Use of a Subtractive Hybridization Approach to Identify New *Medicago truncatula* Genes Induced During Root Nodule Development

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We report the identification of new molecular markers associated with different stages of *Rhizobium*-induced nodule development in the legume *Medicago truncatula*. A cDNA library was constructed from pre-nitrogen-fixing *M. truncatula* nodules, and differentially screened with a polymerase chain reaction–amplified subtracted probe. Twenty-nine new families of nodulin cDNA clones, designated *MtN1* to *MtN29*, were thus identified in addition to clones for several known nodulins. All *MtN* genes were shown by Northern (RNA) hybridization analysis to be induced during nodulation, some of them well before nodule emergence. The *MtN* genes were classified into three groups depending on their expression kinetics. The expression of three *MtN* genes showed a limited induction by Nod factors purified from *Rhizobium meliloti*. Homologies with a variety of proteins were found for the deduced amino acid sequences of 10 of the *MtN* genes.

Additional keyword: symbiosis.

The *Rhizobium*-legume symbiosis requires the formation of specialized organs, the root nodules, which provide the microenvironment necessary to support nitrogen fixation by the bacteria. The emergence of root nodules represents the outcome of a process involving a mutual exchange of specific molecular signals between the plant and the bacteria (for reviews see Fisher and Long 1992; Hirsch 1992; Dénarié and Cullimore 1993; Spaink and Lugtenberg 1994; Mylona et al. 1995). Rhizobia are first attracted by root exudates, and expression of bacterial *nod* genes is then induced by plant compounds (flavonoids and betains). Proteins encoded by these genes are responsible for the synthesis of extracellular lipooligosaccharides known as Nod factors. In turn Nod factors elicit a number of specific responses in different root tissues of the host plant, notably root hair deformations and cell cycle reactivation of cortical cells (Yang et al. 1994). This reactivation leads to nodule primordia formation in the inner cortex through a series of mitoses, and also to the appearance of preinfection structures in outer cortical cells. In contrast, structures directly involved in the infection process usually fully develop only in the presence of bacteria. Shepherd's crooks result from root hair curling and cause bacteria en-

trapment, a prerequisite for the invagination of the plasma membrane and the formation of tubular infection threads that allow the bacteria to enter into the underlying root tissues (for review see Kijne 1992).

Nodules develop from primordia by differentiation of specialized tissues. In *Medicago* and other temperature legumes, mature nodules are cylindrical structures growing continuously from an apical meristem (indeterminate nodules). In *Medicago* nodules four zones have been distinguished in addition to the apical meristem (Vasse et al. 1990): an infection zone (zone II), an interzone II/III (amyloplast-rich), a nitrogen-fixing zone (zone III), and a senescence zone (zone IV).

In some legumes, genuine nodule formation can be observed in the absence of bacteria, in response to specific Nod factors (Truchet et al. 1991; Schultze et al. 1992; Mergaert et al. 1993; Relic et al. 1994; Stokkermans and Peters 1994). In addition, in some lines of *M. sativa*, so-called Nar nodules (nodulation in the absence of *Rhizobium;* Truchet et al. 1989) can arise spontaneously after a period of time under combined nitrogen starvation. These observations establish that all the information necessary for nodule development is carried by the plant genome. The study of nodulation mutants of *Melilotus* (Utrup et al. 1993), and of alfalfa responses to bacterial mutants producing altered Nod factors (Ardourel et al. 1994), indicate that it is possible to uncouple different plant responses to *Rhizobium*, which suggests the existence of several developmental programs during nodulation.

The whole nodulation process is accompanied by the expression of specific genes, known as nodulin genes (reviewed by Sanchez et al. 1991; Franssen et al. 1992). Conventionally, the distinction is made between early nodulin genes, expressed well before the onset of nitrogen fixation, and late nodulin genes, mainly expressed in mature, nitrogen-fixing nodules. Many of the known late nodulins (leghemoglobins, for example) are proteins involved with nodule physiology, especially nitrogen fixation and related metabolic fluxes, or with the structure and formation of the membranes surrounding the bacteria. By contrast, early nodulins are considered to be related to the infection process or to the morphogenetic and organogenetic plant responses. cDNA clones for early nodulins were originally identified by differential screening of nodule libraries (Franssen et al. 1987; Scheres et al. 1990; Kouchi and Hata 1993; Perlick and Pühler 1993) and some subsequently reisolated from other legumes by heterologous

cloning. The sequences of some early nodulins (Enod 2, 5, 12, for example) suggest that they could be cell wall proteins, including the family of proline-rich proteins (PRPs). In *Medicago*, genes for PRPs have been identified using *Psenod12* and *Psenod2* probes and designated *Msenod10* (Löbler and Hirsch 1993), *Mtenod11* (D. Barker, personal communication), *Mtenod12* (Pichon et al. *1992*), *Msenod12A/Msenod12B* (Allison et al. *1993*) and *Mtprp4* (Wilson et al. 1994). Three non-proline-rich early nodulins have also been identified in *Medicago:* Enod8 (Dickstein et al. 1993), Ms/MtEnod40 (Asad et al. 1994; Crespi et al. 1994a), and a peroxidase called Rip-1 (Cook et al. 1995).

Known nodulins have already proved very fruitful as markers, notably to define cell- or tissue-specific responses. For example, a parallel study of the expression of *Psenod5* and *Psenod12* in pea (Scheres et al. 1990) has revealed distinct induction patterns, since *Psenod5* transcripts are detected only in cells containing infection threads, whereas *Psenod12* expression is induced in advance of infection. In *Medicago*, a study of *Mtenod12* (Pichon et al. 1992; Journet et al. 1994) and *rip-1* (Cook et al. 1995) expression has led to the visualization of the region of the root (the zone of differentiating epidermal cells behind the growing root tip), which is activated early in response to *R. meliloti*, perhaps to prepare epidermal cells for subsequent infections. Furthermore, in situ hybridizations with *enod40* probes have revealed that the pericycle is involved in early stages of nodulation (Kouchi and Hata 1993; Yang et al. 1993; Crespi et al. 1994a). Finally, several early nodulin genes (*enod5, enod11, enod12, enod40*, and *rip-1*) have been shown to be induced by specific Nod factor treatments (Horvath et al. 1993; Bauer et al. 1994; Crespi et al. 1994a; Journet et al. 1994; Cook et al. 1995; Vijn et al. 1995; D. Barker, personal communication).

