The Early Nodulin Gene *MtN6* Is a Novel Marker for Events Preceding Infection of *Medicago truncatula* Roots by *Sinorhizobium meliloti*

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MtN6 belongs to a series of cDNA clones representing *Medicago truncatula* genes transcriptionally activated during nodulation by *Sinorhizobium meliloti* (P. Gamas, F. de Carvalho Niebel, N. Lescure, and J. V. Cullimore, *Mol. Plant-Microbe Interact.* 9:233-242, 1996). We show here by in situ hybridization that *MtN6* transcripts specifically accumulate first at very localized regions in the outer root cell layers, corresponding to outer cortical cells containing preinfection threads. At later stages, *MtN6* expression is observed ahead of growing infection threads, including in the infection zone of mature root nodules. Interestingly, regulation of *MtN6* is clearly distinct from that of other early nodulins expressed in the same region of the nodule, in terms of response to bacterial symbiotic mutants and to purified Nod factors. We thus suggest that *MtN6* represents the first specific marker of a pathway involved in preparation to infection, which is at least partly controlled by Nod factors. Finally, we discuss the intriguing sequence homology shown by *MtN6* to a protein from *Emericella* (*Aspergillus*) *nidulans*, FluG, that plays a key role in controlling the organogenesis of conidiophores (B. N. Lee and T. H. Adams, *Genes Dev.* 8:641-651, 1994).

Additional keywords: development, legume, symbiotic interaction.

The rhizobia-legume symbiotic interaction represents a very rich experimental system for several major issues of plant developmental biology, such as perception/transduction of molecular signals or control of organogenesis processes. Indeed, following the exchange of specific signals between the macro- and microsymbionts, a new organ can develop in legumes, the root nodule, in which atmospheric nitrogen fixation can take place (for review see Mylona et al. 1995). Critical in this process are the recognition by the plant of lipo-chitoooligosaccharides produced by the bacteria, the Nod factors (see reviews by Dénarié et al. 1996; Heidstra and Bisseling 1996; Long 1996; Schultze and Kondorosi 1996; Spaink 1996), and

the nutritional status of the host plant, which must be nitrogen starved to be fully responsive (Heidstra et al. 1997 and references therein).

Different stages can be distinguished during nodulation (for reviews see Hirsch 1992; Kijne 1992). During the preinfection stage (approximately the first 2 days following *Sinorhizobium meliloti* inoculation, in *Medicago truncatula*) root hair, epidermal, cortical, and pericycle cells respond to Nod factors (Heidstra and Bisseling 1996), notably by root hair deformation, and reactivation of the cell cycle in cortical cells (Yang et al. 1994). In temperate legumes, such as *Pisum sativum* or *M. truncatula*, cortical cell activation leads to the formation of division foci, the nodule primordia, located in the inner cortex, and of cytoplasmic bridges, also called preinfection threads (van Brussel et al. 1992), in the outer cortex. This takes place at defined locations in the root, opposite protoxylem poles, suggesting the involvement of positional cues derived from the stele (for review see Cohn et al. 1998). A following stage (about 3 days after inoculation) is the establishment in the primordium of a meristem, responsible for the development of the nodule structure. In the mean time, bacterial infection takes place by tubular structures, the infection threads, that form by invagination of the root hair cell wall about 2 days after inoculation and then grow down toward the nodule primordium, following the pathway defined by the cytoplasmic bridges. Infection is not required for nodule organogenesis, since empty (noninfected) nodules can be formed in response to bacterial *exo* mutants (deficient for exopolysaccharide production; Cheng and Walker 1998 and references therein), or spontaneously in some genotypes of nitrogen-starved *M. sativa* plants (NAR nodules, for nodulation in the absence of *Rhizobium*; Truchet et al. 1989). Infection is important, however, for the differentiation of several of the central tissues found in mature indeterminate nodules, which consist of, successively, the distal meristematic zone I, the infection zone II, the amyloplast-rich interzone II-III, the nitrogen-fixing zone III, and finally the proximal senescence zone IV. These tissues are surrounded by peripheral tissues, which include the vascularized nodule parenchyma, the nodule endodermis, and the nodule cortex (Vasse et al. 1990).

The nodulation process is accompanied by the induction of certain genes, termed nodulin genes, classified as early or late

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depending on their kinetics of expression (for review see Pawlowski 1997). Many of these genes have been identified in several legume species, and cloned by a variety of approaches (see for recent publications Cook et al. 1995; de Carvalho Niebel et al. 1998; Frugier et al. 1998; Gamas et al. 1996; Goormachtig et al. 1995; Greene et al. 1998; Heidstra et al. 1997; Szczyglowski et al. 1997). Several of them have been exploited to characterize different nodulation stages essentially at the regulation level (see for recent publications Bauer et al. 1997; Coda de la Peña et al. 1997; Heidstra et al. 1997; Gamas et al. 1998; Goormachtig et al. 1998; Pingret et al. 1998; van Rhijn et al. 1997), the challenge for coming years being to identify key players and understand their mode of action.

