Four Genes of *Medicago truncatula* Controlling Components of a Nod Factor Transduction Pathway

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Rhizobium nodulation (Nod) factors are lipo-chitooligosaccharides that act as symbiotic signals, eliciting several key developmental responses in the roots of legume hosts. Using nodulation-defective mutants of *Medicago truncatula*, we have started to dissect the genetic control of Nod factor transduction. Mutants in four genes (*DMI1*, *DMI2*, *DMI3*, and *NSP*) were pleiotropically affected in Nod factor responses, indicating that these genes are required for a Nod factor–activated signal transduction pathway that leads to symbiotic responses such as root hair deformations, expressions of nodulin genes, and cortical cell divisions. Mutant analysis also provides evidence that Nod factors have a dual effect on the growth of root hair: inhibition of endogenous (plant) tip growth, and elicitation of a novel tip growth dependent on (bacterial) Nod factors. *dmi1*, *dmi2*, and *dmi3* mutants are also unable to establish a symbiotic association with endomycorrhizal fungi, indicating that there are at least three common steps to nodulation and endomycorrhization in *M. truncatula* and providing further evidence for a common signaling pathway between nodulation and mycorrhization.

INTRODUCTION

Symbiotic bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium*, collectively referred to as rhizobia, are able to elicit on their leguminous hosts the formation of specialized organs, called nodules, capable of fixation of atmospheric nitrogen. Among the earliest visible manifestations of symbiotic development are the infection of root hair cells by bacteria, contained in plant-derived infection structures called infection threads, and the induction of cortical divisions to form the nascent nodule primordium (Mylona et al., 1995; Schultze and Kondorosi, 1998).

Genetic analysis of rhizobia has led to identification of *nod* genes, which are involved in the control of host specificity, infection, and nodulation. *nod* gene expression is under the control of plant signals, essentially flavonoids, excreted in the rhizosphere. Once activated, *nod* genes specify the synthesis of nodulation (Nod) factors, which are lipo-chitooligosaccharides (Dénarié et al., 1996). Purified Nod factors are capable of eliciting in the roots of the legume hosts

many of the plant responses characteristic of the bacteria themselves (Dénarié and Cullimore, 1993): root hair deformations, activation of the plant genes that are specifically induced during early stages of nodulation (early nodulin genes), initiation of cortical cell division, and triggering of a plant organogenic program that leads to nodule formation. Nod factors thus act as symbiotic signaling molecules for initiating nodule development. They also play a key role in the control of specificity of infection and nodulation, the result of particular substitutions present on the basic backbone of the Nod factor molecule (reviewed in Long, 1996; Cohn et al., 1998; Schultze and Kondorosi, 1998).

One of the most challenging areas of research in the study of the Rhizobium–legume symbiosis is dissecting the mechanisms by which the Nod factor signal is perceived and transduced by host plants. Because biological responses are induced by very low Nod factor concentrations (10^{-9} to 10^{-12} M) and have precise Nod factor structural requirements, Nod factors are probably perceived by specific receptors. A high-affinity binding site for *Sinorhizobium meliloti* Nod factors, NFBS2, has recently been identified in plasma membrane–enriched fractions of Medicago cell suspension cultures (Gressent et al., 1999), and a lectin nucleotide phosphohydrolase isolated from roots of the legume *Dolichos biflorus* also has been reported to have Nod factor binding activity (Etzler et al., 1999). However, whether either

of these Nod factor binding components plays a role in Nod factor signaling has not been established.

After the perception of Nod factors, numerous rapid plant responses in root hairs have been detected (reviewed in Downie and Walker, 1999). Within seconds of Nod factor addition, fluxes of Ca^{2+}, H^+, Cl^-, and K^+ ions occur (Felle et al., 1996, 1998, 1999a), together with membrane depolarization (Ehrhardt et al., 1992; Felle et al., 1995, 1998; Kurkdjian, 1995). Minutes after addition of Nod factors, changes in cytoplasmic Ca^{2+} concentrations are observed, including transient and plateau-like increases (Gehring et al., 1997; Cárdenas et al., 1999; Felle et al., 1999a) and calcium spiking (Ehrhardt et al., 1996), and evidence suggests a role for Ca^{2+} as a secondary messenger in signal transduction of Nod factors (Felle et al., 1999b). Nod factor–induced changes to the actin cytoskeleton also occur in root hairs and may, in association with certain Ca^{2+} concentration changes, be involved in the inhibition and subsequent reinitiation of root hair tip growth (Cárdenas et al., 1998; de Ruijter et al., 1999; Miller et al., 1999). Gene expression is induced by Nod factors in different cell layers of host roots and with different temporal expression patterns. For example, the early nodulin genes *ENOD11*, *ENOD12*, and *rip1* are induced in the epidermis within 2 hr of addition of Nod factors, whereas *ENOD20* and *ENOD40* are induced later in the cortex and in both the pericycle and cortex, respectively (Asad et al., 1994; Crespi et al., 1994; Journet et al., 1994; Cook et al., 1995; Vernoud et al., 1999; D. Barker, J.L. Pingret, M. Chaboud, and E.P. Journet, unpublished results). Nod factor–induced changes in gene expression are spatially and temporally correlated with cellular and developmental changes in the plant. For example, Nod factors trigger reorganization of the microtubular cytoskeleton and induce cell divisions, first in the root pericycle and then in the cortex (Timmers et al., 1999). These events are correlated with induction of *ENOD40* in the root pericycle and the subsequent induction of several nodulin genes (e.g., *ENOD20*, *rip1*, and *ENOD40*) in the nascent nodule primordium (Peng et al., 1996; Charon et al., 1997, 1999).

The high level of complexity involved in Nod factor signal transduction, leading as it does to cellular, molecular, and developmental responses in several cell layers of the root, means that a genetic approach is needed to determine the sequence of events and fully dissect the mechanisms involved. Genetic analysis of pea nodulation mutants has identified one element of a Nod factor–induced signal transduction pathway, the gene *SYM8*, which is essential for the induction of two epidermal nodulin genes, *PsENOD5* and *PsENOD12A* (Albrecht et al., 1998). To identify further genes involved in Nod factor perception and signal transduction, we have exploited nodulation mutants of the model legume *Medicago truncatula* (Barker et al., 1990; Cook et al., 1997; Cook, 1999). Our strategy was to characterize mutants that were pleiotropically affected in Nod factor responses. Because the majority of such responses are associated with events occurring before infection by Rhizobium, we first set

out to identify mutants that were blocked at early stages of the symbiotic interaction. After large-scale mutagenesis of *M. truncatula* with ethyl methanesulfonate (EMS) (Penmetsa and Cook, 2000) and γ-ray (Sagan et al., 1995, 1998), we identified mutants that were devoid of both rhizobial infection and induction of nodule primordia. Here, we report the results of further mutant screens and the genetic assignment of nine independent mutants into four complementation groups, each of which is required for early symbiotic responses. After detailed analysis of Nod factor–induced responses (root hair deformation, cortical cell divisions, and early nodulin gene expression in the root epidermis, pericycle, and cortex), we have determined that each of these mutants is simultaneously affected in multiple Nod factor responses, which is consistent with a role for the corresponding genes in perception/transduction of the Nod factor signal.