In view of the interesting information obtained from a limited number of nodulin genes, we thought that it would be informative to study a larger range of markers to characterize nodule development. We thus decided to set up a sensitive procedure to identify clones for genes induced during nodulation in the model legume *M. truncatula* Gaertn. (Barker et al. 1990).

RESULTS

Differential screening of a *M. truncatula* nodule cDNA library.

The strategy used for the identification of new nodulins is based on a subtractive hybridization approach as presented in Figure 1. To increase the chance of finding early nodulins expressed before nitrogen fixation, a cDNA library was constructed from pre-nitrogen-fixing, 4-day-old *M. truncatula* nodules. A first characterization of the library showed that, as expected, clones representing the late nodulin leghemoglobins MtLb1 and MtLb2 (Gallusci et al. 1991) were less abundant than those corresponding to the early nodulins MtEnod11, and MtEnod12 (Pichon et al. 1992; D. Barker, unpublished data), which were both found to be present at a frequency of about 1 per 1,000 clones (data not shown).

Subtracted probes were generated from cDNA of 4-day-old nodules RNA, after hybridization with RNA from *M. truncatula* roots inoculated with a nonnodulating (*nodA⁻*) strain of *R. meliloti* (control roots). The average length of cDNA mole-

cules was limited by using a mixture of random hexamers and oligodeoxythymidine as primers for cDNA synthesis. This procedure should reduce undesirable subtractions caused by homologies present in limited domains and hence should allow the identification of clones for members of multigene families that are differentially regulated. Single-stranded cDNA made from nodule polyA⁺ RNA was incubated with an excess of control biotinylated polyA⁺ RNA; hybridizing cDNA molecules, corresponding to noninduced material, were subsequently eliminated (Sive and St. John 1988). It was verified that subtracted probes gave a substantially stronger hybridization signal with *Mtenod11* and *Mtenod12* cDNA clones than did nonsubtracted probes (data not shown).

To increase the screening sensitivity we introduced a polymerase chain reaction (PCR) amplification step following the subtraction. The limited size of the cDNAs should allow homogeneous PCR amplification. To establish that this was so, the amplified and nonamplified cDNA samples were analyzed for their size distribution as well as their hybridization pattern to several thousands of random clones from the nodule cDNA library; both criteria showed that the probes were unaffected by the amplification step (data not shown).

Thirty thousand cDNA clones were screened with the PCR-amplified control and subtracted probes: 473 clones responded differentially, of which 109 could be detected with a mixture of probes corresponding to a variety of nodulins (MtLb1, MtLb2, MtEnod11, and MtEnod12, and two *Medicago* nodulins related to PsEnod5). The remaining differential clones were isolated and classified into independent families by a series of successive cross-hybridizations, using individual cDNA inserts as probes. Thirty different families were thus identified, among which 29 turned out to be novel (see below) and will be referred to as *MtN* (*M. truncatula* Nodulin) clones (Fig. 2). Five families were highly represented (from 16 to 58 clones); the abundance of the others was either moderate (from 4 to 11 clones for 7 families) or low (1 to 3 clones for the remaining 17 families). The last family (of 34 clones) showed homology to *enod40* (see below).

Expression analysis of *MtN* clones.

In a first series of analysis three questions were addressed, aimed at determining whether MtN proteins are more likely to be involved in nodule organogenesis or nodule function: (i) Is the expression of *MtN* clones induced during nodulation?; (ii) Is this induction maximal before or after the onset of nitrogen fixation?; and (iii) Is *MtN* gene expression detectable in *M. sativa* Nar nodules?

Figure 3 shows a Northern (RNA) hybridization analysis of total RNA for a representative selection of nodulins and *MtN* clones. A compilation of various data concerning all the identified *MtN* clones and some known nodulins is presented in Figure 2. The first four lanes of Figure 3 correspond to control RNA samples of different origins, including a stress control to establish that gene inductions were not provoked by wounding caused by nodule harvesting. The next four lanes represent RNA extracted from plants inoculated with wild-type *R. meliloti* and harvested at different times after inoculation. The last lane shows RNA isolated from 3-week-old Nar nodules.

A parallel study of expression of known nodulins in our experimental conditions provides a useful framework for

evaluating the expression data of *MtN* clones. From Figures 2 and 3, different patterns of expression can readily be observed among the known early nodulins. *Mtenod11* and *Mtenod40* expression is maximal in young nodules but *Mtenod40* is also highly expressed in Nar nodules in contrast to *Mtenod11*. *Mtenod2* transcripts are clearly more abundant in mature and Nar nodules although easily detectable in young nodules. The expression of these early nodulin genes can be distinguished from those encoding late nodulins such as MtLb1 whose transcripts are abundant only in mature nodules. Interestingly, *Mtlb1* transcripts, which had not previously been detected in either 4-day-old wild-type nodules or in Nar nodules (Truchet et al. 1989; Gallusci et al. 1991), were detected in this analysis, demonstrating that we were employing very sensitive hybridization conditions.

This analysis clearly establishes that the expression of *MtN* genes is induced during nodulation. In comparison with control roots, 17 *MtN* genes are expressed virtually exclusively in nodulated roots, whereas the remaining genes display either low (eight genes) or moderate (four genes) basal expression levels. For only one clone (*MtN23*) some expression is detected in the stress control, albeit at a level significantly lower than in the nodule samples (data not shown). In three cases (*MtN1, 2,* and *22*), two nodulation-induced transcripts can be detected (see Figures 2 and 3). In one case (*MtN12*), the probe hybridizes to several transcripts, among which only one (shown in Figure 2) is induced during nodulation. Whereas the expression of most *MtN* genes is higher in young nodules (group I; Fig. 2), six genes exhibit expression kinetics similar to those of *Mtenod2* (group II; expression highest in mature nodules but detectable in young nodules), and three genes behave as late nodulin genes since their transcripts are readily detectable only in mature nodules (group III). Finally, it is possible to further subclassify the genes according to their

expression in Nar nodules with about half the genes from groups I and II being expressed in these noninfected nodules. For *MtLb1*, *MtN11*, *MtN16,* and *MtN17* the detected RNA is of a different size in Nar nodules compared with *M. truncatula* nodules, which may reflect a difference between *M. sativa* and *M. truncatula*.