We previously published the identification of a series of cDNA clones representing *M. truncatula* genes transcriptionally activated during nodulation by *S. meliloti* that were designated *MtN* genes (Gamas et al. 1996). We decided to characterize in more detail one of them, *MtN6*, because partial sequencing revealed an intriguing homology to a protein from *Emericella* (*Aspergillus*) *nidulans*, FluG, that plays a key role in the control of a particular developmental process, the organogenesis of conidiophores (Lee and Adams 1994, 1995). We show in this paper that *MtN6* represents a novel marker of nodulation associated with preinfection events, and its regulation appears to be distinct from those described for other known early nodulins.

RESULTS

MtN6 is homologous to the N-terminal domain of the *Emericella nidulans* FluG protein.

Figure 1A shows the complete DNA sequence of the *MtN6* cDNA clone, found to be 1,454 bp long and thus approximately full size, considering the length of transcripts detected with an *MtN6* probe on Northern (RNA) blots (about 1.6 kb). We were able to find a corresponding genomic clone (partly shown in Figure 1B), 7.5 kb long and containing 8 introns, by screening an *M. truncatula* library (kindly provided by David Barker). Southern analysis suggested that in addition to this gene another *MtN6*-related gene exists in *M. truncatula* (data not shown).

Figure 1 also indicates that the *MtN6* sequence contains one long open reading frame (ORF), encoding a putative protein of 419 amino acids (see below, however). The extent of the homology to FluG, originally discovered with partial sequencing data (Gamas et al. 1996), was enhanced with this complete sequence, as reflected by the BLAST score shown in Table 1. Indeed, a high amino acid conservation was observed, covering 90% of the *MtN6* deduced sequence, but corresponding only to the first N-terminal half of FluG (411 out of 865 amino acids). The overall amino acid composition of the homologous regions of these two proteins was found to be similar, as well as their theoretical isoelectric point (5.8 for *MtN6* vs 5.5 for FluG). BLAST searches of nonredundant gene and protein data bases (Table 1) revealed only one other convincing homology, with a bacterial hypothetical protein called Rv1879, discovered by sequencing of the *Mycobacterium tuberculosis* genome (Cole et al. 1998), and also found to be homologous to the N-terminal half of FluG. However, searches of expressed sequence tag data bases (dbest; Table 1)

also revealed strong homologies with three plant sequences, shown in the multiple alignment analysis presented in Figure 2.

There are some uncertainties regarding the size of the *MtN6*-encoded protein(s). Indeed, three possible starting points can be envisaged, two located within the cDNA and the

Fig. 1. A, DNA and translated sequence of the *MtN6* cDNA clone. **B**, A portion of the *MtN6* gene, containing the putative translational start site(s). Possible start codons are in bold and underlined.

Table 1. Homologies found for *MtN6* by interrogation of nr and dbest data bases^a

Homologous sequences ^b	Identities	Similarities	E value ^c
<i>Emericella nidulans</i> FluG protein (P38094)	119/412 (28%)	207/412 (49%)	2 ^e -41
<i>Mycobacterium tuberculosis</i> hypothetical protein Rv1879 (Z97193)	83/256 (32%)	116/256 (44%)	9 ^e -19
<i>Oryza sativa</i> partial sequence S4994_1A (D41960)	71/123 (57%)	94/123 (75%)	7 ^e -35
<i>Arabidopsis thaliana</i> cDNA clone 64D11T7 (T41701)	54/108 (50%)	75/108 (69%)	2 ^e -22
<i>Zea mays</i> cDNA clone 6c02d01 (T18405)	44/86 (51%)	64/86 (74%)	2 ^e -17

^a blastx or tblastn (Altschul et al. 1990) searches were carried out, with default parameter values.

^b Accession numbers are indicated in parentheses.

^c Probability of finding the indicated homologies by chance.

third on the additional 5' sequence present on the cloned genomic DNA, all of them in the same reading frame. Figure 1A shows the cDNA sequence with its two possible start codons in bold, the second being 38 residues downstream from the first, at a position similar to the one proposed for FluG. Figure 1B also indicates the third possible start codon, located only 4 amino acid residues upstream of the beginning of the longest cDNA ORF. It should be pointed out that although in eukaryotes the preferential initiation site is generally the one located closest to the 5' end of the RNA, start codons deviating at one or more crucial positions from the optimal

sequence context may allow the passage of ribosomes to further downstream start sites (leaky scanning; Fütterer and Hohn 1996). Interestingly, in the case of *MtN6*, the two nucleotide residues considered to be crucial around the AUG codon (a purine at -3, and a guanine at +4; Fütterer and Hohn 1996; Kozak 1997) are present only in the most downstream potential start site. Moreover, it should be mentioned that coupled in vitro transcription-translation of the *MtN6* cDNA with wheat germ extracts produced two proteins in similar amounts, whose sizes were consistent with the two possible start codons found in the cDNA (data not shown).

Fig. 2. Multiple alignment analysis of sequences homologous to the *MtN6* putative protein. M.t.1879 = *Mycobacterium tuberculosis* sequence; A.t. EST, O.s. EST, and Z.m. EST = translated EST (expressed sequence tag) sequences from *Arabidopsis thaliana*, rice, and maize, respectively. This alignment was generated with the Multalin program (Corpet 1988) with default parameter values, and the longest open reading frame found in the *MtN6* cDNA. Homologies are defined here as identities, and similarities with a score equal or superior to +2 in the Multalin alignment matrix (blossom 62).

