haeze W, van Maesaelse E, Anthonio E, Van Montagu M, Holsters M, Verecke D (2005) Lipopoly saccharides as a communication signal for progression of legume symbiosis. Proc Natl Acad Sci USA 102: 2655–2660

McGeehan G, Burkhat W, Anderegg R, Becherer JD, Gillikin JW, Mathis R, van Gijsegem F, De Rycke R, D'Haeze W, van Maelsaeke E, Pichon M, Journet EP, Dedieu A, de Billy F, Truchet G, Barker DG, Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi (1999) Matrix metalloproteinase family. Plant Physiol 99: 1179–1183

Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A, Kondorosi E (2003) A novel family in Medicago truncatula consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. Plant Physiol 132: 161–173

Mergaert G, Uchiimi T, Alunni B, Evanno G, Cheron A, Catrice O, Mausset AE, Barloy-Hubler F, Gilibert F, Kondorosi A, et al (2006) Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-legume symbiosis. Proc Natl Acad Sci USA 103: 5230–5235

Mitra RM, Long SR (2004) Plant and bacterial symbiotic mutants define three transcriptionally distinct stages in the development of the Medicago truncatula/Sinorhizobium meliloti symbiosis. Plant Physiol 134: 595–604

Monahan-Giovanelli H, Pinedo CA, Gage DJ (2008) Architecture of infection thread networks in developing root nodules induced by the symbiotic bacterium Sinorhizobium meliloti on Medicago truncatula. Plant Physiol 140: 661–670

Morzhina EV, Tsynanov VE, Borisov AV, Leksby VK, Tikhonovich IA (2000) Four developmental stages identified by genetic dissection of pea (Pisum sativum L.) root nodule morphogenesis. Plant Sci 155: 73–83

Nagase H, Woessner FJ Jr (1999) Matrix metalloproteinases. J Biol Chem 274: 21491–21494

Niehaus K, Kapp D, Pübler A (1993) Plant defence and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPS) deficient Sinorhizobium meliloti mutant. Planta 190: 415–425

Niehaus K, Lagares A, Pübler A (1998) A Sinorhizobium meliloti lipopolysaccharide mutant induces effective nodules on the host plant Medicago sativa (alfalfa) but fails to establish a symbiosis with Medicago truncatula. Mol Plant Microbe Interact 11: 906–914

Novak K, Pesina K, Nebesar Jovova J, Skrdlata V, Lisa L, Nasinec V (1995) Symbiotic tissue degradation pattern in the ineffective nodules of three nodulation mutants of pea (Pisum sativum L.). Ann Bot (Lond) 76: 303–313

Oldroyd GE, Downie JA (2004) Calcium, kinases and nodulation signalling in legumes. Nat Rev Mol Cell Biol 5: 566–576

Pak JH, Liu CY, Huanggu J, Graham JS (1997) Construction and characterization of the soybean leaf metalloproteinase CDNA. FEBS Lett 404: 283–288

Pauly N, Pucciariello C, Mandon K, Innocenti G, Jamet A, Baudouin E, Herouart D, Fredra P, Puppo A (2006) Reactive oxygen and nitrogen species and glutathione: key players in the legume-Rhizobium symbiosis. J Exp Bot 57: 1769–1776

Pemtessa RV, Cook DR (1997) A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. Science 275: 527–530

Pemtessa RV, Frugoli JA, Smith LS, Long SR, Cook DR (2003) Dual genetic pathways controlling nodule number in Medicago truncatula. Plant Physiol 131: 999–1008

Perotto S, Brewin NJ, Kannenberg EL (1994) Cytological evidence for a host defense response that reduces cell and tissue invasion in pea nodules by lipopolysaccharide mutants of Rhizobium leguminosarum strain 3841. Mol Plant Microbe Interact 7: 99–112

Pichon M, Journet EP, Dedieu A, de Billy F, Truchet G, Barker DG (1992) Rhizobium meliloti elicits transient expression of the early nodulin gene ENOD12 in the differentiating root epidermis of transgenic alfalfa. Plant Cell 4: 1199–1211

Rae AL, Bonfante P, Brewin NJ (1992) Structure and growth of infection threads in the legume symbiosis with Rhizobium leguminosarum. Plant J 2: 385–395

Rathbun EA, Naldrett MJ, Brewin NJ (2002) Identification of a family of extensin-like glycoproteins in the lumen of rhizobium-induced infection threads in pea root nodules. Mol Plant Microbe Interact 15: 350–359

Riely BK, Ame JM, Pennetsa RV, Cook DR, Sherrier DJ, Dickstein R (2004) nsp, a symbiotic Medicago truncatula mutant that forms root nodules with aberrant infection threads and plant defense-like response. Plant Physiol 136: 3692–3702

Wisniewski JP, Rathbun EA, Knox JP, Brewin NJ (2000) Involvement of diamine oxidase and peroxidase in insolubilization of the extracellular matrix in nodules of Sinorhizobium meliloti. Mol Plant Microbe Interact 13: 413–420

Yang C, Signer ER, Hirsch AM (1994) Nodules initiated by Sinorhizobium meliloti exopolysaccharide mutants lack a discrete, persistent nodule meristem. Plant Physiol 98: 143–151

Yano K, Tanzengco ML, Hio T, Higashi K, Murooka Y, Imaizumi-Anraku H, Kawaguchi M, Hayashi M (2006) New nodulation mutants responsible for infection thread development in Lotus japonicus. Mol Plant Microbe Interact 19: 801–810