Figure 3 shows that, for a few RNA species, expression can be detected as early as 48 h after inoculation with *R. meliloti*. To further explore this early expression and to increase the sensitivity of detection, an additional Northern analysis was carried out on polyA$^+$ RNA prepared from samples harvested 3, 24, and 48 h after inoculation (see Figures 2 and 4). The amount of RNA loaded in each track was controlled with a

clone name	number of isolates	mRNA (kb)	mRNA expression data					Nar nod.
			basal level	24h. roots	48h. roots	4d. nod.	14d. nod.	
Mtenod11		0.8	0	+	+		+	+-
Mtenod40	34	0.8	-	+	+		+-	+-
Mtenod2		1.6	0	0	0	+		+-
Mtlb1		0.6	0	--	--	-		-
MtN1	16	0.6	0	-	+-		+-	0
		1.0	0	--	-	+	-	0
MtN2	4	1.8	0	--	+-		+-	0
		3.0	0		-	+	-	+
MtN3	3	1.3		+-	+-			
MtN4	18	1.5	+-		+		+-	+-
MtN5	3	0.8	+-	+	+		+	+-
MtN6	4	1.7	--	-	+-		+-	+-
MtN7	1	1.1	0	--	-	+	+-	0
MtN8	1	1.1	--	-	-	+		
MtN9	2	1.2	0	0	+-		--	--
MtN10	58	1.1	-	-	+			
MtN11	25	0.5	0	0	--		+	
MtN12	30	1.0						
MtN13	11	0.7	0	0	0			+
MtN14	2	0.9	-	-	-	+	-	+
MtN15	1	0.6	0	0	0	+	-	0
MtN16	10	0.6	0	0	0	+-	-	
MtN17	2	0.5	0	0	0	+-	-	-
MtN18	2	2.8	0	0	0	+	-	+-
MtN19	1	1.3	--	--	--			--
MtN20	3	0.8	0	0	0			
MtN21	2	1.4	--	-	-			0
MtN22	9	0.8	0	--	-			0
		2.0	0	0	0	-	+	0
MtN23	3	1.8	+-	+-	+-			
MtN24	2	0.9	0	-	-			0
MtN25	7	0.7	0	0	0			0
MtN26	3	0.7	0	0	0			--
MtN27	2	0.6	0	0	0	-	+	0
MtN28	1	0.5	0	0	0	+-		0
MtN29	8	2.8	0	0	0	-		0

(Groups I, II, and III are indicated to the left of the respective clone-name blocks.)

Fig. 2. Summary of results obtained for known nodulin genes and *MtN* clones. Indicated, from left to right, are the assigned expression class of the *MtN* sequences (see text), the number of cross-hybridizing cDNA clones isolated from the nodule cDNA library, and the size of corresponding transcript(s) detected by Northern (RNA) hybridization analysis. The mRNA expression data indicate the basal level of expression in control roots, and the level of induction in *Rhizobium meliloti*–inoculated roots or isolated nodules, and in spontaneous Nar *M. sativa* nodules. Levels of expression are depicted by signs (0, --, -, +-, +, ++, +++, reflecting undetectable to strong expression) and by shading intensity (the darker corresponding to the stronger expression). Indicated results were obtained by analyzing entire root systems (harvested 24 and 48 h after infection), and 4- or 14-day-old nodules. Nar nodules were harvested from *M. sativa* cv. Gemini clone F (Truchet et al., 1989) 3 weeks after the start of nitrogen starvation.
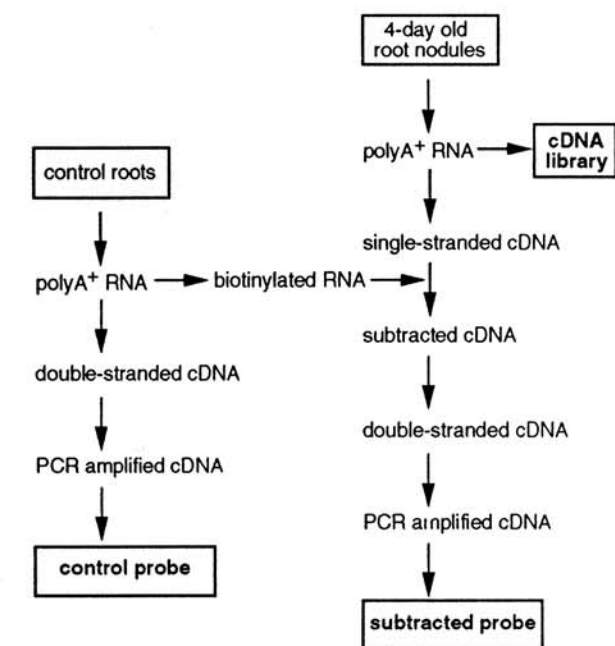