***MtN6* is a nodulin gene activated early during *S. meliloti*-induced or spontaneous (NAR) nodule organogenesis.**

Initial characterization of *MtN6* expression established that this gene is transcribed at a low background level in roots, and induced in nodules as well as in *S. meliloti*-inoculated *M. truncatula* roots, as early as 24 h post inoculation (p.i.; Gamas et al. 1996). In this study we explored whether expression could also be observed in other organs. Figure 3A shows that no induction could be detected by Northern blotting analysis in stems, flowers, and leaves. Moreover, we found that *MtN6* expression was not triggered in leaves by wounding or inoculation with compatible or incompatible bacterial pathogens (*Xanthomonas campestris* pv. *alfalfae* and *Pseudomonas syringae* pv. *pisi*, respectively; data not shown).

Fig. 3. Northern (RNA) analysis of *MtN6* expression. Approximately 6 µg of total RNA was loaded per lane. Bottom lane shows the ethidium bromide-stained 28S rRNA band, after transfer of total RNA onto the blotting membrane. **A**, RNA extracted from different *Medicago truncatula* organs. **B**, RNA extracted from the *M. sativa* cortical cell division/nodulation in the absence of *Rhizobium* (CCD/NAR)⁺ line A279, and from the (CCD/NAR)[−] line 1F. First five lanes are an analysis of root samples; the t₀ lane corresponds to samples harvested when switching to the nitrogen-free medium; 3 and 14 dps correspond to samples harvested after 3 and 14 days of nitrogen starvation. Emerging NAR nodules were manually removed from the 14-day root sample, to focus the analysis on nodule primordia. Last two lanes show an analysis of the RNA extracted from isolated spontaneous NAR nodules, harvested 14 or 24 days after switching to the nitrogen-free medium.

Gamas et al. (1996) also showed that *MtN6*-like transcripts accumulate in spontaneous NAR nodules harvested after 33 days of nitrogen starvation. We thought that it would be of interest to study the kinetics of *MtN6* expression during early stages of NAR induction. We carried out a time course analysis in an *M. sativa* line called A279, particularly active for spontaneous cortical cell divisions (CCDs) and NAR organogenesis (Grosjean and Huguet 1997), two processes that are found in the same region of the root and probably represent two successive developmental stages. We used as a control a second alfalfa line, called 1F, in which spontaneous nodules or division foci are rarely observed (Grosjean 1997). Four types of samples were harvested and analyzed: (i) root samples collected 3 days after nitrogen starvation, at a time when only a few division foci could be observed in the root inner cortex; (ii) root samples at 14 days of starvation, when numerous nodule primordia were present in A279; (iii) isolated NAR nodules after 14 days of starvation, i.e., when they were still small (about 1 mm in diameter), just emerging from the roots; and (iv) NAR nodules after 24 days of starvation, when they were well developed and often multilobed.

Using this material, we compared the expression patterns of the *MtN6* *M. sativa* homologue with those of two well-characterized early nodulin genes expressed in NAR nodules, namely, *ENOD40* (Crespi et al. 1994) and *MscA1* (Coba de la Peña et al. 1997), orthologous to *MtN10* (Gamas et al. 1996). Figure 3B shows that the *M. sativa* *MtN6* homologue was expressed very early in the context of spontaneous CCD/NAR organogenesis, since out of the three tested markers it was the only one exhibiting clear transcript accumulation in root tissues following 3 days of nitrogen starvation.

***MtN6* is a novel marker for root cortical cell activation and the nodule preinfection zone.**

Having established that *MtN6* is an early nodulin gene, we then decided to determine the tissue specificity of its expression. To explore the early stages of the symbiotic interaction, we made use of *S. meliloti* spot inoculation coupled with in situ hybridization. Cytological analysis indicated that in our conditions *M. truncatula* roots responded similarly to spot-inoculated alfalfa (Dudley et al. 1987). Root samples were collected approximately 24, 55, and 96 h p.i., and 7-µm-thin sections were hybridized with a ³⁵S-labeled *MtN6* antisense riboprobe.

At 24 h p.i., the first foci of dividing cells could be observed in the inner cortex (data not shown), and in the pericycle (as mentioned in Hirsch and LaRue 1997, and in agreement with a detailed analysis recently carried out in our laboratory: A. Timmers, M.-C. Auriac, and G. Truchet, *personal communication*). No sign of infection could yet be detected. Figure 4A–C shows that at this time point a strong *MtN6* hybridization signal was observed at very defined locations, exclusively in the outer cell layers of the root (Fig. 4B, double arrow head). Examination of serial root sections established that this signal was limited to a few cells, which were obviously not dividing but nevertheless showed features of cell activation, namely, a large central nucleus and amyloplast accumulation (data not shown). To make sure that this highly localized pattern of expression was not due to the fact that the bacteria were spot inoculated, a series of root samples were analyzed 24 h after whole root system inoculation. Although a

much lower proportion of sections showed symbiotic responses, compared with spot inoculation, it is important to note that regions of *MtN6* induction were also found to be highly localized (data not shown).