RESULTS

Identification of Infection-Defective Mutants of *M. truncatula*

To identify mutants of *M. truncatula* Jemalong blocked at early stages of the symbiotic interaction, we first selected mutants that were unable to form nodules (Nod⁻) after rhizobial inoculation—an easy, visual screen that can be performed on large numbers of plants. After an initial round of screening ∼50,000 EMS-mutagenized plants, ∼50 putative Nod⁻ mutants were selected, of which 25 were confirmed by subsequent testing (see Methods). Nod⁻ mutants were then characterized cytologically to define the block. Thus, infection studies of these 25 mutants and of 26 other EMS-induced Nod⁻ mutants (R.V. Penmetsa and D. Cook, unpublished results), using a strain of *S. meliloti* carrying a constitutively expressed *lacZ* gene (GMI6526), revealed two main groups of mutants: 26 mutants completely blocked for infection initiation and 25 mutants showing aborted infection, in which infection threads aborted either in root hair cells or in cortical cells. Close examination of mutants in the first group revealed that eight mutants showed limited cortical cell divisions and that the remaining 18 mutants were defective for cortical cell divisions (Ccd⁻). In the second group of mutants, however, Ccd was in certain cases extensive, giving rise to visible bumps.

The mutants chosen for further study were those blocked at the earliest stage of the symbiotic interaction, that is, the mutants of group 1 that were defective for induction of cortical cell divisions. The final choice of mutants was based both on results of genetic analysis, which enabled us to eliminate possible siblings (allelic mutants from the same bulk), and on practical considerations, such as the stage of backcrossing. The analysis of six mutants from group 1

(B85, B129, C54, C71, P1, and Y6) is described in this article, along with three previously described γ-ray Nod⁻ mutants of *M. truncatula* Jemalong (TR25, TR26, and TRV25) (Table 1; Sagan et al., 1995, 1998). The shoot and root systems of plants of all nine Nod⁻ mutants developed normally when grown in the presence of added nitrate (data not shown).

Wild-type *M. truncatula* plants grown on agar medium showed marked root hair curling (Hac⁺) and infection thread formation (Inf⁺) within 4 days of inoculation by *S. meliloti* GMI6526 (Figures 1A and 1B) and were nodulated (Nod⁺) within 7 days. When mutants were studied in the same conditions at different times up to 3 weeks after inoculation by this strain, no marked root hair curling (Hac⁻), no infection threads (Inf⁻), and no nodules could be seen on any of the mutants. The absence of induced cortical cell divisions in these mutants was confirmed by examining semi-thin sections of roots spot-inoculated by *S. meliloti* GMI6526 (data not shown). However, root hair deformations were induced in all mutants within 4 days of inoculation. For B85 and C54, only a few root hairs were deformed, and those showed branched or moderately curled root hair tips (Figure 1C); all the other mutants showed a large proportion of swollen root hair tips (Figure 1D).

To exclude the possibility that the *M. truncatula* mutants were Nod⁻ because of an inability to secrete rhizobial *nod* gene–inducing flavonoids, we inoculated plant mutants with the constitutive Nod factor–producing strain GMI6390 (*S. meliloti* carrying the regulatory genes *nodD3* and *syrM*; Table 1). No nodules were observed on roots of any of the mutants 3 weeks after inoculation (data not shown). To

Table 1. Bacterial Strains, Plasmids, and Plants Used in This Study

| Designation	Relevant Characteristics	Reference/Source
S. meliloti		
ABS7	Nod⁺Fix⁺ on *M. truncatula*	Bekki et al. (1987)
GMI6526	2011(pXLGD4), Nod⁺Fix⁺ on *M. truncatula*	Ardourel et al. (1994)
GMI6390	2011(pMH682)	Roche et al. (1991b)
GMI3198	2011(pGMI3194)(pXLGD4)	This study
GMI6628	2011Δ(*nodF*)13*nodL*::Tn5, Pm^Ra	Ardourel et al. (1994)
GMI6630	2011Δ(*nodF*)13*nodL*::Tn5(pXLGD4)	Ardourel et al. (1994)
GMI3125	2011Δ(*nodF*)13*nodL*::Tn5*nodC*::Sp	This study
GMI6702	2011*nodA*::Tn5#2208(pXLGD4)	Debellé et al. (1986)
Plasmids		
pXLGD4	pGD499 prime (IncP) carrying a *hemA*::*lacZ* fusion, Tc^R	Leong et al. (1985)
pMH682	pWB85a prime (IncP) carrying *nodD3* and *syrM* of *S. meliloti*, Tc^R	Honma et al. (1990)
pGMI1394	pML132 prime (IncQ) carrying *nodD1* of *S. meliloti*, Gm^R	Demont et al. (1994)
pRmM57	pRMSL26 (IncP) *nodC*::Sp, Tc^R	Mulligan and Long (1985)
pR751-pMG2	IncP plasmid, Tra+, Gm^R	Jacoby et al. (1976)
pRK2013	Helper plasmid for mobilization of IncP and IncQ plasmids, Km^R	Ditta et al. (1980)
M. truncatula		
Jemalong A17	Wild type, Nod⁺Fix⁺ with *S. meliloti*	Penmetsa and Cook (1997)
Jemalong (*MtENOD11–GUS*)	Transgenic wild type carrying a fusion between the promoter of *MtENOD11* and the β-glucuronidase (*GUS*) gene	D.G. Barker, J.-L. Pingret, M. Chabaud, and E.-P. Journet, unpublished results
TR25	γ-ray Nod⁻ mutant of Jemalong	Sagan et al. (1995)
TR26	γ-ray Nod⁻ mutant of Jemalong	Sagan et al. (1995)
TRV25	γ-ray Nod⁻ mutant of Jemalong	Sagan et al. (1998)
B85	EMS Nod⁻ mutant of Jemalong	R.V. Penmetsa and D. Cook, unpublished results
B129	EMS Nod⁻ mutant of Jemalong	R.V. Penmetsa and D. Cook, unpublished results
C54	EMS Nod⁻ mutant of Jemalong	R.V. Penmetsa and D. Cook, unpublished results
C71	"*domi*," EMS Nod⁻ mutant of Jemalong	Penmetsa and Cook (1997)
P1	EMS Nod⁻ mutant of Jemalong	This study
Y6	EMS Nod⁻ mutant of Jemalong	This study