Fig. 1. Strategy for cloning *Medicago truncatula* nodulin cDNAs. The subtracted and control probes were used to screen the cDNA library differentially.
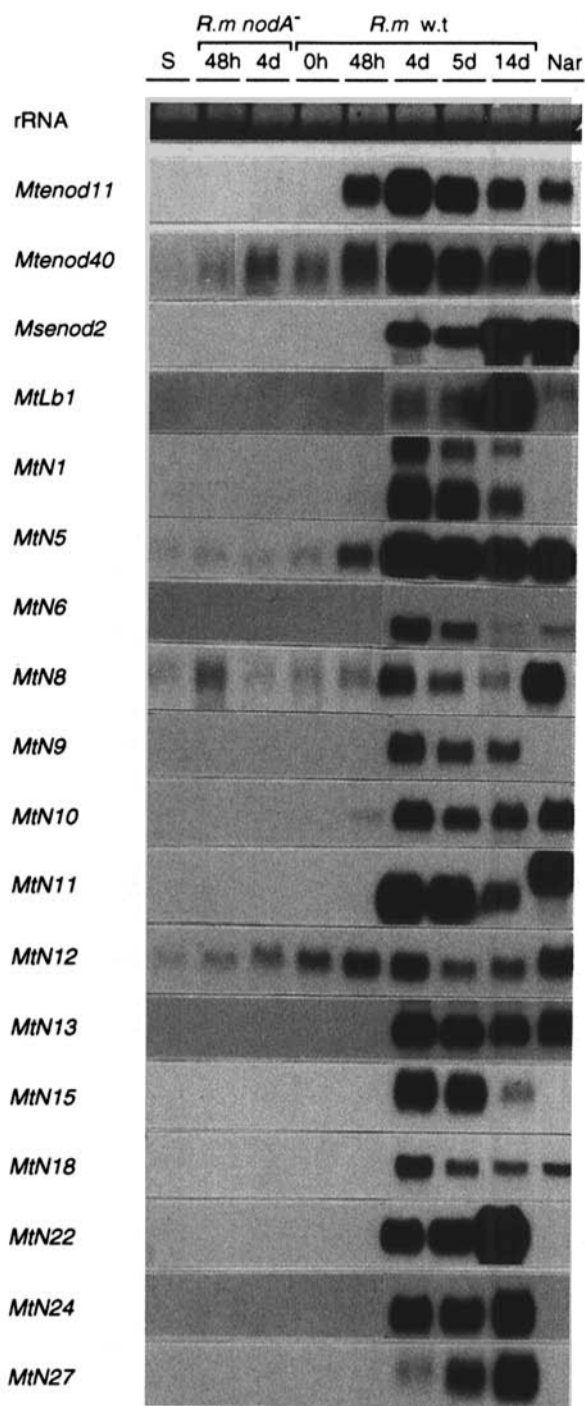
Flowchart (Fig. 1):

4-day old root nodules → polyA$^+$ RNA → cDNA library

polyA$^+$ RNA → single-stranded cDNA

control roots → polyA$^+$ RNA → biotinylated RNA → subtracted cDNA

biotinylated RNA → double-stranded cDNA → PCR amplified cDNA → control probe

subtracted cDNA → double-stranded cDNA → PCR amplified cDNA → subtracted probe

Fig. 3. Expression analysis of representative nodulin and *MtN* clones. Northern (RNA) hybridization analysis of 8-µg samples of total RNA with the indicated radioactive probes was performed as described in Material and Methods. Autoradiography exposures vary, depending on the clone, from a few hours to several days. A picture of a representative membrane showing ethidium bromide–stained 28 S rRNA depicts the evenness of the RNA loaded and transferred. The left lane, labeled S, represents a stress (wound) control, which corresponds to RNA prepared from segments of roots from noninoculated, nitrogen-starved plants, cut in the same way as for nodule harvesting. RNA was extracted either from total root systems (first five lanes) or from nodules (next four lanes) harvested at different times following inoculation with either wild-type *R. meliloti* (*R.m* w.t) or a nonnodulating mutant (*R.m nodA⁻*). Spontaneous Nar nodules were harvested from roots after 3 weeks of combined nitrogen starvation.

probe corresponding to the translation elongation factor *EF-Iα* gene (Axelos et al. 1989) since expression of this gene did not appear to be induced at these early time points, although moderately induced in young nodules (data not shown), as reported for broadbean by Perlick and Pühler (1993). From this analysis, 14 *MtN* genes (*MtN1* to *MtN12*, *MtN22*, and *MtN24*) can be considered as induced before nodule emergence, although for three of them (*MtN3, 4,* and *5*) there may also be a transient induction following inoculation by the nonnodulating strain.
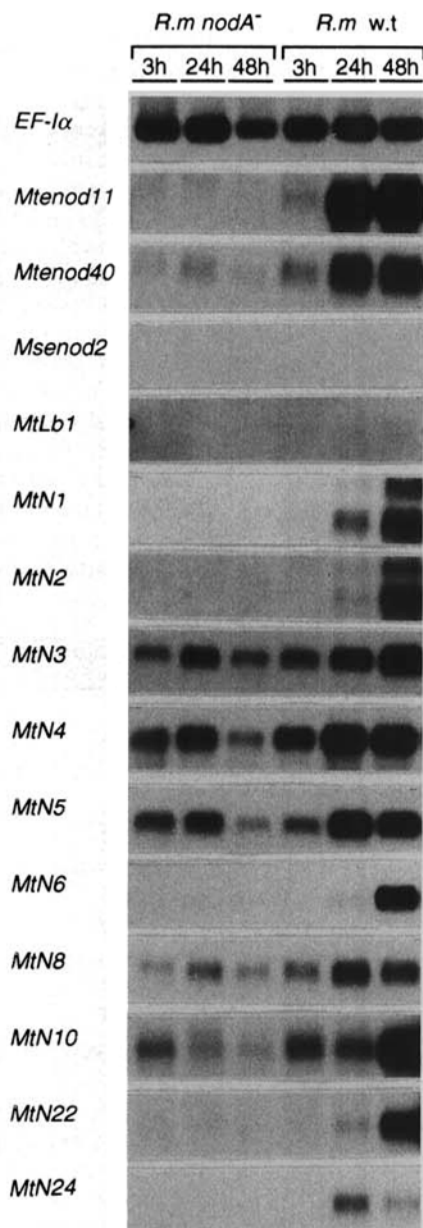

Fig. 4. Early induction of a subset of *MtN* genes after *Rhizobium* inoculation. Autoradiographs of Northern (RNA) blots obtained with approximately 500 ng of polyA⁺ RNA from entire roots harvested at indicated times after inoculation with mutant (*R.m nodA⁻*) or wild-type (*R.m* w.t) *R. meliloti*.

Effects of NodRm treatment on expression of *MtN* genes.

Previous work in our laboratory has established that specific *Rhizobium* Nod factors cause root hair deformations and cortical cell divisions in *M. truncatula* (P. Gamas, unpublished data), as reported for *M. sativa* and many other legumes (see above). It has also been demonstrated that Nod factors from *R. meliloti* (NodRm factors) induce expression of the *M. truncatula* early nodulin genes *Mtenod11*, *Mtenod12* (Journet et al. 1994; D. Barker, personal communication), and *rip-1* (Cook et al. 1995).