Figure 4D–I depicts three serial sections of a sample harvested at about 55 h p.i., representative of the expression pattern observed in several independent samples. At this stage, newly formed infection threads can be observed in the outer

Fig. 4. In situ hybridization analysis of 7- μ m-thin root sections, with 35 S-labeled *MtN6* antisense riboprobe. **A, C, D, F, and H,** Dark-field microscopy, with the hybridization signal appearing as white dots. **B, E, G, and I,** Bright-field microscopy, with the hybridization signal appearing as black dots. Bars = 50 μ m, except in **G** and **I**, where bars = 25 μ m. **A,** Transverse section of a *Sinorhizobium meliloti* spot-inoculated root, 24 h post inoculation (p.i.). A strong signal is seen at a very localized region, in the outer cell layers of the root. **B** and **C,** Longitudinal section of an *S. meliloti* spot-inoculated root, 24 h p.i. Cell showing the *MtN6* hybridization signal is marked with a double arrowhead in **B**. **D–I,** Serial longitudinal sections of an *S. meliloti* spot-inoculated root, 55 h p.i. Arrow in **E** shows an infection thread just forming in the outer root cell layers. Arrowheads in **G** and **I** indicate cytoplasmic structures likely to correspond to preinfection threads. **D** and **E,** The same section observed by dark- or bright-field microscopy, respectively, as well as **F–G** and **H–I.** Magnification is doubled in **G** and **I**, compared with **F** and **H**.

root cell layers, as shown in Figure 4E (arrow). It was found that *MtN6* transcripts accumulate essentially one or two cell layers ahead of the extremity of these infection threads (Fig. 4D–I), and thus with time become proportionally more abun-

dant in the inner part of the root (compare Figure 4B–C and D–I), as the infection thread grows and ramifies (data not shown). Consequently, the hybridization signal, originally restricted to a few cells in the outer cortex, broadens to reach the

Fig. 5. In situ hybridization analysis of 7- μ m-thin longitudinal nodule sections, with *MtN6* antisense 35 S-labeled riboprobe. **B, C, and F,** Dark-field microscopy, with the hybridization signal appearing as white dots. **A, D, and E,** Bright-field microscopy of the same sections, with the hybridization signal appearing as black dots. Nodule meristem is indicated by a star; infection threads by arrows. **A–D,** Four-day-old nodules. Residual, nonhybridizing infection threads can be seen in the outer cortex. Bars = 50 μ m. **E–F,** Fourteen-day-old nodule. Hybridization signal is exclusively found in the infection zone II of the nodule. Bars = 83 μ m.

dividing cells of the inner cortex, seen in the lower part of Figure 4F–I. From these series of observations, we came to the conclusion that *MtN6* is expressed in cells about to be infected, which was supported by a close examination of outer cortical cells showing a strong *MtN6* signal, such as those presented in Figure 4F–I. Indeed, cytoplasmic structures (marked with an arrowhead) are found in the center of these cells, in the vicinity of a large nucleus, and certainly correspond to pre-infection threads (pits; van Brussel et al. 1992).

That the *MtN6* signal observed in these experiments was nodulation specific and not simply related to cell activation was confirmed by the fact that no such signal could be detected in the small cells of emerging lateral roots (data not shown). An *MtN6* sense riboprobe, systematically used as a negative control (at least 3 slides per time point), never gave a detectable hybridization signal (data not shown).

Figure 5 shows representative examples of in situ hybridization analyses carried out at later stages of the symbiotic interaction, with immature and mature root nodules. The young nodules (approximately 4 days old) presented in Figure 5A–D essentially consist of an apical meristem (marked with a star) and a large infection zone. At this stage, *MtN6* transcripts are now absent from the outer cortical cells, and it is quite clear from Figure 5A–B that they are also excluded from the meristem. In contrast, the infection zone is strongly labeled, but it can be noted that the strongest signal is not found in cells containing infection threads, but rather in the vicinity of these cells (Fig. 5C–D, and data not shown). In more developed (2- and 4-week-old) nodules with a zone I to zone IV differentiation, *MtN6* transcripts are essentially observed in the so-called preinfection zone (Pichon et al. 1992), i.e., in the distal part of the zone II, close to the meristem and above the infection threads (Fig. 5E–F). Some hybridization signal is also detected at a lower level in the proximal part of the zone II, whereas none is found in any other nodule tissue (data not shown).

MtN6 displays a novel pattern of regulation.

A key question raised by the study of molecular markers of development is the nature of the signal(s) activating gene expression. Thus, in *M. truncatula* it has been demonstrated that Nod factors are able to induce different early nodulin genes, such as *MtENOD12*, *MtENOD40*, or *MtAnn1* (Crespi et al. 1994; de Carvalho Niebel et al. 1998; Journet et al. 1994). In addition to treatments with purified Nod factors, the use of several symbiotic mutants of *S. meliloti* can also be informative in helping to define requirements for gene activation (Gamas et al. 1998; de Carvalho Niebel et al. 1998). We tested *MtN6* induction in response to the following four bacterial mutants: the *S. meliloti nodA* polar mutation completely inhibits Nod factor production and known symbiotic plant responses (Debellé et al. 1986); the *S. meliloti nodH* strain synthesizes Nod factors lacking the sulfate group on the reducing sugar and elicits very limited symbiotic responses in *Medicago* (V. Vernoud, E.-P. Journet, and D. Barker, *personal communication*); the double mutant *nodFL* produces Nod factors with an altered acyl chain and without an *O*-acetyl group, and triggers strong epidermal and cortical activation, but neither efficiently infects nor induces meristem and nodule formation, within the time scale used in our experiments (Ardourel et al. 1994; P. Gamas, *unpublished observations*);