ᵃR, resistant.
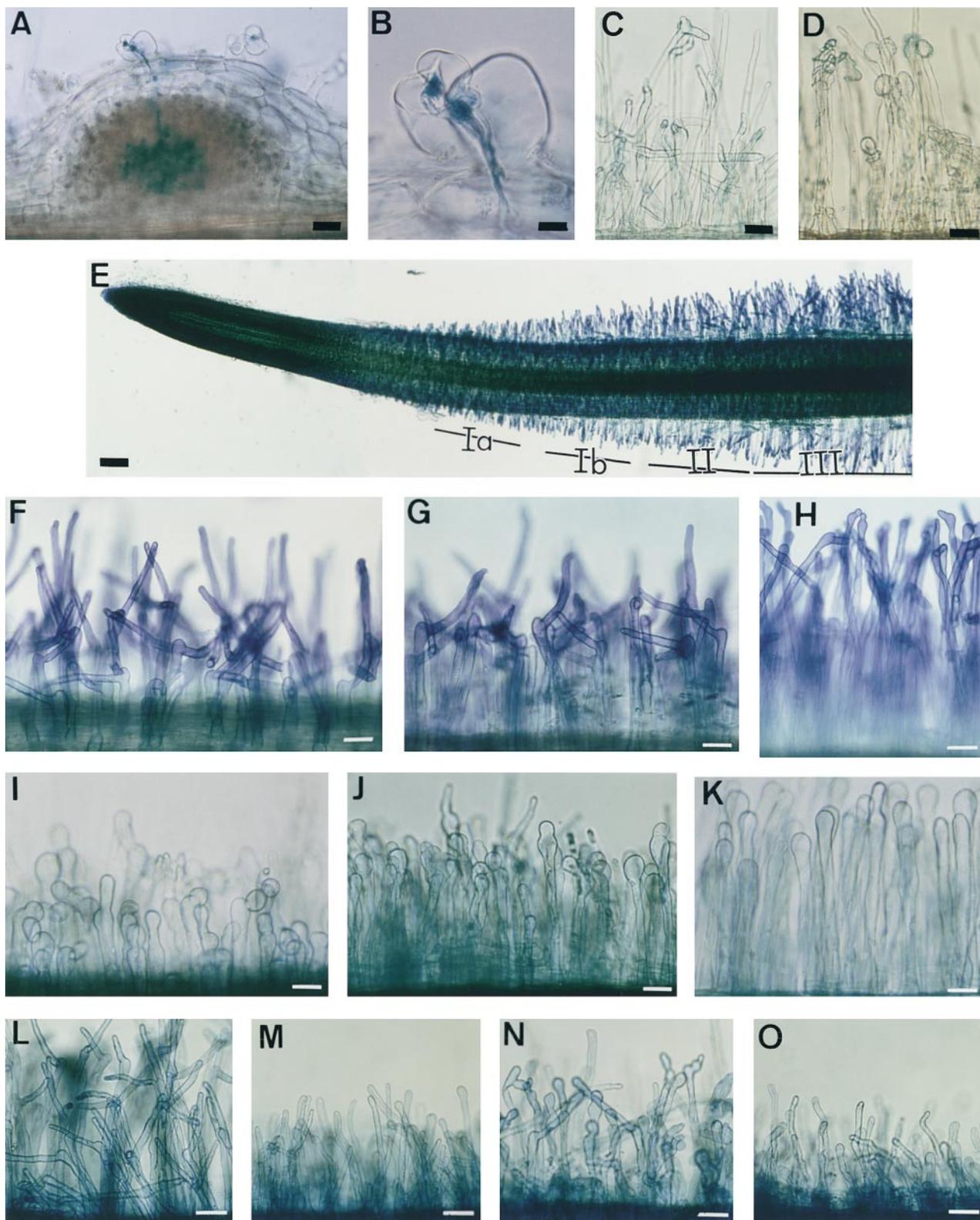
Figure 1. Root Hair Deformation Phenotypes of Wild-Type and Mutant Plants.

determine whether the infection and nodulation deficiency of Nod⁻ mutants resulted from decreased sensitivity to Nod factors, we inoculated mutants with the Nod factor–overproducing strain GMI3198 (*S. meliloti* carrying the regulatory gene *nodD1* for Nod factor overproduction and a constitutively expressed *lacZ* reporter gene for bacterial visualization; see Table 1). No infection-related events could be detected in any of the Nod⁻ mutants 7 days after spot inoculation, although mutants B85 and C54 showed more root hair deformations than they did after inoculation with a wild-type *S. meliloti* strain (data not shown).

Identification of Four Complementation Groups of *M. truncatula* Infection-Defective Mutants

The mutations responsible for the Nod⁻ phenotypes of TR25, TR26, TRV25, and C71 are monogenic and recessive (Sagan et al., 1995, 1998; R.V. Penmetsa and D. Cook, unpublished results). The remaining five mutants (B85, B129, C54, P1, and Y6) were crossed to wild-type plants (see Methods). As shown in Table 2, F_1 plants of these crosses all had a wild-type Nod⁺ phenotype, indicating that all the mutations were recessive, and the F_2 segregation data are consistent with the mutations all being nuclear and monogenic. The results of allelism tests, performed to define complementation groups, are presented in Table 3. TR25, TR26, and TRV25 had already been shown to fall into two complementation groups, one consisting of TR25 and TR26, the other TRV25 (Sagan et al., 1998); here, we determined that P1 is in the same complementation group as TR25, but no mutants could be placed in the same complementation group as TRV25. All the other five mutants fell into two new complementation groups: one containing B129, C71, and Y6; the other, B85 and C54. Pending further characterization of mutants, the four complementation groups were designated locus 1 (B129, C71, and Y6), locus 2 (TR25, TR26, and P1), locus 3 (TRV25), and locus 4 (B85 and C54).

Nod Factors Induce a Wild-Type Root Hair Deformation Phenotype in Mutants in Locus 4

The absence of any infection-related events (no Hac or Inf) in all the mutants studied suggested impaired abilities to respond to Nod factors. An early, characteristic response to Nod factors is root hair deformation, that is, swelling of the root hair tip and subsequent reinitiation of polar growth from the swelling (Heidstra et al., 1994; de Ruijter et al., 1998). Results presented in Table 4 show that homologous *S. meliloti* sulfated Nod factors (at 10^{-7} to 10^{-11} M), but not nonsulfated Nod factors, were able to induce root hair deformations, in the form of branched root hairs (the Hab phenotype, for hair branching), in wild-type *M. truncatula* plants. Deformation started by a swelling of the root hair tip, visible within 1 hr after treatment; a new outgrowth produced after 2 to 3 hr continued to grow, giving rise to branches that were observed after 16 hr (Figures 1F to 1H).

To facilitate comparison with previous work performed on vetch (Heidstra et al., 1994), we adopted the same three root zones they used, corresponding to different stages of root hair growth: (I) growing, (II) terminating growth, and (III) fully grown root hairs (Figure 1E). As in vetch, root hairs of zone II exhibited the Hab phenotype in response to Nod factors, and those of zone III did not. However, in contrast to vetch, in *M. truncatula* the whole root hair–growing region responded by branching. To refine the analysis of the Hab phenotype, therefore, we divided zone I into Ia and Ib, given the clear differences in the forms of branched root hairs between zones Ia and Ib (Figure 1): In zone Ia, long branches are produced on very short root hairs (Figure 1F), whereas in zone Ib, shorter branches are produced on longer root hairs (Figure 1G). In zone II, root hairs show very short branches or, less frequently, have swollen tips without branches (Figure 1H). Thus, branch length decreased progressively from root hairs that were the shortest at the time of treatment to those that had almost reached their full-grown length when Nod factors were added.

Figure 1. (continued).

(A) to **(D)** Root hair responses to *S. meliloti*. Roots were inoculated with *S. meliloti* GMI6526 and stained for β-galactosidase activity 4 days after inoculation. **(A)** and **(B)** Wild type, showing an infected nodule primordium **(A)** and a close-up of the infected root hair **(B)**. **(C)** B85, showing root hair deformations in the form of branched root hair tips. **(D)** B129, showing root hair deformations in the form of swollen root hair tips.

(E) Untreated wild-type root stained with methylene blue, showing the zones Ia, Ib, II, and III, corresponding to different stages of root hair growth, as referred to in the text.

(F) to **(O)** Root hair responses to Nod factors. Roots were treated with sulfated *S. meliloti* Nod factors and stained with methylene blue after 16 hr.

(F) to **(H)** Wild type treated with 10^{-8} M Nod factors and showing the Hab phenotype: zone Ia **(F)**, zone Ib **(G)**, and zone II **(H)**.

(I) to **(K)** Y6 (locus 1) treated with 10^{-8} M Nod factors and showing the Has phenotype: zone Ia **(I)**, zone Ib **(J)**, and zone II **(K)**.

(L) to **(O)** Roots treated with *S. meliloti* Nod factors at 10^{-10} M after growth of plants in the absence (**[L]** and **[N]**) or presence (**[M]** and **[O]**) of 10 mM ammonium nitrate for 7 days. **(L)** Wild type, showing the Hab phenotype; **(M)** wild type showing the reduced Hab phenotype; **(N)** B129 (locus 1) showing the Has phenotype; and **(O)** B129 (locus 1) showing the reduced Has phenotype.