To investigate whether purified Nod factors could induce expression of *MtN* genes we used two different sets of conditions. In the first one, plants were grown for 9 days in growth pouches, and treated for 48 h with 10^{-7} M NodRm. In the second one, *M. truncatula* was grown aeroponically for about 5 weeks, and treated for 24 h with 10^{-8} M NodRm. Under both conditions *Mtenod11* expression was substantially induced by Nod factors (Fig. 5). The effect upon *Mtenod40* was not as obvious, but was nonetheless apparent in comparison with noninduced controls (*EF-1α* and *Mtcyc*). Only three of our *MtN* genes (*MtN5, 8,* and *12*) showed some level of induction by Nod factors. These genes all belong to group I, two of them (*MtN8* and *12*) corresponding to PRPs (see below).

Sequence analysis of *MtN* clones.

The whole cDNA insert was sequenced for the nine shortest clones (*MtN5, 11, 13, 15, 16, 17, 25, 27,* and *28*). About 250 to 500 nucleotides (nt) were sequenced from the 5′ end of the remaining clones, and the presence of a polydT tract was verified by sequencing about 200 nt at the 3′ end. Homology searches indicated that among all the differential clones identified during this screening (apart from *Mtenod11*, *Mtenod12*, *Mtlb1*, and *Mtlb2*, and the two *M. truncatula* clones related to *Psenod5*, which were eliminated at an early stage), only one, *Mtenod40*, was already present in the data bases (Crespi et al. 1994a). Interestingly, the *Mtenod40* cDNA that we sequenced contains an additional 92 bp before the polydT tract but was otherwise identical over the regions sequenced to the cDNA of Crespi et al. (1994a). Partial homologies were found for 10 clones (see Table 1), whereas the last 19 clones were not seemingly related to known DNA or protein sequences. Surprisingly, no cDNA clones corresponding to *enod2* were isolated, even though strong signals were detected on Northern blots with a *Msenod2* probe. Either these clones are poorly represented in our library or in our subtracted probe, or they were screened out through cross-hybridization to probes for other PRPs (such as Mtenod11).

The translated sequences of two clones, *MtN8* and *MtN12*, display features of (hydroxy)proline-rich structural proteins, with a proline content higher than 30% (as well as a high proportion of histidine and tyrosine) and a repetitive structure (Fig. 6). On the basis of partial sequence analysis they differ one from another by their repeat organization and differences in amino acid content. The MtN8 putative protein appears to be related to the PRP family, whereas the MtN12 amino acid sequence contains the SPPPP repeat characteristic of the extensin family (for reviews see Showalter 1993; Kieliszewski
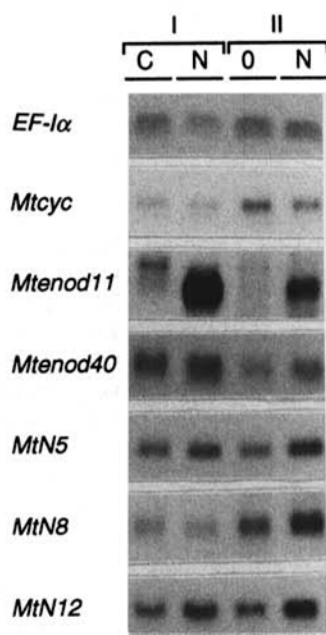

Fig. 5. Effect of NodRm treatments upon *MtN* gene expression. Approximately 20-µg samples of total RNA were subjected to Northern (RNA) hybridization analysis with indicated probes. Probes corresponding to control, non-induced genes were prepared from an *Arabidopsis thaliana EF-1α* gene and a *Medicago truncatula* cytochrome c cDNA. The two left lanes (condition I) correspond to plants grown in growth pouches and treated for 48 h with 10^{-7} M NodRm (lane N) or with growth medium only (lane C). The right lanes (condition II) represent plants grown in aeroponic conditions; lane N corresponds to plants treated for 24 h with 10^{-8} M NodRm, and the lane 0 to the t_0 time point (plants harvested just before the treament).

Table 1. Sequence homologies detected for *MtN* clones

Clone	Sequence 5′ (bases)	Best homology[a]	Program[b]	Statistical significance[c]
MtN3	493	*Arabidopsis thaliana* EST	FASTA	63 % in 341 bp
MtN6	387	*Emericella nidulans* FluG protein	BLASTX	$4.8 \; 10^{-7}$
MtN8	311	*Pisum sativum* Enod2	BLASTX	$2.5 \; 10^{-24}$
MtN9	350	*Glycine max* metalloendoproteinase 1	BLASTX	$1.6 \; 10^{-19}$
MtN10	235	*Pisum sativum* carbonic anhydrase	BLASTX	$2.6 \; 10^{-10}$
MtN12	235	*Lycopersicon esculentum* HRGP	BLASTX	$5.4 \; 10^{-28}$
MtN13	620	*Pisum sativum* disease resistance response protein (DRRG49-c)	BLASTX	$1.7 \; 10^{-45}$
MtN18	293	*Arabidopsis thaliana* cycloartenol synthase	BLASTX	$6.8 \; 10^{-41}$
MtN22	388	*Vicia faba* Nvf-30 mRNA	FASTA	71.4 % in 84 bp
MtN23	341	*Nicotiana tabacum* ascorbate oxidase	BLASTX	$1.3 \; 10^{-38}$

[a] From search of "nonredundant" protein data base (BLASTX) or GenBank and EMBL DNA data bases (FASTA). Only the best homology is indicated.

[b] BLASTX (Altschul et al. 1990) and FASTA (Pearson and Lipman 1989) programs, which search on translated open reading frames and DNA sequences respectively, were used with default parameters values. These two programs generally gave similar results except in a couple of cases where gaps had to be authorized to optimize homologies (which is possible with FASTA program only).

[c] The probability of finding the homology by chance (BLASTX), or the percent identity in the homologous region (FASTA), is shown.

and Lamport 1994); as shown in Figure 6 this motif is included within a larger repeat of 13 amino acids.

The sequence of a third clone, *MtN22*, presents homologies in its 5′ end to the late nodulins Nvf-30/Nvf-32 from broadbean (Küster et al. 1994) and Nms-25 from alfalfa (Kiss et al. 1990). Nvf-30/Nvf-32 and Nms-25 show two short regions of homology, one near the N terminus and the other near the C terminus (Küster et al. 1994). No significant homologies were found with the 3′ end of *MtN22*, but the sequenced region (262 nt) was essentially noncoding.

