The *Medicago truncatula* *MtAnn1* Gene Encoding an Annexin Is Induced by Nod Factors and During the Symbiotic Interaction with *Rhizobium meliloti*

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Here we report the characterization of a new Nod factor-induced gene from *Medicago truncatula* identified by mRNA differential display. This gene, designated *MtAnn1*, encodes a protein homologous to the annexin family of calcium- and phospholipid-binding proteins. We further show that the *MtAnn1* gene is also induced during symbiotic associations with *Rhizobium meliloti*, both at early stages in bacterial-inoculated roots and in nodule structures. By in situ hybridization, we demonstrate that *MtAnn1* expression in nodules is mainly associated with the distal region of invasion zone II not containing infection threads, revealing *MtAnn1* as a new marker gene of the pre-infection zone. Moreover, analyses of *MtAnn1* expression in response to bacterial symbiotic mutants suggest that the expression of *MtAnn1* during nodulation requires biologically active Nod factors and is independent of the infection process.

Additional keywords: supernodulating mutant line, symbiosis.

The symbiotic association between leguminous plants and rhizobia (*Azorhizobium*, *Bradyrhizobium*, and *Rhizobium*) culminates in the formation of a new root organ known as the nodule, in which the bacteria reside and convert atmospheric nitrogen into ammonium, which is then assimilated by the host plant. Nodule development in temperate legumes such as *Medicago* spp. is initiated by the specific attachment of *Rhizobium* bacteria to plant root hairs leading to hair deformation (Had) and curling (Hac), morphological changes characteristic of the symbiotic stage preceding bacterial infection. Within the curled root hairs, the invading bacteria induce the formation of new tubular structures, the infection threads (ITs), within which *Rhizobium* bacteria progress toward the inner root cortical cells. Concomitantly, these inner cortical cells divide, resulting in the formation of a nodule primordium. Once the ITs reach the primordium, bacteria are released into the plant cells and an apical meristem is formed. Subsequent growth and differentiation results in the development of the nodule structure of indeterminate type, composed of a persis-

tent apical meristem (zone I) followed by different zones corresponding to distinct developmental stages of bacterial and plant differentiation. In a mature nodule structure, the meristematic zone I is followed successively by invasion zone II (where infection of the plant cells takes place), the amyloplast-rich interzone II-III, the fixation zone III (where *R. meliloti* differentiates into bacteroids that fix nitrogen) and the senescence zone IV (where bacteria cells degenerate) (for review see Mylona et al. 1995).

The initiation and development of the nodulation process require bacterial-secreted lipochitooligosaccharide signal molecules, called Nod factors (reviewed in Dénarié et al. 1996). Nod factors produced by various rhizobia are composed of a backbone of four or five β -1,4-linked glucosamine residues, N-acylated on the nonreducing sugar and N-acetylated on the other residues. Further modifications on the reducing and nonreducing sugars of the Nod factors are specific for each rhizobia species and can be important determinants of host specificity. For example, *R. meliloti* bacteria that nodulate plants of *Medicago* spp. produce mainly tetrameric Nod factors decorated by a sulfate group on the reducing sugar and an *O*-acetate and a C16:2 acyl chain on the nonreducing sugar (reviewed by Dénarié et al. 1996). Purified Nod factors can induce early symbiotic responses in the roots of host plants, and in some plant species they can also induce the formation of nodule structures (reviewed by Dénarié et al. 1996; Heidstra and Bisseling 1996; Long 1996; Schultze and Kondorosi 1996; Spaink 1996). Examples of early responses induced by Nod factors include membrane depolarization, increased cytoplasmic pH, calcium spiking, and Had, in epidermal cells; the activation and division of root cortical cells; and the induction of specific plant gene expression, in epidermal, cortical, and pericycle tissues.

Plant genes that are expressed during the nodulation process are referred to as nodulin genes and are classified as early or late, according to their timing of expression during the nodulation process (for review see Mylona et al. 1995; Heidstra and Bisseling 1996). Late nodulin genes are mainly expressed in mature nodule structures around the onset of nitrogen fixation. Early nodulin genes are generally expressed during the early stages of nodulation and are thus believed to be involved in the infection process and/or nodule organogenesis. Although nodulin genes were initially considered to be specifically expressed during symbiotic associations with rhizobia,

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many of them are likely to participate in other plant developmental processes, since they are also expressed in other plant tissues (see for example Crespi et al. 1994; Matvienko et al. 1994; Cook et al. 1995; Bauer et al. 1996; Papadopoulou et al. 1996).

Studies on early nodulin gene expression have revealed these genes to be valuable molecular markers for studying early stages of the symbiotic process. Several of these genes are induced by purified Nod factors and hence are also useful markers for studies of Nod-factor-induced plant responses (Horvath et al. 1993; Journet et al. 1994; Vijn et al. 1995; Minami et al. 1996). Although homologies to known gene products have been found for a number of early nodulin genes (Cook et al. 1995; Goormachtig et al. 1995; Bauchrowitz et al. 1996; Gamas et al. 1996; Coba de la Pena et al. 1997; Heidstra et al. 1997), the function of specific gene products in the symbiotic process remains to be determined.