Bars in **(A)**, **(C)**, and **(D)** = 55 μm; bar in **(B)** = 13 μm; bar in **(E)** = 135 μm; bars in **(F)** to **(H)** and **(L)** to **(O)** = 50 μm; bars in **(I)** and **(K)** = 40 μm; bar in **(J)** = 45 μm.

Table 2. Genetic Analysis of Nod[−] Mutants

Mutants[a]	F$_1$[b]		F$_2$[b,c]		
	Nod[+]	Nod[−]	Nod[+]	Nod[−]	χ[2d]
B85	16	0	76	24	0.02
B129	6	0	134	43	0.05
C54	5	0	74	21	0.01
P1	6	0	86	28	0.01
Y6	2	0	73	27	0.21

[a] Mutants were crossed to Jemalong (*MtENOD11–GUS*) Nod[+] plants, which were used in place of wild-type plants to provide a marker for crosses (see Methods).

[b] Nodulation was scored in plants of the F$_1$ and F$_2$ generations, 3 weeks after inoculation with *S. meliloti* GMI6526; numbers indicate the number of plants found to be Nod[+] or Nod[−]. In most cases, F$_1$ results presented are from two or more independent crosses.

[c] F$_2$ results are given for a single F$_2$ population but were confirmed in each case by analyzing at least one additional, independent F$_2$ population derived from a different F$_1$ plant.

[d] χ^2 calculated for a 3:1 ratio of Nod[+]/Nod[−] phenotype; $P > 0.05$ when $\chi^2 < 3.84$.

To determine whether the Nod[−] mutants still respond to Nod factors by root hair branching, we observed root hairs of mutants 16 hr after Nod factor treatment. Root hair growth and morphogenesis were normal in untreated roots of all mutants (data not shown). For mutants in locus 4, sulfated but not nonsulfated Nod factors induced the Hab phenotype in zones Ia, Ib, and II, the same as for the wild type (data not shown). We then tested the sensitivity of this response; as the results in Table 4 show, for C54, the Hab phenotype could be detected for concentrations of Nod factors down to 10^{-11} M, whereas for B85, it could be detected for concentrations as low as 10^{-10} M. Except for this slight difference in sensitivity, the root hair branching response of mutants in locus 4 was therefore no different from the wild-type response.

Lasting Inhibition of Tip Growth Is Observed in Response to Nod Factors in Mutants in Loci 1 to 3

In contrast to wild-type plants and mutants in locus 4, root hair tips of mutants in loci 1, 2, and 3 all became swollen in the presence of 10^{-8} M sulfated Nod factors, without producing the long branches typical of the wild-type Hab phenotype (Figures 1I to 1K). To distinguish this new phenotype from the Hab phenotype, we have adopted the term Has (for hair swelling). This Has phenotype seen after Nod factor treatment resembled, but was more pronounced than, the swollen root hair tip phenotype seen in these same mutants

after inoculation with *S. meliloti* (Figure 1D). As with the Hab phenotype in the wild-type plants, zones Ia, Ib, and II exhibited the Has phenotype in mutants in loci 1, 2, and 3. The youngest root hairs in zone Ia had a tendency for swollen tips and short, stumpy branches (Figure 1I); zone Ib root hairs were a combination of root hairs with swollen tips and root hairs with short, irregular-shaped deformations (Figure 1J); and the majority of root hairs in zone II showed swollen tips without such branches (Figure 1K). We then tested the Nod factor specificity and sensitivity of this response by looking at the ability of nonsulfated Nod factors to induce the Has phenotype in all mutants of loci 1 to 3 and by testing Nod factor dilutions. For one representative of the Has phenotype, B129 (locus 1), a range of sulfated Nod factor concentrations were tested (10^{-7} to 10^{-12} M); for all other mutants, sulfated Nod factors were tested at 10^{-9} or 10^{-10} M. Results in Table 4, and data not shown, revealed that the Has phenotype was dependent on sulfated Nod factors and was just as sensitive to low Nod factor concentrations as the Hab phenotype in wild-type plants.

To further characterize the Nod factor structural requirements of the Has phenotype, we investigated the ability of non-*O*-acetylated Nod factors with a modified acyl substituent to induce root hair deformations in mutants in loci 1 to 3. We therefore compared the response of mutants to the double *nodFL* mutant of *S. meliloti*, GMI6630 (Table 1), which produces such Nod factors and induces marked root hair deformations on wild-type plants (Ardourel et al., 1994), with the response induced by a *nodFL* mutant rendered incapable of producing Nod factors by introduction of a mutation in the *nodC* gene (GMI3125; see Table 1 and Methods). One mutant representative of each locus was tested: B129 (locus 1), TR25 (locus 2), and TRV25 (locus 3). The Has plant mutants responded to GMI6630 but not to GMI3125 by swollen

Table 3. Allelism Tests on Nod[−] Mutants[a]

	Group 1			Group 2		Group 3	Group 4	
	Y6	B129	C71	P1	TR25	TRV25	B85	C54
Y6		− (22)	− (7)	+ (4)	+ (14)	+ (8)	+ (11)	ND[b]
B129			− (9)	+ (10)	+ (11)	+ (19)	+ (12)	+ (7)
C71				+ (9)	+ (7)	+ (11)	ND	ND
P1					− (12)	+ (15)	+ (5)	ND
TR25						+[c]	+ (14)	+ (8)
TRV25							+ (13)	+ (12)
B85								− (17)

[a] Represented are the nodulation phenotype of F$_1$ plants and the number of F$_1$ individuals tested (figures in parentheses). (+), Nod[+]; (−), Nod[−].

[b] ND, not determined.

[c] The Nod[+] phenotype of F$_1$ individuals from a cross between TR25 and TRV25 was demonstrated by Sagan et al. (1998).

Table 4. Effect of Sulfated and Nonsulfated *S. meliloti* Nod Factors at Different Concentrations on Root Hair Deformation of Wild Type and Mutants

| | | Nod Factor Concentration | | | | | | | |
| | | Sulfated | | | | | | Nonsulfated | |
Plant	Phenotype[a]	10^{-7} M	10^{-8} M	10^{-9} M	10^{-10} M	10^{-11} M	10^{-12} M	10^{-7} M	10^{-9} M
Wild type	Hab	+[b]	+	+	+	+	−	−	−
B129	*Has*	+	+	+	+	+	−	−	−
B85	Hab	ND[c]	+	+	+	−	−	−	−
C54	Hab	ND	+	+	+	+	−	−	−

[a] Hab indicates root hair branching; *Has* indicates root hair swelling.
[b] Samples were scored for the percentage of plants showing deformed root hairs and then compared with control samples. (+) indicates a significantly (P = 0.01) higher percentage of responding plants; and (−) indicates no significant difference (P = 0.01) compared with control plants. At least two independent experiments were performed on 10 to 15 plants/sample, all of which were scored double blindly 16 hr after Nod factor treatment. Analysis of variance was conducted with Fisher's Exact test (Kendall and Stuart, 1976).
[c] ND, not determined.

root hair tips (data not shown), indicating that the Has response is not stringent with respect to modifications at the nonreducing end of the Nod factor molecule.