and, finally, the *exoA* strain is defective for exopolysaccharide production, and elicits small empty nodules because of infection thread abortion in the root hairs (Yang et al. 1992). Figure 6A shows that differential responses to these various *Sinorhizobium* strains allowed us to distinguish *MtN6* from other well-known early nodulin genes, even though all of them are expressed in the infection zone II of mature nodules. Thus, in contrast to *MtENOD11* (a gene whose pattern of expression is qualitatively similar to that of *MtENOD12*, but more strongly expressed and thus easier to study by Northern analysis; D. Barker, *personal communication*), *MtAnn1* (de Carvalho Niebel et al. 1998), or *Mtenod40* (data not shown), *MtN6* was not found to be activated by a *nodFL* mutant. *MtN6* was not induced by *nodA* or *nodH* strains, but could be expressed in response to the *exoA* mutant, indicating that successful infection is not critical for its induction, which is consistent with the strong expression observed in NAR nodules (Fig. 3B). The experiment shown in Figure 6 was conducted with a super-

Fig. 6. Analysis of *MtN6* pattern of induction in *Medicago truncatula* TR122, in response to (A) various *Sinorhizobium* strains or (B) purified Nod factors. *MtN6* expression was compared with that of *MtENOD11* or *MtENOD12*, two early nodulin genes showing similar regulation features. A, Northern (RNA) analysis with approximately 6 μ g of total RNA per lane. *M. truncatula* RNA was isolated from whole root systems, harvested 6 days after inoculation with wild-type (w.t.) or mutant strains of *S. meliloti*. Bottom lane shows the ethidium bromide-stained 28S rRNA band, after transfer of total RNA onto the blotting membrane. B, Reverse-transcription-polymerase chain reaction (RT-PCR) analysis of RNA extracted from roots treated for 2 or 15 days with 10^{-7} M NodRm-IV(Ac, S, C16:2) (lanes 2d NF and 15d NF), or with water (lanes 2d C and 15d C). Right lane shows the signal obtained with *S. meliloti*-inoculated roots (6 days post inoculation). Amplification products, obtained after 25 (*MtN6* and *MtENOD12*) and 20 (*MtPR10-1*) PCR cycles, were detected by hybridization with the corresponding 32 P-labeled probe, after Southern blotting. No signal was detected with control samples from which reverse-transcriptase was omitted.

nodulating mutant of *M. truncatula*, TR122 (Sagan et al. 1995), which shows enhanced induction of nodulins in response to wild-type, *exoA*, and *nodFL* strains of *Sinorhizobium* without qualitative modification, and is thus quite useful for Northern analysis (de Carvalho Niebel et al. 1998). The same experiments were also carried out with qualitatively similar results on wild-type plants of *M. truncatula* (data not shown).

We then investigated *MtN6* response to purified Nod factors (NodRm-IV(Ac, S, C16:2), 10^{-7} M). Since our initial studies with Northern analysis failed to show induction (Gamas et al. 1996; P. Gamas, *unpublished data*), we therefore used a reverse-transcription polymerase chain reaction (RT-PCR) assay, with *MtPR10-1* and *MtENOD12* as positive controls for the quality of the cDNA and for Nod factor induction, respectively (Pingret et al. 1998). Figure 6B is a representative example of several independent RT-PCR experiments, from which it appears that a very low level of *MtN6* induction was occasionally detected after 48 h of treatment, i.e., at a time when strong *MtENOD12* transcript accumulation was observed. Due to the basal level of *MtN6* expression in roots, it was not possible to further increase the sensitivity of detection. However, after 2 weeks of growth in the presence of Nod factors *MtN6* showed a weak but more convincing induction of its expression, whereas the *MtENOD12* signal substantially decreased in comparison to earlier time points (Fig. 6B and data not shown). At such a late stage, plant responses included CCDs as well as massive amyloplast accumulation, mainly in lateral roots (data not shown). In contrast to alfalfa (Truchet et al. 1991), no real nodule-like structure could be observed under our experimental conditions.

DISCUSSION

A nodulin gene is particularly useful as a tool in nodulation studies when its expression can be clearly associated to a defined developmental stage or process. In this study, we show that *MtN6*, a gene recently identified from *M. truncatula* (Gamas et al. 1996), represents an early nodulin gene likely to be directly associated with the capacity for a cell to become infected by *S. meliloti*. *MtN6* shows several novel features that are discussed below, in terms of both regulation and sequence homologies.

MtN6 is a novel marker for events preceding infection in *M. truncatula*.