We are studying the symbiotic interaction between the legume *Medicago truncatula* and the bacterial symbiont *Rhizobium meliloti*. To extend our knowledge concerning the initiation and development of the nodule organogenesis program in *M. truncatula*, we decided to isolate new plant genes induced during the early stages of symbiosis. To this end we used the differential display approach to identify Nod-factor-induced genes likely to be associated with these early symbiotic stages.

Here we report the characterization of one of the Nod-factor-induced cDNA clones coding for a putative calcium- and phospholipid-binding annexin protein. We have demonstrated that the new gene, named *MtAnn1* for *Medicago truncatula* *Annexin 1*, corresponds to a new early nodulin gene expressed both at early stages of the symbiotic process and also at the distal end of the early symbiotic zone II of nodules. Additionally, our results indicate that *MtAnn1* expression during symbiosis requires biologically active Nod factors and is independent of the infection process.

RESULTS

Identification by differential display of a new Nod-factor-induced *M. truncatula* gene.

To identify new *M. truncatula* genes induced by purified *R. meliloti* Nod factors (NodRm factors), we used the sensitive polymerase chain reaction (PCR)-based technique of differential display (Liang et al. 1994). With this method, differentially expressed mRNAs are detected in different RNA samples by comparing the patterns of partial cDNAs generated after reverse transcription and PCR amplification with arbitrary primers. This strategy therefore has the advantage of allowing the detection of relatively low-abundant mRNA species.

We have compared gene expression patterns with RNAs isolated from *M. truncatula* roots after treatment either with 10^{-7} M NodRm factors or with the growth medium only (controls). These RNA samples were used to synthesize first-strand cDNAs with anchored oligo dT 3' primers, which were subsequently amplified by PCR with the same oligo dT 3' primer and arbitrary 10-mer 5' primers (see Materials and Methods). The cDNA subpopulations from NodRm-treated and control samples obtained in this way were compared on polyacrylamide gels, where differential bands could be identi-

fied and isolated. We first analyzed samples that were harvested 2 and 24 h after NodRm or growth medium treatments. No differential bands were identified for these samples although a relatively large number of RNA species were analyzed (about 4,000 and 2,400, respectively). To increase the chances of finding Nod-factor-induced genes, we decided to use root samples that had been nitrogen-starved for a longer period (10 days, instead of 6 days as for the previous samples) and treated for a longer time (48 h) with NodRm factors. Four cDNA bands showing enhanced amplification in the 48-h NodRm-treated samples were identified with fewer primer combinations (20) than the previous experiments (51 and 31). After excision from the gels, re-amplification, and cloning, the four clones, named *ddA*, *ddB*, *ddC*, and *ddD* of, respectively, 373, 295, 402, and 284 bp, were used as probes on RNA gel blots to verify the induction by Nod factors. This was confirmed for *ddA* and *ddB*, although hybridizing signals were weak (data not shown), while the expression of *ddC* and *ddD* was undetectable following RNA gel blot analysis.

This paper focuses on the characterization of the differential display clone *ddA*, represented in Figure 1A, for which a full-length cDNA clone was isolated to improve the sensitivity of the expression analysis. DNA gel blot analysis at high stringency conditions with the entire *ddA* cDNA as a probe revealed one major hybridizing band, suggesting that *ddA* is a single-copy gene in *M. truncatula* (data not shown). To better evaluate the Nod factor induction of *ddA*, RNA gel blot analysis was carried out with total RNA from *M. truncatula* roots treated for different times with 10^{-7} M NodRm. As shown in Figure 1B, increased levels of *ddA* transcripts were clearly detected in *M. truncatula* roots treated for 48 h with NodRm factors. A similar induction was also detected on NodRm-treated roots of another *M. truncatula* genotype, the supernodulating mutant TR122 (Sagan et al. 1995) (see Materials and Methods) (Fig. 1B). RNA gel blot analysis of Nod-factor induction of *ddA* expression at earlier time points (3, 6, 12, and 24 h) was not conclusive due to the variation in the basal levels of *ddA* expression observed in control roots (data not shown).

The Nod-factor-induced *M. truncatula* *MtAnn1* gene encodes an annexin-like protein.

The *ddA* cDNA clone is 1,202 bp long with 216 bp of untranslated sequence preceding the polyA tail. The transcript and the cDNA insert size of about 1.2 kb is consistent with the *ddA* cDNA clone being about full length. Sequence analysis of the *ddA* cDNA revealed a single open reading frame encoding a protein of 313 amino acids with a predicted molecular mass of 35.6 kDa and an isoelectric point of 7.8. The PSORT program (version 6.4; Nakai and Kanehisa 1992) predicts the *ddA* encoded protein to be cytoplasmic (with a confidence of 0.65). Data base searches revealed that the putative encoded protein shows homology to the annexin family of calcium- and phospholipid-binding proteins, with the highest homologies to plant annexin sequences, as shown in Table 1. The *ddA* clone was then designated *MtAnn1*, for *Medicago truncatula* *Annexin 1*.