These data show that the Hab response in the wild type and the Has response in mutants of loci 1, 2, and 3 occurred in the same root hair zones, was observed in the same Nod factor concentration range (10^{-7} to 10^{-11} M), and had the same Nod factor structural requirements. These findings strongly suggest that the same mechanism for perception of Nod factors is involved in the induction of the Hab and Has phenotypes. In Has mutants, polar tip growth appears to be inhibited, the process of effective reinitiation of polar root hair tip growth is defective, and root hairs become markedly swollen, producing only short, deformed branches—indicating that root hairs of Has mutants are no longer capable of sustained polar growth after treatment with Nod factor. Furthermore, root hairs remained swollen for at least 1 week after treatment (data not shown), showing that this response has a lasting effect.

Finally, the symbiotic significance of the Has phenotype was investigated by testing the effect of combined nitrogen, which is inhibitory for nodulation and certain Nod factor–induced responses, including root hair deformation (Carroll and Mathews, 1990; Heidstra et al., 1994, 1997). In the case of wild-type *M. truncatula* grown in the presence of 10 mM ammonium nitrate, nodule formation was completely inhibited (data not shown) and the Hab response was greatly reduced, although root hairs were shorter after treatment with Nod factor, indicating that some inhibition of polar growth still occurs (Figures 1L and 1M). Similarly, for B85 (Hab$^+$) and B129 (Has) mutants, Hab and Has phenotypes were also greatly decreased by growing the plants in the presence of 10 mM ammonium nitrate (illustrated for B129 in Figures 1N and 1O). We note that this inhibition was observed after 7 days of growth in the presence of ammonium

nitrate but not after 24 hr. A preincubation period >24 hr is also needed in vetch (Heidstra et al., 1994).

All Four Loci Are Involved in Controlling Epidermal Nod Factor–Induced Nodulin Gene Expression

Nodulin genes are molecular markers induced during the symbiotic interaction (Mylona et al., 1995). In contrast to root hair deformation, nodulin gene expression is detected in different cell layers and with different temporal expression patterns. Thus, the study of their expression in mutants should help elucidate whether we have identified genes involved in Nod factor signal transduction. Two early epidermal nodulin genes expressed at the preinfection stage and for which expression can be induced by purified Nod factors were studied: *MtENOD11* (D. Barker, J.L. Pingret, M. Chaboud, and E.P. Journet, unpublished results) and *rip1* (Cook et al., 1995).

To determine whether Nod factors could induce *MtENOD11* in mutants, we treated plants with *S. meliloti* Nod factors (10^{-8} M) and analyzed *MtENOD11* expression by reverse transcription–polymerase chain reaction (RT-PCR). No *MtENOD11* expression was detected in mutants in locus 1 (B129 and C71), locus 2 (TR25 and TR26), locus 3 (TRV25), or locus 4 (B85 and C54) (illustrated for TR25 and B85 in Figure 2A). Because transgenic plants carrying promoter–reporter gene fusions are a very sensitive means of analyzing gene expression, we analyzed transgenic plants carrying a fusion between the promoter of *MtENOD11* and the β-glucuronidase (*GUS*) reporter gene. These mutant transgenic plants were generated by crossing mutants to wild-type plants carrying the construction *MtENOD11–GUS* (D.G. Barker, J.-L. Pingret, M. Chabaud, and E.-P. Journet, unpublished results; see Methods and Table 1). Strong
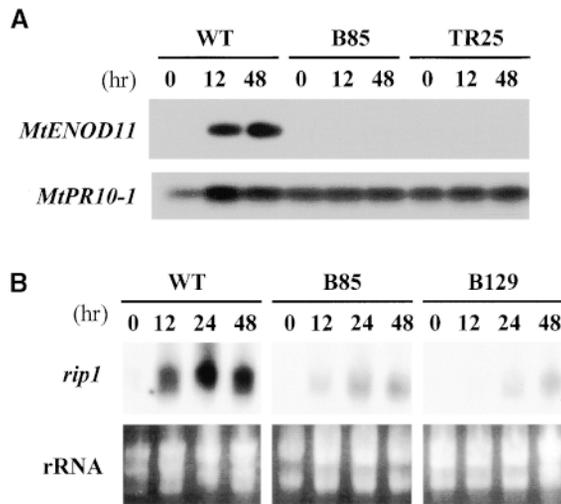
Figure 2. Analysis of Early Nodulin Gene Expression in Wild-Type and Mutant Plants in Response to Nod Factors.

(A) RT-PCR analysis of *MtENOD11* expression in wild type (WT) and mutant (B85, locus 4, and TR25, locus 2) roots treated with Nod factors at 10^{-8} M and harvested at the indicated times (see Methods). *MtPR10-1* RNA was amplified as a control for the quality and quantity of the RNA samples.

(B) RNA gel blot of *rip1* expression in wild type (WT) and mutant (B85, locus 4, and B129, locus 1) roots treated with *S. meliloti* Nod factors at 10^{-8} M and harvested at the indicated times (see Methods). rRNA concentrations are shown to control for equal loading.

induction of *MtENOD11* was observed in wild-type transgenic plants in response to 10^{-8} M *S. meliloti* Nod factors (Figure 3A), but no *MtENOD11* expression could be detected in the epidermis in response to 10^{-8} M *S. meliloti* Nod factors in mutants in locus 1 (B129, C71, and Y6) or locus 2 (P1) (illustrated for B129 in Figure 3B). As a control, GUS activity could be detected in root caps, lateral root primordia, and aerial parts of treated and nontreated plants, exactly as for wild-type plants (Figure 3D; data not shown), indicating that the nonsymbiotic pattern of *MtENOD11* expression (D. Barker and V. Gianinazzi, unpublished results) was unaffected in these mutants. These results are similar to those found for mutants in other locus 2 alleles (TR25 and TR26) and for the locus 3 mutant TRV25 (D.G. Barker, J.-L. Pingret, M. Chabaud, and E.-P. Journet, unpublished results). For mutants in locus 4 (B85 and C54), analysis of transgenic plants revealed that *MtENOD11* was induced in the epidermis by *S. meliloti* Nod factors but to a lesser extent than in wild-type plants and with a smaller responding zone (Figures 3A and 3C to 3E). This difference was quantified by testing transgenic plants with a series of Nod factor dilutions (from 10^{-9} to 10^{-12} M). Results presented in Figure 4 show that the *MtENOD11* expression response is at least 100-fold less sensitive to Nod factors in B85 and C54 (locus 4) than in wild-type plants.

The ability of Nod factors to induce expression of another early nodulin gene, *rip1*, was studied by RNA gel blot analysis in the following mutants: B129 and C71 (locus 1), TR25 (locus 2), TRV25 (locus 3), and B85 and C54 (locus 4). We found that although *rip1* was strongly induced in wild-type
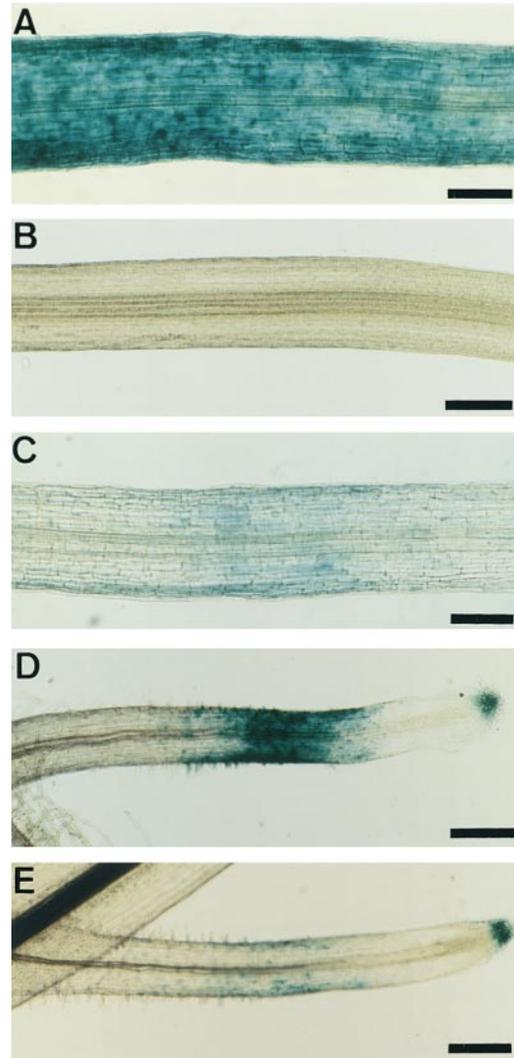

Figure 3. Histochemical Localization of GUS Activity in Roots of Transgenic Plants Carrying a Fusion between the Promoter of *MtENOD11* and the *GUS* Reporter Gene after Treatment with *S. meliloti* Nod Factors for 6 hr.