The remaining *MtN* clones shown in Table 1 are likely to represent nonstructural proteins. The translated sequence of one of them, *MtN10*, shows strong homologies to carbonic anhydrases of various species, an enzyme that was recently reported to be induced in *Medicago* nodules (Crespi et al. 1994b). The others are homologous to sequences not yet described in the context of the *Rhizobium*-legume symbiotic association.

DISCUSSION

We report in this paper an experimental approach, based on PCR-amplified subtracted probes, that allowed us to isolate a number of cDNA clones for *M. truncatula* genes induced during root nodule development. Indeed, 29 distinct families of cDNA clones were identified after a differential screening of a nodule library. Sequence determinations suggest that most of them correspond to genes not previously described.

MtN8:
```
caccaaattataacccaccgcgtcatgaaaagccaatgccaaagtatcaa 50
    P N Y N P P R H E K P M P K Y Q

cctcctcatgaaaaactaccaccagtgtataaatcgccttatgtgaaaac 100
P P H E K L P P V Y K S P Y V K T

acctccaccacaagcatatcatccacccccaccaatttaccatcatccac 150
P P P Q A Y H P P P P I Y H H P P

cctttcacccacctcctcatgtgaaaccacctgtctacgagacacctcta 200
 F H P P P H V K P P V Y E T P L

gctaaggtaccacaaatgaagaaaccaacacgctacaatactcgaccttt 250
A K V P Q M K K P T R Y N T R P F

tggccattatccaccatataacaacaatccaataatgacaccactgaaga 300
G H Y P P Y N N N P I M T P L K N

atgtggcgtat 311
V A Y
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MtN12:
```
agtctaccactctcctccaccccagttcatcacacctaccctaaaccag 50
V Y H S P P P P V H H T Y P K P V

tctaccactctcctccaccccagttcacacctaccctcaccctaaacca 100
 Y H S P P P P V H T Y P H P K P

gtctaccactctccaccaccccagttcacacttaccctaaaccagtcta 150

V Y H S P P P P V H T Y P K P V Y

ccactccccaccaccaccagttcacacctacgtxcctcaccctaagccag 200

 H S P P P P V H T Y V P H P K P V

tctaccactctccaccaccaccagttcacacctat 235
 Y H S P P P P V H T Y
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Fig. 6. Nucleotide and predicted amino acid sequence of proline-rich MtN sequences. The figure shows sequences from the 5′ ends of cDNA clones corresponding to MtN8 and MtN12 (containing inserts of 1.4 kb and 0.7 kb respectively). The amino acid sequences of the longest open reading frames are presented. PPXXX repeats of MtN8 are underlined, whereas perfectly conserved motifs of 13 amino acids (found in MtN12) are doubly underlined.

In addition, clones were also identified for *Mtenod11*, *Mtenod12*, *Mtenod40*, *Mtlb1*, and *Mtlb2*, and two *M. truncatula* clones hybridizing to *Psenod5*. In contrast to what we observed for several of the known early nodulins, many of our *MtN* clones were not highly represented in our cDNA library, thus confirming the utility of using a sensitive screening technique.

On the basis of Northern analysis we have distinguished three main classes among our *MtN* clones. Classes I and II correspond to early nodulins, expressed well before the onset of nitrogen fixation. These clones can be subdivided into a first group (class I, *MtN1* to *21*) in which transcripts are of lower abundance in mature nodules, and a second group (class II, *MtN22* to *26*) in which transcripts are at least as abundant in mature nodules as in pre-nitrogen-fixing ones. Class III contains three clones (*MtN27* to *29*) that show readily detectable expression only in mature nodules. Interestingly, about half of our *MtN* genes (all from classes I and II) are also expressed in uninfected Nar nodules, which suggests that their encoded proteins play a role in nodule organogenesis or structure rather than in the processes related to nitrogen fixation or interactions with the bacteria. The different expression patterns of *MtN* genes suggest that they should provide useful markers for studying nodule organogenesis. The fact that a number of them are expressed well before nodule emergence whereas others are expressed in mature nodules indicates that they are related to different stages of development. This illustrates the advantage of having used indeterminate nodules as the biological starting material for cloning, since successive developmental stages coexist within such nodules. It is now important to determine the spatial expression patterns of these genes using in situ hybridization.

In a first series of experiments aimed at identifying the signals required for eliciting *MtN* gene expression, we investigated the effect of the exogenous addition of Nod factors. None of the *MtN* genes was found to be substantially induced by Nod factor treatment, under conditions in which *Mtenod11* was well expressed. Nevertheless our results suggest a limited induction for three *MtN* genes, including *MtN8* and *MtN12*, that encode putative (hydroxy)PRPs. A larger range of conditions will now have to be tested to examine Nod factor induction in greater detail, but it is likely that the number of nodulin genes that are induced early by Nod factors is quite limited.

Two *MtN* clones belong to well-characterized families of (hydroxy)PRPs. The MtN8 putative protein is structurally related to Enod2 (Franssen et al. 1987; Van de Wiel et al. 1990), but is expressed much earlier and more transiently. The *MtN12* gene encodes an extensin-like protein (see Showalter 1993; Kieliszewski and Lamport 1994). Northern results revealed several differently regulated transcripts related to *MtN12;* it will be interesting to determine whether these transcripts are derived from a single or multiple genes. It is worth mentioning that Perlick and Pühler (1993) also cloned an extensin-like nodulin from *Vicia faba*. It should be recalled that many (hydroxy)PRPs are located in the plant cell wall, a structure that plays a major role during different stages of nodulation (root hair curling, infection thread formation, cell division and differentiation, and emergence of nodules through the root cortex).

One clone (*MtN22*) presents at its 5′ end a region of homology to cDNAs representing late nodulins of unknown

function, Nms-25 and Nvf-30/Nvf-32, identified in *M. sativa* (Kiss et al. 1990) and *V. faba* (Perlick and Pühler 1993; Küster et al. 1994), respectively. Alignment of the Nvf-32 and Nms-25 amino acid sequences reveals only two short homologous stretches, one at the N termini of the putative proteins and the other at the C termini (Küster et al. 1994). Strong homologies observed between these proteins and the MtN22 amino acid sequence also cover the same N-terminal region, which essentially corresponds to proposed signal peptides (Kiss et al. 1990; Küster et al. 1994). The fact that *MtN22*, *Nms-25*, and the related genes from *V. faba* exhibit similar expression kinetics suggests that this homology is not fortuitous. Interestingly, two transcripts hybridize to the *MtN22* probe, indicating the possible existence of two genes, perhaps including a closer *M. truncatula* homolog of *Nms-25*.