Annexins have been described in many eukaryotic organisms, including animals, plants, and protists. Annexins proteins are composed of a variable N-terminal region and a highly conserved C-terminal core formed by four (and some-

times eight) repeats of about 70 amino acids that are also found in the *MtAnn1* sequence, as indicated in Figure 2. Within the repeats, we find a 17-amino-acid consensus sequence or the “endonexin fold” that is generally highly conserved in repeats 1, 2, and 4 of animal sequences (Fig. 2). In

the *MtAnn1* sequence, the “endonexin fold” region is highly conserved in repeats 1 and 4 (93 and 80% similarity to consensus sequence, respectively, with the blosum 62 amino acid matrix with a score +1 or higher), and sequence conservation decreases successively in repeat 3 (67%) and repeat 2 (47%). The major sites for calcium binding defined as type II are characterized by a discontinuous sequence corresponding to G-X-G-T-(X₃₈)-(D,E) where X is any residue (for reviews see Raynal and Pollard 1994; Clark and Roux 1995). The entire type II calcium-binding site is only conserved in repeat 1 of *MtAnn1*. However, the glutamic acid (E) or the aspartic acid (D) residues located in the C-terminal position within the conserved motif are also conserved in repeats 3 and 4 of the plant sequences (Fig. 2).

The Nod-factor-induced *MtAnn1* gene is also expressed during symbiotic interactions with *R. meliloti*.

To evaluate whether the induction of *MtAnn1* expression by NodRm factors is related to the nodulation process, *MtAnn1* mRNA levels were examined in *M. truncatula* roots at different times following inoculation with *R. meliloti*. In our experimental conditions nodules can be initially visualized as small bumps 3 to 4 days post-inoculation. As shown in Figure 3A, induction of *MtAnn1* expression is detected at different stages of nodulation, in inoculated roots (48 h) and in roots containing both young (4-day-old) and mature (16-day-old) nodule structures. Subsequent analysis revealed that substantial levels of *MtAnn1* mRNA are found in both young and mature isolated nodules (Fig. 3A). *MtAnn1* mRNA levels detected in roots that were inoculated with a non-nodulating (*nod A*) mutant of *R. meliloti*, which does not produce Nod factors, were generally close to the basal levels of *MtAnn1* expression.

To further characterize *MtAnn1* expression at early stages of the nodulation process, RNA gel blot analysis was carried out with poly A⁺ RNA isolated from roots harvested 3, 24, and 48 h after inoculation with either wild-type *R. meliloti* or the *nod A* mutant as a negative control. As shown in Figure 3B, high levels of *MtAnn1* mRNA were readily detected 24 h after root inoculation with wild-type *R. meliloti*. No induction was detected for *MtAnn1* 3 h after inoculation, although we cannot exclude that low level induction may be masked by the basal level of expression in roots.

To examine whether *MtAnn1* is expressed in other tissues of the plant, RNAs from flowers, stems, and leaves were analyzed by RNA gel blots. As shown in Figure 3C, *MtAnn1*

Fig. 1. Induction of the *Medicago truncatula* annexin gene *MtAnn1* by *Rhizobium* Nod factors. **A**, Area of the differential display gel showing the NodRm-enhanced polymerase chain reaction (PCR) band corresponding to the *MtAnn1* annexin gene. Two independent reverse transcription (RT)-PCR reactions of RNA from control (C1 and C2) and NodRm-treated (N1 and N2) roots were resolved on a 6% denaturing polyacrylamide gel. Arrow indicates the 373-base *dda* PCR fragment. **B**, RNA gel blot analysis of Nod factor-treated roots. Ten micrograms of total RNA from roots of wild type (*Mt wt*) and from the supernodulating TR122 line (*Mt TR122*) of *M. truncatula* were loaded in each lane. Roots were treated for 48 h with growth medium only (C) or growth medium plus 10⁻⁷ M NodRm factors (N). Blots were hybridized with ³²P-labeled, full-length *dda* riboprobes. Ethidium bromide-stained 28S rRNA on the blotted membrane reveals the uniformity of RNA loading.

mRNA was barely detectable in flower tissues, and undetectable in leaves and stems. *MtAnn1* expression was also examined in leaves in response to different stress conditions such as wounding and infection by the respective incompatible and compatible pathogenic bacteria *Pseudomonas syringae* pv. *pisi* and *Xanthomonas campestris* pv. *alfalfae*. No induction of *MtAnn1* expression was observed under conditions in which the stress-related genes chalcone synthase and the *M. truncatula* pathogenesis-related PR-10 gene (*MtPR10-1*) (Gamas et al. 1998) were strongly induced (data not shown).