Primary roots (**[A]** to **[C]**) and secondary roots (**[D]** and **[E]**), which generally show more intense GUS staining than do the primary roots.
(A) Wild type treated with 10^{-8} M.
(B) B129 (locus 1) treated with 10^{-8} M.
(C) C54 (locus 4) treated with 10^{-8} M.
(D) Wild type treated with 10^{-9} M.
(E) B85 (locus 4) treated with 10^{-9} M.
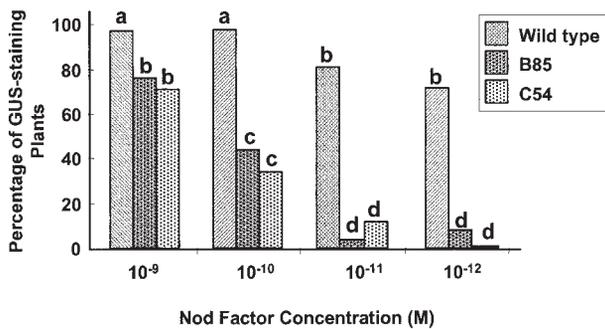Bars in **(A)** to **(E)** = 275 μm.

Figure 4. Effect of Different Nod Factor Concentrations on *MtENOD11* Expression in Transgenic Lines of Wild Type, B85, and C54 (Both at Locus 4).

At least two independent experiments were performed with 15 to 20 plants per sample, all of which were scored 6 hr after treatment with *S. meliloti* Nod factors. Values with different letters (a, b, and c) differ significantly at P = 0.05. d, values that were not significantly different from control, untreated plants at P = 0.05. Analysis of variance was conducted with Fisher's Exact test (Kendall and Stuart, 1976).

plants within 12 hr after 10^{-8} M *S. meliloti* Nod factors was added, induction of *rip1* was substantially reduced in all of the mutants tested (illustrated for B129 and B85 in Figure 2B).

Taken together, these results indicate that all four loci are required for Nod factor–induced expression of *MtENOD11* and *rip1* in the root epidermis and are consistent with the function of the respective proteins in perception or transduction of the Nod factor ligand.

All Four Loci Are Involved in Controlling Nod Factor–Induced *MtENOD40* Expression in the Pericycle and Cortex

ENOD40 is an early nodulin gene induced by Nod factors, first in the pericycle and subsequently in dividing cortical cells and in all differentiating cells of the growing nodule primordium (Asad et al., 1994; Crespi et al., 1994; Gamas et al., 1996; Fang and Hirsch, 1998). Accumulating evidence supports an important role for *ENOD40* in primordium formation during nodulation. In particular, overexpression and bombardment of *ENOD40* induce dedifferentiation and division of root cortical cells in legumes (Charon et al., 1997); transgenic lines of *M. truncatula* having reduced amounts of *MtENOD40* transcripts form only a few modified, nodule-like structures (Charon et al., 1999). The absence of cortical cell divisions in mutants in the four loci provided an opportunity to examine the correlation between *MtENOD40* induction and cortical cell division. The ability of Nod factors to induce *MtENOD40* expression was studied by comparing plants inoculated with either a wild-type *S. meliloti* strain (GMI6526) or an *S. meliloti nodA*::Tn*5* mutant (GMI6702) unable to produce Nod factors (Table 1).

MtENOD40 expression was analyzed by in situ hybridization after spot inoculation of wild-type and mutant plants. Although a very low background amount of *MtENOD40* expression was detected throughout the pericycle of many roots, *MtENOD40* was clearly induced in wild-type plants by GMI6526, in dividing cells of the pericycle, the inner cortex at 24 hr (data not shown). At 48 hr, when the infection process is just starting, *MtENOD40* expression was observed in dividing cells of the pericycle and both the inner and middle cortex and in overlying cells of the external cortex (Figures 5A and 5B). No induction of *MtENOD40* expression by the *S. meliloti nodA*::Tn*5* mutant could be detected (data not shown), indicating that *MtENOD40* expression in *M. truncatula* depends on the presence of Nod factors.

MtENOD40 gene expression was then analyzed in mutants in locus 1 (B129), locus 2 (TR25), locus 3 (TRV25), and locus 4 (B85 and C54). No induction was detected in the pericycle or cortex of any of these mutants 48 hr after inoculation by the wild-type *S. meliloti* strain (illustrated for B85 in Figures 5C and 5D). RNA gel blot analysis has also shown that Nod factors can no longer induce *MtENOD40* expression in the mutant C71 (locus 1) (S.K. Ramu, R.V. Penmetsa, and D. Cook, unpublished results). The requirement for Nod factor–producing rhizobia for *MtENOD40* induction, together with the absence of *MtENOD40* induction in mutants in loci 1 to 4, indicates that these four loci are required for the Nod factor signal transduction that leads to *MtENOD40* expression.

Single Mutations Are Responsible for the Pleiotropic Defects in Nod Factor Responses

The preceding results show that all the nine mutants we have studied are affected in more than one Nod factor response. Four mutants (B129, C71, TR25, and TRV25) are affected for root hair branching; *MtENOD11*, *rip1,* and *MtENOD40* expression; and cortical cell divisions. Three mutants (Y6, TR26, and P1) are affected for root hair branching, *MtENOD11* expression, and cortical cell division. In the remaining two (B85 and C54), Nod factors are no longer able to induce *MtENOD40* expression, cortical cell divisions are absent, and induction of *MtENOD11* and *rip1* is greatly reduced. Because most, if not all, mutagenic treatments commonly used on plants, including the γ-ray and EMS mutageneses used in this study, can induce more than one mutation in each plant (Feldmann et al., 1994), it was important to provide evidence that single mutations were responsible for these pleiotropic phenotypes. Such genetic evidence can be provided either by studying independent allelic mutants or by analyzing the cosegregation of two or more phenotypes in an F_2 population of plants.

For three of the loci described, we studied more than one allelic mutant. Thus, for locus 1 we studied B129, C71, and Y6; for locus 2, TR25, TR26, and P1; and for locus 4, B85 and C54. The allelic mutants were found to be altered in

Nod factor responses and in infection all in the same manner (see previous results), strongly indicating that they were the result of a mutation in the same gene in each case. Interestingly, only one case of allelic variation was detected, with B85 and C54 (locus 4) differing slightly for the Hab phenotype at low Nod factor concentrations. For locus 1, we supplemented the evidence that a single mutated gene is responsible for the pleiotropic defects in Nod factor responses by using cosegregation analysis. We thus checked an F_2 population of B129 homozygous for the transgene *MtENOD11–GUS* for the cosegregation of three phenotypes: Nod factor induction of the Has phenotype, absence of Nod factor–induced GUS activity (*MtENOD11*⁻), and absence of nodulation (Nod⁻). In a population of 284 F_2 plants, the three phenotypes cosegregated completely; that is, all 67 Nod⁻ plants displayed the Has phenotype and were *MtENOD11*⁻, whereas all 217 Nod⁺ plants were Hab⁺ *MtENOD11*⁺.