For seven other *MtN* sequences, homologies have been found with diverse proteins, including carbonic anhydrase (see Raines et al. 1992), ascorbate oxidase (Ohkawa et al. 1989), cycloartenol synthase (an enzyme involved in plant steroids biosynthesis; Corey et al. 1993), a metalloendoproteinase (McGeehan et al. 1992), *Aspergillus nidulans* FluG protein (Lee and Adams 1994), and members of the family of pathogenesis-related proteins, PR10 (for review see Van Loon et al. 1994). Among these only carbonic anhydrase has previously been reported to be induced during nodulation (Crespi et al. 1994b).

It is premature to speculate on the role of these putative MtN proteins, but it is clear that the variety and novelty of the detected genes promise to open up new perspectives for nodulation studies. In the short term, more detailed analysis will need to be carried out to determine cell and tissue specificities of *MtN* gene expression, and also to establish whether *MtN* gene induction is restricted to nodulation or whether it can be induced in other contexts during plant development.

MATERIALS AND METHODS

Plant material.

For nodule production, plants of *M. truncatula* cv. *Jemalong* genotype J5 were grown aeroponically as described in Gallusci et al. (1991), except that they were nitrogen starved for 5 days before bacterial inoculation. The plants were 4 to 5 weeks old when inoculated with *R. meliloti* RCR 2011 (GMI51). Under these conditions, hundreds of young nodules could be visually detected on each root system, approximately three and a half days after infection. Nodules were harvested, by dissection, at indicated times, immediately frozen in liquid nitrogen and stored at −70°C.

Control plants were grown and starved in the same way, but inoculated with *R. meliloti* nodA::Tn5#2208 (GMI5382; Debellé et al. 1986); whole root systems were harvested as indicated. For the stress control, segments of roots from noninoculated, nitrogen-starved plants were cut as for nodule harvesting, over approximately the same period.

For Nod factor induction studies, preliminary experiments established the conditions for obtaining a good induction of *Mtenod11*. To test the *MtN* clones, one Nod factor treatment (condition II in Fig. 5) was carried out on aeroponically grown plants; after the usual periods of growth and nitrogen starvation, the growth chamber was emptied, rinsed, and then refilled with fresh medium supplemented with 10^{-8} M NodRm

factor and whole root systems were harvested 24 h later. A second Nod factor treatment (condition I in Fig. 5) was performed on plants grown in pouches, as described in Journet et al. (1994), except that the medium was the same as for aeroponic cultures. Segments approximately 3 cm long, measured from the root tips, were harvested 48 h after treatment (from about 160 plants), which corresponded to the newly grown tissues in addition to approximately 1 cm already present at the time of treatment. NodRm factors (mainly NodRm-IV(Ac, S, C16:2)) were purified from luteolin-induced *R. meliloti* 2011 pGMI149, as described by Lerouge et al. (1990).

RNA analysis.

Total RNA was purified by the procedure of Jackson and Larkins (1976). When necessary, polyA$^+$ RNA was then extracted with magnetic beads (Dynabeads oligodT$_{25}$ from Dynal, Oslo, Norway), following the manufacturer's instructions. RNA (in a loading buffer containing 15 μg of ethidium bromide per ml) was loaded on a formaldehyde 1% agarose gel and fractionated by electrophoresis. It was then transferred overnight by capillary blotting onto a Nytran Plus membrane (Schleicher & Schuell, Dassel, Germany), following standard procedures (Sambrook et al. 1989).

Riboprobes were generated by in vitro transcription in the presence of ^{32}P-labeled CTP, with T7 RNA Polymerase (Promega, Madison, WI) from *Eco*RI-restricted pBluescript SK(-) plasmids (resulting from in vivo excision from Lambda Zap II vector, Stratagene, La Jolla, CA). After riboprobe synthesis, DNA templates were eliminated by RQ1 DNAse hydrolysis (Promega), which was then inactivated by phenol-chloroform extraction. RNA probes were finally purified with "microspin 200 HR" columns (Pharmacia, Uppsala, Sweden). Hybridizations were carried out for about 20 h, at 60°C, in 50% formamide, 6× SSPE, (1× SSPE is 0.18 M NaCl, 10 mM NaPO$_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's, 0.1% sodium dodecyl sulfate (SDS), 100 μg salmon sperm DNA per ml, 5% dextran sulfate. Membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 0.1% SDS (15 min at room temperature, then 20 min at 65°C) then in 0.1× SSC 0.1% SDS for 20 min at 65°C.

DNA probes were also generated from clones (provided by D. Barker) for known nodulin genes *Msenod2*, *Mtenod12*, *Mtlb1*, and *Mtlb2*, and two *M. truncatula* clones related to *Psenod5*. DNA radioactive probes were prepared with the "Ready-To-Go" kit (Pharmacia) and purified on "microspin 200 HR" columns (Pharmacia). Hybridizations were carried out as with riboprobes, but at 37°C. The DNA probe generated from an *EF-1α* gene of *A. thaliana* (Axelos et al. 1989) was used under less stringent conditions (hybridization in 40% formamide, 37°C; final wash in 1× SSC, 65°C). The *M. truncatula* cytochrome c clone was isolated in this work as a noninduced species and the corresponding probe was used at high stringency.

DNA analysis.

cDNA inserts were prepared by PCR amplification from phage stocks, followed by direct purification using the Promega "Wizard PCR prep" kit or by gel purification with Genomed (Research Triangle Park, N.C.) "Jetsorb" kit. Plasmid DNA was extracted following an alkaline lysis procedure (Silhavy et al. 1984), or with the Promega "Wizard

miniprep" kit. Southern blotting was performed onto Nytran Plus membranes (Schleicher and Schuell), following standard procedures (Sambrook et al. 1989). DNA probes were prepared and used as previously described.

Preparation of subtracted and control probes.