To better understand the specificity of *MtAnn1* expression in relation to the symbiotic process, RNA gel blot analysis was performed with RNA extracted from *M. truncatula* roots inoculated with various symbiotic mutants unable to produce genuine nodule structures. The bacterial mutants tested were: *nodH* (producing Nod factors lacking the host specificity determinant sulfate group on the reducing sugar), *nodFL* (producing Nod factors modified on the non-reducing sugar, lacking the *O*-acetyl group and with a C18:1 instead of a C16:2 acyl chain), and *exoA* (deficient in a surface acidic polysaccharide). In *M. truncatula* plants, *nodH* *R. meliloti* (as

Table 1. Comparison of MtAnn1 with other annexin sequences

Annexin sequences ^a	BLASTX (probability) ^b	BESTFIT ^c		AA no.	Accession nos.
		% S	% I		
Strawberry cDNA clone (FaAnn)	1.1e-126	79.1	69.4	268	U19941
Maize annexin p33 cDNA clone (ZmAnnp33)	9.7e-103	62.5	52.2	312	X98244
<i>Arabidopsis thaliana</i> cDNA clone (AtAnn)	1.1e-102	59.4	49.5	313	U28415
Alfalfa cDNA clone (MsAnn)	1.4e-102	61.4	51.5	303	X74947
Maize annexin p35 cDNA clone (ZmAnnp35)	1.1e-100	61.2	52.6	312	X98245
Cotton cDNA clone (GhAnn)	1.6e-100	62.7	54.0	300	U73747
Bell pepper cDNA clone (CaAnn)	1.0e-98	59.2	50.8	313	X93308
Chicken Annexin V (GgAnnV)	6.3e-56	46.0	35.7	311	P17153
Human Annexin XIII (HsAnnXIII)	1.4e-54	46.4	34.4	308	P27216

^a The indicated sequences correspond to the nine best homologies found with the BLASTX program, using the nonredundant protein data base (Altschul et al. 1990).

^b The probability of finding these homologies by chance.

^c Percentage of sequence similarities (% S) and identities (% I) at the amino acid level found with the BESTFIT GCG program. AA no. corresponds to the number of amino acid residues for which homologies were found.

Fig. 2. Amino acid sequence alignment of MtAnn1 to other annexin sequences. MtAnn1 is aligned with the deduced amino acid sequences of plant (FaAnn, AtAnn, CaAnn, MsAnn, GhAnn, ZmAnnp33, and ZmAnnp35) and animal (GgAnnV and HuAnnXIII) annexins. Annexin sequence abbreviations are defined in Table 1. Numbers 1 to 4 above the MtAnn1 sequence refer to the start of the characteristic annexin repeat domains according to Raynall and Pollard (1994). Boxed areas correspond to the 17 amino acid-endonexin fold region with its characteristic KGHTDEXXLIpLApR motif (h = hydrophobic residue; X = any residue; p = polar residue). Black dots below the HuAnnXIII sequence indicate expected position of the four first and last residues within the consensus type II calcium-binding site defined as G-X-G-T-(X38)-(D/E), where X can be any residue. Alignment was obtained with the CLUSTAL W 1.7 program (blosum matrix; Thompson et al. 1994). Identical residues for at least six of the 10 sequences are shaded.

nodA) is non-nodulating, and does not elicit any of the early morphological symbiotic responses. The *nodFL* mutant is neither able to infect the plant roots nor to elicit nodules but is still able to induce some of the primary symbiotic responses such as root hair deformation and cortical cell activation (Ardourel et al. 1994). Finally, *exoA* bacteria are blocked during the infection process but can still induce the formation of bacteria-free nodule structures (Yang et al. 1992). In a number of independent experiments, the induction of *MtAnn1* expression was only clearly observed in *exoA*-inoculated roots (data not shown). In order to improve sensitivity we chose to use a supernodulating mutant line of *Medicago truncatula*, TR122 (Sagan et al. 1995), characterized by an enhanced number of nodules formed throughout the root system. *MtAnn1* expression levels were higher in TR122 than in wild-type *M. truncatula* *R. meliloti*-inoculated roots (not shown). As shown in Figure 3D,

MtAnn1 expression was clearly induced by *exoA* and *nodFL* bacterial mutants in TR122-inoculated roots. As expected, no significant induction of *MtAnn1* expression was observed in response to the bacterial mutants *nodA* and *nodH*.

MtAnn1 mRNA is localized to distal zone II of *M. truncatula* nodules.

To determine the tissue specificity of *MtAnn1* expression during nodule development, in situ hybridizations were carried out on sections of *M. truncatula* nodules harvested at different times following inoculation with *R. meliloti* bacteria.

MtAnn1 expression was first analyzed in wild-type *R. meliloti*-induced nodules of *M. truncatula*. At a relatively early stage of nodule development (6 days after inoculation), as shown in Figure 4A and 4B, an apical meristematic region followed by a central region with ramifying infection threads penetrating the plant cells is observed. *MtAnn1* transcripts were exclusively detected within central tissues, but with the highest hybridization signal associated with the more distal part of this region, adjacent to the meristematic cells (Fig. 4B). At higher magnifications, it can be seen that the highest density of silver grains is localized to the region where infection threads are not yet present (Fig. 4D and E). A control nodule section hybridized with sense *MtAnn1* probes is shown in Figure 4C. As shown in Figure 4F and G, a similar but more restricted hybridization pattern is observed in more mature nodules (18 days post-inoculation), where *MtAnn1* transcripts were mainly localized to the distal part of invasion zone II adjacent to the meristem and lacking infection threads, a region that has been termed the pre-infection zone (Pichon et al. 1992). The more localized expression of *MtAnn1* in mature nodules correlates with a more clearly defined zone II, indicating that *MtAnn1* transcripts are associated with the same region throughout nodule development. Signals were detected neither in the proximal region of zone II nor in zone III, containing bacteria-infected plant cells. Furthermore, signals were found neither in the peripheral nodule tissues nor in root tissues surrounding the nodule structures.