Only a single allele, TRV25, has been isolated for locus 3.

Therefore, we analyzed the cosegregation of the same three phenotypes (absence of nodulation, absence of *MtENOD11* induction, and the Has response) to test whether the phenotypes were linked and were therefore likely to result from the same mutation. Because this was done in an F_2 population of 201 plants showing segregation for the transgene *MtENOD11–GUS*, only those individuals carrying the transgene (GUS⁺) were scored for *MtENOD11* expression in response to Nod factors. All Nod⁺ GUS⁺ plants were *MtENOD11*⁺, and all Nod⁻ GUS⁺ plants were *MtENOD11*⁻. Among this population, 106 plants were scored for root hair branching; of these, 88 were Nod⁺, all of which were Hab⁺, and 18 were Nod⁻ and all displaying the Has phenotype. These cosegregation data suggest that a single mutation in locus 3 is responsible for the pleiotropic phenotype of TRV25 but does not exclude the possibility that the mutation affects more than one gene. However, we hypothesize that a single mutated gene is responsible for the pleiotropic phenotype of TRV25, given that we were unable to distinguish
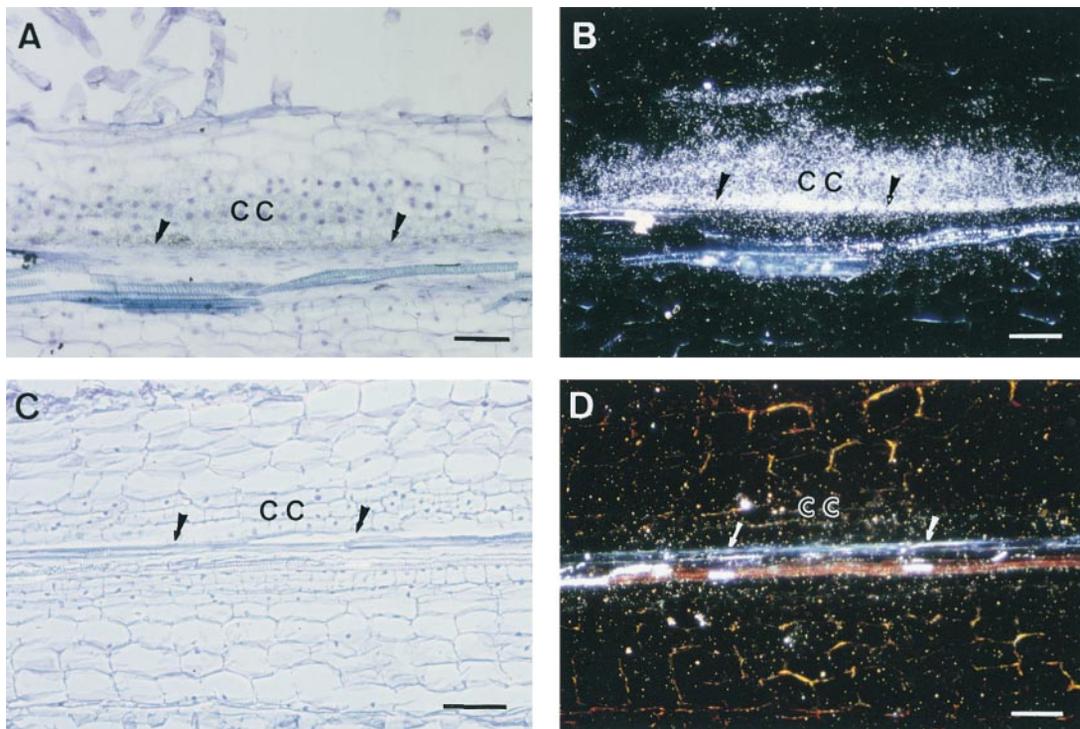

Figure 5. In Situ Hybridization Analysis of *MtENOD40* mRNA in Roots of Wild-Type and B85 (Locus 4) Plants in Response to *S. meliloti*.

Roots were hybridized with α-³⁵S-UTP–labeled *MtENOD40* RNA probes 48 hr after spot inoculation with *S. meliloti* GMI5626. Data are presented only for the antisense probes; no hybridization signals were detected with sense probes.
(A) and **(C)** Bright-field microscopy; hybridization signals are visible as dark spots.
(B) and **(D)** Dark-field microscopy; hybridization signals are visible as white dots.
(A) and **(B)** Longitudinal section of a wild-type root showing *MtENOD40* RNA localized in the cortical and pericycle dividing cells of a nodule primordium.
(C) and **(D)** Longitudinal section of a B85 root, showing neither cell divisions nor *MtENOD40* expression.
CC, cortical cells; arrows, pericycle. Bars in **(A)** to **(D)** = 65 μm.

the pleiotropic phenotype of TRV25 from those of mutants in loci 1 and 2, for which we did have strong evidence for the involvement of single genes.

The gene corresponding to mutant C71 (*domi*; Penmetsa and Cook, 1997) has been given the name *dmi1* (for <u>d</u>oesn't <u>m</u>ake <u>i</u>nfections) (R.V. Penmetsa and D. Cook, unpublished results). This name is based on the fact that C71 not only is defective for infection by rhizobia but also is unable to establish a symbiotic interaction with endomycorrhizal fungi (Myc⁻ phenotype). The two other mutants (B129 and Y6) in this gene, which corresponds to locus 1, have also been found to be Myc⁻ (M. Harrison, personal communication, for B129; V. Gianinazzi-Pearson and D. Morandi, personal communication, for Y6). Mutants in loci 2 and 3 are similarly Myc⁻ (Sagan et al. [1995, 1998] for TR25, TR26, and TRV25; V. Gianinazzi-Pearson and D. Morandi, personal communication, for P1). Because of this, and because the mutants in loci 1, 2, and 3 are indistinguishable phenotypically, the corresponding genes have been named *dmi2* (for locus 2 mutants) and *dmi3* (for locus 3 mutants). Mutants in locus 4 (B85 and C54), however, can establish a normal, effective endomycorrhizal symbiotic interaction (M. Harrison, personal communication). The gene corresponding to this mutant locus has been named *nsp* (for <u>n</u>odulation <u>s</u>ignaling <u>p</u>athway) to reflect that this gene appears to control a step of Nod factor transduction specifically involved in the signaling leading to nodulation. The different alleles of these four genes have been distinguished as follows: *dmi1-1* (C71); *dmi1-2* (B129); *dmi1-3* (Y6); *dmi2-1* (TR25); *dmi2-2* (TR26); *dmi2-3* (P1); *dmi3-1* (TRV25); *nsp-1* (B85); and *nsp-2* (C54).