The use of spot inoculation coupled with in situ hybridization was very fruitful in exploring *MtN6* expression at early stages of the symbiotic interaction with *S. meliloti*. It revealed that *MtN6* expression is quite strong as early as 24 h post bacterial inoculation, in very localized regions in the outer root cell layers, in cells presenting cytological features of cell cycle activation. Examination of later stages (about 55 h p.i.) has established that these regions correspond to sites shortly to be infected. Indeed, at this time point, newly formed infection threads are observed at these sites, with the *MtN6* signal being maximum just ahead of these infection threads, in outer cortical cells presenting structures likely to correspond to pits (van Brussel et al. 1992). Interestingly, this signal rapidly disappears from outer cortical cells traversed by the infection thread, indicating that accumulation of *MtN6* transcripts is

transient, and suggesting that *MtN6* plays a role before the infection thread penetrates the cell. Consequently, a gradual displacement of the hybridization signal toward inner cell layers can be observed during the first 3 days of the symbiotic interaction. The signal broadens when reaching the inner cortex, which can be interpreted as an increasing number of cells preparing for infection, ahead of branching infection threads. In mature nodules *MtN6* is expressed exclusively in the infection zone II, and preferentially in its apical part, i.e., in the region located ahead of the infection threads growing toward the nodule meristem. This localization is therefore consistent with that observed at earlier symbiotic stages.

We thus believe that *MtN6* induction is a specific marker for pre-infection events, including pit formation. Pits were originally described in pea and vetch (van Brussel et al. 1992), but since then have been observed in *Medicago* spp., with kinetics and localization properties consistent with the pattern of *MtN6* activation (A. Timmers, M.-C. Auriac, and G. Truchet, *personal communication*). Their differentiation is thought to result from cell cycle reactivation events with an arrest at the G2 phase, before the cells divide (Yang et al. 1994). Interestingly, the *MtN6* expression pattern revealed by in situ hybridization is quite similar to the one described for *H4* (a marker for cells in S phase) in the outer cortex (Yang et al. 1994). However, in contrast to *H4*, at early stages *MtN6* is not expressed in inner cortical cells preparing for division, which is consistent with the idea that it is specifically associated with preparation for infection, and not simply a marker of cell activation. This conclusion is also supported by *MtN6* expression in response to two *Sinorhizobium* mutants. Indeed, an *exoA* strain, which elicits abortive infection threads but normal pit formation (A. Timmers, M.-C. Auriac, and G. Truchet, *personal communication*) is able to induce *MtN6*. In contrast, a *nodFL* double mutant that elicits strong epidermal and cortical cell activation but no pit formation (A. Timmers, M.-C. Auriac, and G. Truchet, *personal communication*) does not trigger *MtN6* expression.

It seems that, at early symbiotic stages, the *MtN6* expression pattern presents unique features, clearly distinct from that of *ENOD12*, a well-known early nodulin gene described in several legumes, and generally postulated to play a role in the preinfection processes (Bauer et al. 1994; Pichon et al. 1992; Scheres et al. 1990a; see Bauer et al. 1997 for an alternative view of *MsENOD12A* function). In *M. truncatula*, *ENOD12* is first transcribed in all epidermal cells of the nodulation-competent region (Pichon et al. 1992). This type of pattern is also observed with another early nodulin gene involved in pre-infection events, *Mtrip-1* (Cook et al. 1995), but was never seen with *MtN6* (11 independent spot-inoculated samples, i.e., at least 300 sections, see Materials and Methods), which was exclusively activated at infection sites, even when spread inoculation was used instead of spot inoculation (data not shown). In pea, *ENOD12* also differs from *MtN6* since it is expressed in the dividing inner cortical cells, at a stage when infection threads are not yet developed (Scheres et al. 1990a). It should be noted that the *MtN6* pattern of expression is also distinct from that of early nodulins strictly associated with the infection process, such as *PsENOD5* (Scheres et al. 1990b) or *MtN1* (Gamas et al. 1998), none of them being expressed before the infection threads are formed. In contrast, at later stages of nodulation, the *MtN6* pattern is reminiscent of that of

ENOD12 (Pichon et al. 1992; Scheres et al. 1990a) and of the annexin-encoding gene *MtAnn1* (de Carvalho Niebel et al. 1998) expressed ahead of growing infection threads in roots and in the infection zone II of mature nodules.

Control of the *MtN6* activation pathway.

One reason for characterizing a variety of early nodulin genes is to determine the number and the nature of the signals involved in the control of the nodulation. In that light, *MtN6* is interesting because it may represent the first specific marker associated with pit formation. In this study, we first analyzed *MtN6* expression in responses to *S. meliloti* mutants and established that *MtN6* induction could be elicited by *exoA* mutants, but not by *nodA*, *nodH*, or *nodFL* strains. We then showed that plant treatments with purified Nod factors (mainly NodRm-IV(Ac, S, C16:2)) were able to induce a low level of *MtN6* expression, under conditions in which other nodulin genes such as *MtENOD12* (Fig. 6), *MtENOD11* (data not shown), or *MtAnn1* (de Carvalho Niebel et al. 1998) were strongly activated. The induction of *MtN6* expression by Nod factors was mainly seen at late time points, which could reflect either a slow accumulation of transcripts produced at a low level, or a late induction of transcription.