Fig. 3. *MtAnn1* expression analysis by gel blots. **A**, Induction of *MtAnn1* expression in *Rhizobium meliloti*-inoculated roots (R) and nodules (N). Total RNA isolated from *M. truncatula* roots before (R) and 48 h (48h), 4 days (4d) or 16 days (16d) after inoculation with *R. meliloti* (*Rm wt*); RNA from *M. truncatula* roots 4 days after inoculation with the non-nodulating *Rhizobium* mutant *nodA* (*A-*, R) and RNA from young, 4-day-old (*Rm wt*, 4d N) and mature, 14-day-old (*Rm wt*, 14d N), isolated *R. meliloti*-induced nodules. **B**, Early induction of *MtAnn1* expression in *R. meliloti*-inoculated roots. Poly A+ RNA from entire roots harvested at different times (3, 24, and 48 h) after inoculation with *nodA* mutant (*A-*) or wild-type (*Rm wt*) *R. meliloti*. **C**, Analysis of *MtAnn1* expression in different plant tissues. Total RNA isolated from flowers (F), stems (S), leaves (L), and roots (R) of *M. truncatula*. **D**, Differential induction of *MtAnn1* by *R. meliloti* symbiotic mutants. Total RNA extracted from entire roots of *M. truncatula* TR122 plants 6 days after inoculation with *R. meliloti* (*Rm*) wild type (*wt*) and mutants *exoA* (*exo-*), *nodH* (*H-*), *nodFL* (*FL-*), and *nodA* (*A-*). Control RNA sample (C) was isolated from roots of plants 6 days after treatment with growth medium only. For **A**, **C**, and **D**, 10 μ g of total RNA was loaded per lane, and ethidium bromide-stained 28S rRNA on the blotted membrane is shown below. For **B**, approximately 120 ng of poly A+ RNA was loaded per lane, and RNA loading was controlled by hybridizing blots to a DNA probe to detect translation elongation factor EF-1 α transcripts. Riboprobes were used to detect *MtAnn1* transcripts.

MtAnn1 expression was also examined during nodule development in *M. truncatula* TR122 plants. Because of the supernodulation phenotype we were able to analyze, in the same section, nodules of different developmental stages. In sections of TR122 roots 6 days post-inoculation, certain nodules were at a similar developmental stage as wild-type *M. truncatula* nodules of the same age (Fig. 4H, upper part) but younger

stages of nodule development were also observed (Fig. 4H, bottom). *MtAnn1* hybridization signals in immature TR122 nodules were located in the same region as in wild-type nodules of the same age (compare Figure 4B and H), demonstrating that the pattern of *MtAnn1* expression seen in wild-type nodules is conserved in TR122 nodules. Earlier developmental stages revealed in sections of TR122-nodulated

Fig. 4. Cell-specific localization of *MtAnn1* mRNA during nodule development with in situ hybridization. Longitudinal sections (7 μ m) were hybridized with 35 S-labeled *MtAnn1* antisense and sense riboprobes. Toluidine blue-stained sections are visualized in bright field images (A, D, and F), where dark spots represent radioactive labeling. In the dark field images (B, E, G, and H), radioactive labeling appears as bright spots. Sections of young (6-day-old), isolated nodules of wild type *Medicago truncatula* hybridized with sense (C) and antisense (A, B, D, and E) *MtAnn1* riboprobes. Remaining root tissues (cortex and epidermis) can be detected on the top of these young nodule sections. Section of a mature nodule (18-day-old) of wild-type *M. truncatula* hybridized with antisense *MtAnn1* riboprobes (F and G). Section of young, developing nodules from TR122 *M. truncatula* plants hybridized with antisense *MtAnn1* riboprobes (H). Symbols: star = meristematic zone; arrows = infection threads; asterisk = nodule invasion zone II. Horizontal bars = 80 μ m.

roots showed that *MtAnn1* transcripts accumulate at a high level and homogeneously in all nodule primordia cells (Fig. 4H, bottom).

DISCUSSION

In this paper we report the identification and characterization of a new gene from *M. truncatula* induced by *R. meliloti* Nod factors. This gene, designated *MtAnn1*, encodes a protein structurally homologous to the annexin family of calcium- and phospholipid-binding proteins. We show here that Nod factor induction of *MtAnn1* expression is related to the symbiotic process since *MtAnn1* is also expressed in *R. meliloti*-inoculated roots and in *R. meliloti*-induced nodules. Thus the annexin-like *MtAnn1* gene constitutes a novel Nod factor-induced early nodulin gene from *M. truncatula*.