Three micrograms of polyA$^+$ RNA from 4-day-old nodules was reverse-transcribed with SuperscriptII RTase (GIBCO-BRL, Gaithersburg, MD), in the presence of tracer radioactive dATP, following the manufacturer's instructions; a mixture of oligodT$_{12-18}$ and random hexamers (approximately 500 ng and 150 ng, respectively, per μg of polyA$^+$ RNA) was used to prime the cDNA synthesis. The reaction was stopped with 20 mM EDTA, extracted with phenol-chloroform, and desalted on a Sephadex G50 spin column. RNA template was hydrolyzed by a 30-min incubation at 65°C in 0.3N NaOH. The cDNA was then repurified on a G50 spin column, then stored at –20°C. An aliquot was analyzed by alkaline gel electrophoresis and autoradiography. It displayed a size distribution of about 200 to 2,000 nt (in contrast to the 500 to 5,000 nt smear observed for cDNAs synthesized from the same RNA sample with oligodT$_{12-18}$ but no hexamer primers).

Fifteen micrograms of control polyA$^+$ RNA was biotinylated twice with photoactivatable biotin (Super XX-PAB; Clontech, Palo Alto, CA), extracted following manufacturer's instructions, and coprecipitated with the nodule cDNA. Subtraction was conducted for 48 h at 42°C, in 50% formamide, 50 mM MOPS (morpholinepropanesulfonic acid) pH 7.3, 2 mM EDTA, 1M NaCl, at a nucleic acid concentration of about 1 μg/μl. Under these conditions both cDNA and RNA were found to be quite stable, as verified by gel electrophoresis analysis. The subtraction was stopped by three streptavidin (GIBCO-BRL)/phenol-chloroform extractions, followed by one phenol-chloroform and one chloroform extraction (Sive and St. John 1988). Resulting cDNA (about 10% of the input cDNA) was ethanol-precipitated in the presence of 1 μg of glycogen (Boehringer Mannheim France S.A., Meylan).

A fraction of the subtracted cDNA (about 30 ng) was converted to double-stranded molecules by Klenow enzyme (Pharmacia) (incubated with 1 u of Klenow for 4 h at 16°C, with 2 μg of random hexamers and 1 mM dNTP, in Klenow buffer). After a phenol-chloroform extraction, small molecules were removed by a sizing column (chromaspin-100, Clontech) and ethanol precipitation. To ensure a complete repair of its extremities, the cDNA was incubated with T4 DNA Polymerase (Pharmacia) (1.5 u with 0.5 mM dNTP in T4 DNA Polymerase buffer; 15 min at 37°C then 15 min at room temperature). The mixture was extracted by phenol-chloroform, then by the "Prep-A-Gene" kit (Bio-Rad Laboratories, Hercules, CA). At this stage about one-third of the starting subtracted cDNA had been recovered, and was ready for ligation to linkers (necessary for subsequent PCR amplification). We chose the linker described by Wang and Brown (1991), with one blunt end (5' phosphorylated) and one staggered. An overnight ligation was carried out at 15°C between about 4 ng of cDNA and 500 ng linkers with 1 u of T4 DNA ligase (Boehringer), in polyethylene glycol containing ligation buffer (GIBCO-BRL). Excess linkers were removed by Prep-A-Gene purification. One-tenth of the resulting material (about 250 pg) was PCR-amplified, in three 100-μl reactions (1 u Stehelin Super Taq, 1× Stehelin buffer, 200 μM dNTP,

0.5 μM primer; 30 cycles of 1 min at 94°C / 1 min at 42°C / 3 min at 72°C, followed by a 10 min chase at 72°C). An aliquot analyzed by gel electrophoresis showed a smear of double-stranded DNA fragments ranging from 200 to about 2,000 bp.

Probes were generated with the Ready-To-Go labeling kit (Pharmacia) from about 20 ng of the different cDNA samples. Control probes were prepared following the same procedure, starting from 3 μg of polyA$^+$ RNA isolated from roots of *M. truncatula* harvested 48 h after inoculation with GMI5382.

Preparation and screening of the cDNA library.

A directional cDNA library was constructed, using the Lambda ZAP II cloning kit (Stratagene) and 5 μg of 4-day-old nodule polyA$^+$ RNA. At least 99% of the clones contained a cDNA insert. About 30,000 plaques were screened at low density (approximately 2,000 plaques per 14 cm plate), by transfer in duplicates onto Nylon membranes (BiodyneA 1.2 μm, PALL, Portsmouth, UK) and hybridization to control or subtracted radioactive probes (about 2.10^6 cpm/ml of hybridization solution) for 48 h at 37°C, in 50% formamide, 5× SSPE, 120 μg denatured salmon sperm DNA per ml, 5× Denhardt's, 0.1% SDS, 5% dextran sulfate. Membranes were washed twice in 2× SSC 0.1 % SDS (10 min at room temperature, 15 min at 60°C), then twice in 0.2× SSC 0.1% SDS at 60°C (15 min each). They were exposed for 3 days. After stripping the membranes, we rehybridized them with a mixture of probes of *Mtenod11, Mtenod12, Mtlb1,* and *Mtlb2,* and two *M. truncatula* clones related to *Psenod5.* New differential clones were spotted again in an organized fashion on a lawn of bacteria. Corresponding cDNA inserts were prepared by PCR amplification (using M13-20 and reverse primers), and hybridized, after Southern blotting, to control and subtracted probes. This allowed us to confirm differential species (pure plaques), or to identify them when they were mixed with nondifferential ones (impure plaques). In the latter case, differential species were gel purified. Probes were prepared from individual inserts and used against the organized library of differential clones, in order to determine families of homologous clones.

cDNA sequencing and analysis.

In vivo excision was carried out from the selected Lambda Zap II cDNA clones, following manufacturer's instructions (Stratagene) and recombinant pBluescript SK(-) containing colonies obtained from phagemid infections of XL1-Blue strain (Stratagene). Plasmid DNA was extracted with the Promega "Wizard miniprep" kit, and double-stranded DNA was used to sequence the inserts from both ends by the dideoxy chain termination method (Sanger et al. 1977) with Sequenase (Amersham France, Les Ulis). Sequence data were analyzed using University of Wisconsin Genetic Computer Group software (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, Madison, WI); homology searches in databases by BLASTX programs were carried out through the NCBI BLAST E-mail Server. Sequences described in this paper are available from the authors. Complete cDNA sequences will be deposited in the EMBL database.

ACKNOWLEDGMENTS

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