We conclude from these experiments that (i) not surprisingly, Nod factors are obligatory components, whether direct or indirect, of bacteria-induced *MtN6* expression, (ii) *MtN6* gene induction is not controlled via the same pathway as *MtENOD11*, *MtENOD12*, *MtENOD40*, or *MtAnn1*. Indeed, in contrast to *MtN6*, these four genes are efficiently induced by the *nodFL* double mutant of *S. meliloti*, as well as by purified Nod factors.

The existence of several (at least two) perception mechanisms of Nod factors has been proposed from studies of situations in which epidermal and cortical cell activation are uncoupled from infections, due to mutations in bacterial *nod* genes (Ardourel et al. 1994) or in the host plant (*sym2* of pea; Geurts et al. 1997). The pathway controlling infections (through the Nod factor “entry receptor”; Ardourel et al. 1994), including the pit formation, is more stringent in terms of Nod factor structure requirements than the pathway leading to root hair deformation, inner cortical cell activation (“signaling receptor”; Ardourel et al. 1994), or *PsENOD12* induction (Geurts et al. 1997; Horvath et al. 1993; van Brussel et al. 1992). It seems to us that the features of *MtN6* expression, in terms of both spatio-temporal pattern and response to *S. meliloti* mutants, make it a good candidate for a marker of the pathway controlling infections. Other early nodulin genes such as *ENOD12*, *ENOD40*, or *MtAnn1* could be induced at early symbiotic stages through the nonstringent signaling receptor, and additionally through the infection pathway at later stages, which might explain why their pattern is similar to that of *MtN6* in nodules. The fact that these genes, as well as *MtN6*, are induced in NAR nodules may indicate that several Nod factor-controlled pathways are activated during NAR organogenesis, even though it is not known whether pits are formed in this situation.

Whether *MtN6* expression is directly controlled by Nod factors remains an open question, especially since *MtN6* is induced only weakly by purified Nod factors. *MtN6* could be activated by a Nod factor-dependent process, rather than by or in addition to Nod factor molecules. One obvious possibility

could be the beginning of infection itself. Indeed, it is likely that various processes are activated well before infection threads form, such as local degradation of the cell wall by secreted bacterial enzymes. However, the high level of *MtN6* expression during NAR organogenesis is not consistent with this hypothesis, unless *MtN6* could be induced via several pathways. The possibility that additional signal molecules are required in combination with Nod factors for strong *MtN6* induction is also conceivable. It should be mentioned here that, in contrast to the situation described in pea and vetch, Nod factor-induced pit formation has not been observed in *M. sativa* or *M. truncatula* (A. Timmers, M.-C. Auriac, and G. Truchet, *personal communication*), which may suggest that an additional factor is required in this legume. Alternatively, it is possible that our Nod factor preparation is not perfectly adequate to activate a stringent receptor. Indeed, it cannot be excluded that the population of Nod factors produced under physiological conditions differs slightly from the molecules that we used, i.e., purified from a luteolin-induced culture of a Nod factor-overproducing strain of *Sinorhizobium* (Lerouge et al. 1990).

What could be the connection between *MtN6* and FluG proteins?

The level of homology between FluG and *MtN6* as well as the major role played by FluG in a developmental program of *E. nidulans* justify an exploration of the possible functional relationships between these two proteins. FluG seems to act through the synthesis of a small diffusible signal, yet to be identified, that is capable of activating a pathway leading to conidiophore formation, via a signal-transducing mechanism and a central regulatory gene, *brlA* (Lee and Adams 1995). It is interesting to note that, as nodulation, conidiation is sensitive to the nutritional status of the organism. Indeed, submerged cultures can be induced to sporulate by nutrient (carbon or nitrogen) starvation, *fluG* being required for responding to nitrogen, but not carbon, starvation (Lee and Adams 1995). Nevertheless, it is not yet established whether *fluG* is involved in sensing the nitrogen status of *A. nidulans*, or, alternatively, whether different signals act through separate but additive pathways to activate conidiation.

The FluG protein is structured in two domains, with an N-terminal domain homologous to the *MtN6* deduced protein, and a C-terminal region in which all the *fluG* mutations identified so far are located, demonstrating its importance for FluG function. We have been able to identify a small gene family in *M. truncatula*, encoding products highly homologous to the C-terminal domain of FluG, and possibly also to the whole FluG product for some of them, based on the size of the detected transcripts (R. Mathis, P. Gamas, and J. Cullimore, *in preparation*). One possibility, which will have to be evaluated in the future, is that *MtN6* is able to interact with either of these protein species, to interfere positively or negatively with their action. It should be borne in mind that homologues of each FluG domain, called Rv 1879 and Rv 1878, also exist in *Mycobacterium tuberculosis* (Cole et al. 1998), and are present in separate ORFs whose expression is likely to be tightly coupled. Indeed, they are separated by only 3 bp, with the potential ribosome-binding site of the downstream ORF being 7 bp before the stop codon of the upstream ORF.

It is premature to speculate more precisely on the biochemical function of *MtN6*, and relate it to a possible role in prepa-

ration to infection. It is worth noting, however, that close homologues to *MtN6* are likely to exist in other legumes, since cross-hybridizing transcripts of similar size were found by Northern analysis in nodule RNA from *Vicia sativa* and *P. sativum* (R. Mathis and P. Gamas, *unpublished results*), as well as in nonlegume plants and bacteria (data base searches; see Results). Future experiments should help us to understand the role played by this family of related proteins in such diverse organisms.