In situ hybridization of nodules has revealed that *MtAnn1* expression is associated with the distal part of invasion zone II, as is the case for the *PsENOD12* (Scheres et al. 1990a, 1990b), *MtENOD12* (Pichon et al. 1992) and *MsENOD12A* (Bauer et al. 1997) early nodulin genes characterized in indeterminate nodules. *PsENOD12* is considered to be an infection-related marker gene since its expression is associated with the distal zone II containing actively growing infection threads (Scheres et al. 1990a, 1990b). *MtENOD12* transcription is also maximal in cells containing infection threads, although it has been shown that expression is initiated within the narrow zone of cells, adjacent to the meristem and lacking infection threads, that is defined as the pre-infection zone (Pichon et al. 1992). *MtAnn1* expression is localized to the same region as *MtENOD12* but the highest transcript levels are observed in the pre-infection zone rather than in the zone containing infection threads. This nodule pre-infection zone was defined on the basis of *MtENOD12* expression in nodules (Pichon et al. 1992) and it has been suggested that the cells within this zone are being prepared for the subsequent infection process, by analogy with the early symbiotic expression of *MtENOD12* in epidermal cells prior to *Rhizobium* infection of root hairs. Thus, *MtAnn1* annexin is likely to play a role in this pre-infection process in nodules. It will now be necessary to determine the pattern of *MtAnn1* expression in root tissues during early symbiotic stages.

To learn more about the expression of *MtAnn1* during nodulation, we made use of a variety of *R. meliloti* mutants. *MtAnn1* can be expressed independently of successful infection since induction of *MtAnn1* mRNA levels was observed in roots that were inoculated either with the *nodFL* *R. meliloti* mutant (absence of infection) or the *exoA* *R. meliloti* mutant (blocked in infection). These data also suggest that *MtAnn1* is more likely to play a role in preparation for infection or nodule organogenesis, rather than in the infection process itself. Interestingly, the pattern of *MtAnn1* expression found in *R. meliloti*-induced nodules adjacent to meristematic cells is similarly found in *exo* nodules (data not shown) and for a *MtAnn1* homologue in spontaneous alfalfa NAR nodules (C. Grosjean, F. de Carvalho Niebel, and P. Gamas, unpublished data), suggesting that this sub-meristematic location of *MtAnn1* expression is not dependent on bacterial infection.

Sequence comparisons have shown that the protein encoded by the *MtAnn1* cDNA is a new member of the annexin protein family. Annexins constitute a family of calcium-dependent,

phospholipid-binding proteins identified in many eukaryotic organisms (reviewed by Raynal and Pollard 1994; Kaetzel and Dedman 1995; Moss 1997). These proteins are amphipathic, soluble proteins, which in the presence of calcium can shift to a membrane-associated state. Annexins generally have a variable N-terminal region and a conserved core region composed of four repeats (except for the animal class VI, containing eight repeats) of about 70 amino acids. The N-terminal region of animal annexins can vary in length and in amino acid composition and is mostly associated with the specific properties of certain annexins (reviewed by Raynal and Pollard 1994; Moss 1997). It is interesting to note that all the reported full-length plant sequences, including *MtAnn1*, have short N-terminal sequences. Molecular phylogenetic analysis reveals at least 27 subfamilies of annexin proteins (Morgan and Fernandez 1997). The well-studied vertebrate annexins are classified within the 10 first subfamilies, sharing approximately 50% amino acid identity. The plant annexins have diverged significantly in comparison to animal annexins and fall into a separate group comprising at least 7 subfamilies represented in one or more plant species (Morgan and Fernandez 1997). Sequence comparison has shown that *MtAnn1* annexin is closer to an annexin from strawberry (Wilkinson et al. 1995) than to an annexin from the closely related species alfalfa (Pirck et al. 1994) (see Table 1), suggesting that *MtAnn1* and the alfalfa annexin belong to separate subfamilies. Given the high degree of sequence conservation observed for a number of genes between *M. truncatula* and alfalfa (Stanford et al. 1993; Crespi et al. 1994), it is likely that a homologue of *MtAnn1* also exists in alfalfa. Indeed, RNA blot analysis at high stringency conditions with *Mtann1* cDNA as a probe demonstrated the induction of expression of an *Mtann1* homologue in alfalfa NAR nodules (C. Grosjean, F. de Carvalho Niebel, and P. Gamas, data not shown).

Members of the annexin family can show different properties, although their actual cellular roles remain elusive (reviewed by Raynal and Pollard 1994; Moss 1997). In animal cells, annexins have been implicated in the transduction pathways of mitogenic signals, in membrane trafficking processes such as exocytosis, in interactions with cytoskeletal elements, or in the formation of voltage-dependent, ion-selective calcium channels (reviewed by Creutz 1992; Demange et al. 1994; Raynal and Pollard 1994). This wide variety of properties, together with varied intracellular localizations (associated with plasma or organelle membranes, cytoplasm, nuclei; see reviews of Gruenberg and Emans 1993; Raynal and Pollard 1994; Clark and Roux 1995) suggest that annexins are specialized for different cellular functions. In plants, annexins have also been reported to be associated with different cellular processes. Annexins purified from plant species such as tomato, maize, pea, cotton, and celery have different characteristics (reviewed by Clark and Roux 1995). For example, a celery annexin (Seals et al. 1994) was identified as a calcium-dependent, vacuole-associated protein whereas a cotton annexin (Andrawis et al. 1993) was associated with the modulation of plasma membrane-located callose synthase activity. Although both tomato and maize annexins show ATPase activity, only the tomato annexins are able to interact with the cytoskeletal element actin (McClung et al. 1994; Calvert et al. 1996). Moreover, a role for annexins in the oxidative stress response has recently been proposed since an *Arabidopsis*

thaliana annexin-encoding cDNA was able to complement an *Escherichia coli* mutant unable to grow in the presence of high concentrations of H₂O₂ (Gidrol et al. 1996). Finally, cDNA sequences to annexin genes showing enhanced expression during fruit maturation have been identified both in strawberry (Wilkinson et al. 1995) and in bell pepper (Proust et al. 1996). Further analysis would help to elucidate which of the reported properties of annexins are common or specific to different members.

The identification of a novel Nod-factor-induced early nodulin gene coding for an annexin opens new perspectives in relation to the molecular events occurring at early symbiotic stages. In particular, the specific Nod factor induction of the *MtAnn1* gene raises the intriguing question of whether the MtAnn1 protein could play a role in the Nod factor signaling pathway(s) that lead(s) to the induction of the early symbiotic responses. At this stage, several of the potential roles attributed to annexins could be envisaged for the MtAnn1 annexin during the symbiotic process, and certain of the recently reported Nod-factor-induced cellular responses could be related to MtAnn1 protein function. For example, Ehrhardt et al. (1996) have recently reported that periodic calcium spiking can be elicited in alfalfa root hairs in response to specific *R. meliloti* Nod factors, suggesting that calcium may be a second messenger in Nod factor signaling. In this context, annexins are calcium-binding proteins and many of their functions are mediated by or dependent on the presence of calcium ions. We can also speculate that MtAnn1 function may be related to the changes occurring in the cellular cytoskeleton during the nodulation process, since certain annexin proteins can interact in a calcium-dependent manner with cytoskeletal elements such as actin (Thiel et al. 1992; Calvert et al. 1996). Changes in the cytoskeletal network associated with different symbiotic events such as cortical cell activation and root hair deformation have been reported (Bakhuizen 1988; Allen et al. 1994). More detailed analyses on the spatial and temporal expression patterns of *MtAnn1* during early symbiotic stages, as well as the analysis of the biochemical properties and sub-cellular location of the MtAnn1 protein, should help to elucidate the cellular role of MtAnn1 in the symbiotic process.

MATERIALS AND METHODS

Plant treatments.

Both wild-type and the supernodulating mutant line TR122 (Sagan et al. 1995) of *Medicago truncatula* cv. Jemalong (genotype J5) were used in this work. For nodulation studies, plants were grown either aeroponically or in pouches as described in Gamas et al. (1996). Bacterial inoculations were performed on roots of nitrogen-starved, 1-month-old, aeroponically grown plants, or 8-day-old pouch-grown plants. The bacterial strains used were as follows: *Rhizobium meliloti* RCR 2011 (GMI6526; Ardourel et al. 1994), *R. meliloti* RCR 2011 *nodA::Tn5* #2208 (GMI5382; Deblle et al. 1986), *R. meliloti* RCR2021 *nodH::Tn5* #2313 (GMI6527; Faucher et al. 1989), *R. meliloti* RCR2021 *nodFL::(nodF)* 13 *nodL::Tn5* (GMI6629; Ardourel et al. 1994), and *R. meliloti* 1021 *exoA32::Tn5* (provided by J. Leigh, Washington University, Seattle). Inoculated roots or isolated nodules were harvested at different times, frozen immediately in liquid nitrogen, and stored at -70C.

Nod factor treatments were performed on 8-day-old pouch-grown plants by adding growth medium only (control) or growth medium supplemented with 10⁻⁷ M NodRm factors to the plant roots. Whole root systems were harvested at different times, frozen in liquid nitrogen, and stored at -70C. The NodRm factors are as described in Gamas et al. (1996). For pathogenesis studies, leaves of 2-week-old, pouch-grown plants were infiltrated with bacterial suspensions containing approximately 10⁸ CFU/ml.

The bacteria used were the incompatible bacteria *Pseudomonas syringae* pv. *lisi* (D. Roby, this laboratory) and the compatible bacteria *Xanthomonas campestris* pv. *alfalfae* (provided by CFBP, French Collection of Phytopathogenic Bacteria, INRA Station de Phytobactriologie, Beaucouz, France). Leaf tissues were harvested after different times post-inoculation: 3, 6, 23, and 48 h for the incompatible interaction, and 6, 23, and 48 h for the compatible bacteria. To evaluate possible wound induction of *MtAnn1*, *M. truncatula* leaves were cut in strips about 1 mm wide and incubated in MS medium (Murashige and Skoog 1962). Uncut control leaves were also incubated in MS medium. Leaf tissues were harvested after incubation for 30 s and 1, 2, 4, and 24 h on MS medium.

M. truncatula plants grown aeroponically were used for the isolation of total RNA from root, leaf, flower, and stem tissues.

RT-PCR differential display.