MATERIALS AND METHODS

Plant growth and treatments.

Three types of plant growth conditions were used for *M. truncatula* cv. Jemalong (Barker et al. 1990), depending on the experiment. For analysis of the RNA extracted from the whole root system (studies of responses to bacterial strains, or Nod factors), plants were grown in pouches as described in Gamas et al. (1998). For spot-inoculation experiments, they were grown on nitrogen-free Fahraeus medium (Fahraeus 1957) on 12.5 × 12.5 cm dishes, whereas aeroponic growth conditions (as in Gamas et al. 1996) were chosen to obtain isolated nodules and aerial tissues. Alfalfa lines (*Medicago sativa* L. 'Gemini') were propagated vegetatively as described in Grosjean and Huguet (1997), and grown aeroponically to collect material, first in nitrogen-containing medium (5 mM ammonium nitrate) and then in nitrogen-free medium (as in Truchet et al. 1989) to trigger spontaneous CCDs and NAR nodule organogenesis. Cytological observations at various time points allowed us to define the portions of roots to be harvested (medium region of the primary root, i.e., excluding the oldest and youngest parts of the root; Grosjean 1997) to enrich our material in nodule primordia.

The *Sinorhizobium* strains used were described in Gamas et al. (1998). Each pouch (i.e., 5 plants) was inoculated, after 8 days of plant growth in the absence of combined nitrogen, with 1 ml of bacteria resuspended at an OD₆₀₀ of 0.015 in the growth medium. Experiments were performed twice with wild-type *M. truncatula* and with the TR122 supernodulating mutant of *M. truncatula* (Sagan et al. 1995). Results were qualitatively similar in both genotypes, with stronger levels of induction in TR122, as observed for all tested nodulin markers (de Carvalho Niebel et al. 1998; M. C. Boudet and P. Gamas, *unpublished results*). Nod factor treatments were carried out on plants of the same age, with 1 ml of solution per pouch. Two different Nod factor preparations (a mixture of Nod factors with a majority of NodRm-IV(Ac, S, C16:2), purified from luteolin-induced *R. meliloti* 2011 pGMI149, as in Lerouge et al. 1990) were used, at a 10⁻⁷ M final concentration, with similar results.

Spot inoculation was carried out on 8-day-old plants, with 0.2 µl of bacteria resuspended in 1% carboxymethylcellulose wt/vol (Stokkermans and Peters 1994) at an OD₆₀₀ of 0.1. Spots were realized at the site of emerging root hairs (i.e., 5 to 10 mm away from the root tip), and marked with charcoal. Root segments covering about 3 mm on each side of the spot were harvested for sections. Approximately 80% of the roots responded to the treatment in our conditions. One control experiment was conducted with spread inoculation, done with 200 µl of a bacterial suspension at the same OD. Root segments (8 mm long) were harvested 24 h p.i. from 16 plants,

sectioned transversally or longitudinally, and analyzed by in situ hybridization. Two of them showed *MtN6* activation.

RNA and DNA analysis.

Total RNA was extracted from frozen plant tissue following the procedure of Jackson and Larkins (1976). Northern and Southern analyses were conducted with Nytran Plus membranes according to the manufacturer's instructions (Schleicher & Schuell, Dassel, Germany), with DNA radioactive probes prepared with the Ready-To-Go kit (Pharmacia, Uppsala, Sweden) and purified on microspin 200 HR columns (Pharmacia).

RT-PCR analysis was carried out as described by Pingret et al. (1998), on DNase-treated RNA samples. The number of PCR cycles was determined for each primer combination in order to analyze amplification products corresponding to the exponential phase of the reaction. For *MtN6*, 25 cycles were carried out, consisting of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C, and with the following primers: 5'-TGGTGATGCT GTTGCTTC-3' (forward); 5'-CATGAATCACGAAAACC-3' (reverse). The cDNA and the gene amplification products could easily be distinguished because of the presence of several introns between the two primer sites (data not shown). Experiments were conducted three times, with similar results. Wild-type *M. truncatula* cv. Jemalong J5 was tested for Nod factor induction, as well as the TR122 supernodulating mutant (Sagan et al. 1995). Induction was weaker with the wild-type than with TR122 plants.

DNA sequencing was carried out on both strands of plasmid DNA, with a Perkin-Elmer/ABI 373A automatic sequencer (Foster City, CA). Sequence data were analyzed with University of Wisconsin Genetic Computer Group (GCG) software. Homology searches were carried out through the NCBI BLAST server. Sequence alignments were done with the Multalin program (Corpet 1988).

In situ hybridization.

In situ hybridization was conducted following the procedure described in Gamas et al. (1998), with a 3- to 4-week exposure. Samples were treated with 0.2% eosin B (in absolute ethanol) just before the ethanol-xylene treatments, in order to visualize them more easily when embedded in paraplast. Sections were observed with a Zeiss axiophot light microscope. Observations were made on samples from two independent experiments, with 7 to 12 slides per time point (each corresponding to an independent sample, and representing at least 30 serial sections).

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