Reverse transcription (RT)-PCR differential display was performed essentially as described by Liang et al. (1994) with certain modifications. Total RNA was extracted from control roots and roots treated with NodRm factors for 2, 24, and 48 h. To eliminate contaminating genomic DNA, DNaseI (Promega, Madison, WI), treatment of RNA samples was carried out as described by Liang et al. (1994). The concentration and integrity of the RNA samples was evaluated by spectrophotometric analysis and gel electrophoresis. DNA-free total RNA (0.2 g) was used in first-strand cDNA synthesis reactions primed with 10 M oligo(dT₁₁) primers with two anchored bases: dT₁₁CC, dT₁₁CG, dT₁₁AC, or dT₁₁GG. Reverse transcription was carried out following the Superscript RT RNase H⁻ protocol (Gibco BRL, Life Technologies, Gaithersburg, MD). After heat inactivation, 10% of the cDNA was amplified by PCR with combinations of anchored oligo dT primers and 10-mer primers (OP: A-08, B-11, J-09, J-13, K-04, K-07, K-12, L-17, M-14, M-15, N-09, or N-12, Operon Technologies, Alameda, CA). Fifty-one, 31, and 20 primer combinations were used for, respectively, the 2-, 24-, and 48-h samples. PCR reactions were performed as described by Liang et al. (1994). Six microliters of each PCR reaction was denatured and analyzed on 6% denaturing polyacrylamide gels. PCR bands that were differential in three independent RT-PCRs were excised from gels and re-amplified by PCR as described by Liang et al. (1994). The re-amplified, blunt-ended cDNA fragments were cloned in a pUC18 vector with the Sure Clone Ligation Kit from Pharmacia Biotech (Uppsala, Sweden). Positive bacterial colonies with the expected insert sizes were selected by PCR amplification with primers to the vector polylinker sequences.

RNA gel blot analysis.

Total RNA was extracted from nodule, root, leaf, stem, and floral tissues as described by Jackson and Larkins (1976).

When indicated, polyA⁺ RNA was extracted from total RNA preparations with magnetic beads (Dynabeads oligo dT25 from Dynal, Oslo, Norway). Total RNA (10 µg) or 120 ng of polyA⁺ RNA was electrophoresed in a 1.5% agarose-formaldehyde gel and blotted on Nytran-plus membranes (Schleicher & Schuell, Dassel, Germany) according to Sambrook et al. (1989). To verify RNA quality and quantity, ethidium bromide (0.25 µg per RNA sample) was added to samples prior to gel electrophoresis, and the RNA samples were photographed after blotting. Plasmids containing *MtAnn1* sequences cloned in the pBluescript II vector (Stratagene, La Jolla, CA) were used to generate α³²P-labeled riboprobes with the Promega transcription kit. To detect EF-1α transcripts, radioactive DNA probes were generated from an *A. thaliana* EF-1α gene (Axelos et al. 1989), using the Ready To Go labeling kit (Pharmacia). Hybridization and washing conditions were essentially performed as described in Gamas et al. (1996).

DNA sequencing and sequence analysis.

Plasmid DNA prepared with the Wizard miniprep kit (Promega) was sequenced on both strands by using the double-stranded dideoxy chain termination method with Sequenase enzyme (Amersham International, Les Ulis, France). Sequence data were analyzed with the University of Wisconsin Computer group (GCG) software. The EMBL accession number for the *MtAnn1* cDNA sequence is Y15036.

cDNA library screening.

The *MtAnn1* cDNA clone was isolated from a directional Lambda ZAP II (Stratagene) cDNA library prepared from RNA isolated from *M. truncatula* roots 6, 24, and 48 h after inoculation with *R. meliloti* (Szybiak-Strozycka et al. 1995). About 270,000 plaques plated onto 15-cm plates (approximately 46,000 plaques per plate) were transferred onto replica reinforced nitrocellulose membranes (Optitran BA-S 85, Schleicher & Schuell) that were then hybridized with ³²P-labeled *dda* DNA probes. Hybridization and washing conditions were the same as for RNA blot hybridizations except that the hybridization temperature was 42°C. Positive phage plaques were directly used for PCR amplification with the 5' M13-reverse primer to Lambda ZAP sequences, and the 3' Fc2 primer (5'TCCCCAAGAGAGTGAGAAG3') to the *dda* sequences. Phage plates that generated PCR products of the expected size for the entire *dda* cDNA sequence were selected for phage purification and DNA in vivo excision following manufacturer's instructions (Stratagene).

In situ hybridization.

In situ hybridizations were performed with 7-µm sections of nodules isolated from pouch-grown plants, as described by van de Wiel et al. (1990) except that slides were exposed for 3 weeks with Amersham LM1 emulsion (Amersham). *MtAnn1* sense and antisense riboprobes were synthesized following the Promega transcription kit protocol and were labeled with 50 µCi of ³⁵S-UTP (1,500 Ci/mmol). Probes were partially degraded to a length of approximately 150 bases before hybridization with tissue sections. Hybridizations were performed in a volume of 100 µl per slide containing 5 × 10⁶ cpm of probe (approximately 3 × 10⁶ cpm/µl).

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