DISCUSSION

Although many individual Nod factor–induced responses have been characterized, the mechanisms by which Nod factors induce these responses and the symbiotic significance of many of the responses are far from understood. The dissection of Nod factor perception/transduction has so far consisted of pharmacological approaches, identifying potential elements of a Nod factor signal transduction pathway that leads to expression of the early nodulin gene *MtENOD12* (Pingret et al., 1998) or the characterization of certain plant mutants. Thus, two genetic loci of pea and one of *Lotus japonicus* have been studied for their role in Nod factor perception: *SYM8* of pea, which is involved in controlling *PsENOD5* and *PsENOD12A* gene expression in response to Nod factors (Albrecht et al., 1998); *SYM2^A* of pea, which is involved in the specific recognition of Nod factors leading to infection but does not control Nod factor–induced root hair deformation or *PsENOD12* expression (Geurts et al., 1997); and *NIN* of *L. japonicus*, which does not control the perception of Nod factors that leads to root hair curling and deformation (Schauser et al., 1999). In addition, an alfalfa Nod⁻ mutant has been characterized that is deficient

for calcium spiking in response to Nod factors, suggesting that this mutant is blocked in an early stage of Nod factor signal transduction (Ehrhardt et al., 1996).

A strategy aimed specifically at identifying genes involved in signal transduction is to isolate mutants that are altered pleiotropically in their response to the signal of interest. This strategy has been used very successfully, for example, to dissect genetically the ethylene signal transduction pathway by isolating "multiple response mutants" and has enabled both identification of the genes controlling steps in the pathway and elucidation of the sequence of events (Chang and Shockey, 1999). Here, we have used this strategy to initiate the genetic dissection of Nod factor transduction in *M. truncatula*. We isolated multiple response mutants by first screening for Nod⁻ mutants and then searching among these Nod⁻ mutants for those pleiotropically altered in their responses to Nod factors. The aim of this study was thus to deliberately identify legume genes involved in Nod factor transduction.

Nine Nod⁻ mutants of *M. truncatula* were characterized and assigned to four loci. All nine mutants were defective in infection by the normally compatible rhizobial strain *S. meliloti*, and this deficiency could not be overcome by using Nod factor–overproducing strains. According to their response to Nod factors, mutants could be divided into two phenotypic classes. One, which included mutants in loci 1, 2, and 3, showed no induction of the early nodulin genes *MtENOD11* and *MtENOD40,* had greatly reduced *rip1* induction, responded with no cell divisions, and were affected in Nod factor–induced root hair branching. The other included mutants in locus 4, which were similarly defective for Nod factor–induced *MtENOD40* induction and cell divisions and showed a greatly reduced presence of *MtENOD11* and *rip1* induction. By being defective in different responses—transcription of early nodulin genes in different tissues (the epidermis, the cortex, and the pericycle) and cortical cell divisions—these mutants clearly can be called pleiotropic and thus they correspond to multiple response mutants. Furthermore, the analysis of different alleles and cosegregation studies provided genetic evidence that a single mutated gene in each locus was responsible for the multiple defects. We have thus hypothesized that these mutations alter genes involved in a signal transduction pathway activated by Nod factors and leading to nodulation. The strategy adopted for identifying such genes has therefore been successful and should enable fine dissection of the nodulation signaling pathway, made possible because nodulation is a facultative trait for legume growth, in the presence of combined nitrogen. Moreover, the identification of such genes in the model legume *M. truncatula* will facilitate their cloning and detailed functional analysis.

A Model for Nod Factor Signal Transduction That Leads to Induction of Symbiotic Responses

Given that several of the symbiotic responses (induction of *MtENOD11, rip1,* and *MtENOD40*; cortical cell divisions;

and nodulation) are altered by all the mutations we characterized in *DMI1, DMI2, DMI3*, and *NSP*, we propose a model in which these four genes intervene in the same Nod factor transduction pathway that leads to nodulation (Figure 6A). The four genes are good candidates to code for components of this signal transduction pathway, although we cannot exclude the possibility that products of these genes could allow the functioning of a signal transduction mechanism without their activity being modulated by signal perception itself. We also cannot exclude that any of the genes code for a Nod factor receptor, although all of the mutants described in this study are still able to specifically perceive Nod factors. Within the pathway, *DMI1, DMI2,* and *DMI3* cannot be ordered because mutations in these three genes result in the same phenotype. Further work will aim at distinguishing *dmi* mutants, for example, by exploring the calcium-spiking response (Ehrhardt et al., 1996) or the induction of *MtENOD11* or *MtENOD12* by Nod factor agonists such as mastoparan (Pingret et al., 1998). *nsp* mutants could be clearly distinguished from the three *dmi* mutants: *nsp* mutants exhibit a wild-type root hair branching phenotype, whereas *dmi* mutants are defective in their ability to reinitiate root hair tip growth after treatment with Nod factor. These data indicate that *NSP* acts downstream of the three *DMI* genes in the pathway and are consistent with the finding that normal expressions of *MtENOD11* and *rip1* are not needed for root hair branching in *M. truncatula*. Expression of *VsENOD5, VsENOD12,* and *VsLb1* in vetch similarly is not required for root hair deformation (Vijn et al., 1995; Heidstra et al., 1997). Although *MtENOD11* and *rip1* expressions are clearly diminished in *nsp* mutants, they are not completely eliminated, which suggests either the involvement of additional regulatory mechanisms or our possible characterization of leaky alleles of *nsp*.

The signaling pathway controlled by *DMI1, DMI2, DMI3,* and *NSP* may coincide, at least in part, with that identified by the gene *SYM8* of pea, which controls the Nod factor signal transduction leading to epidermal expression of nodulin genes (Albrecht et al., 1998). However, we have extended the pathway to include root hair deformation, cortical cell divisions, and expression of *MtENOD11* and *rip1* in the epidermis and *ENOD40* in the pericycle and cortex. Furthermore, the fact that in each mutant a single mutation apparently results in defective infection, the absence of nodulation, and defects in expression of *MtENOD11, rip1,* and *MtENOD40* provides genetic evidence that these early nodulin genes, identified by a molecular approach, are indeed involved in the symbiotic process. Finally, the signaling pathway in which *DMI1, DMI2, DMI3,* and *NSP* intervene is probably part of a host-specific response to Nod factor recognition, given our demonstration that, just as for nodulation of host plants by *S. meliloti* (Lerouge et al., 1990), induction of two of the responses characterized (the Has and the Hab responses) depends on the sulfate group of *S. meliloti* Nod factors.
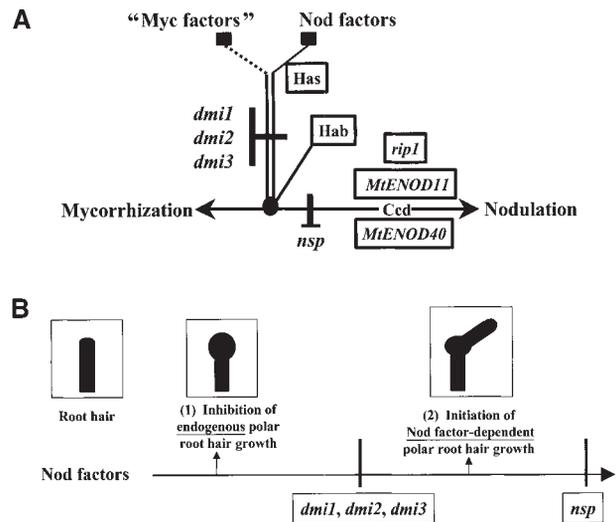

Figure 6. Models for the Intervention of *DMI1*, *DMI2*, *DMI3,* and *NSP1* in Nod Factor Signal Transduction.

(A) Model for roles in a Nod factor signal transduction pathway leading to *MtENOD11*, *rip1*, and *MtENOD40* expression; cortical cell division; and nodulation. As deduced by the phenotypes of *dmi1*, *dmi2*, *dmi3,* and *nsp1* mutants, *DMI1*, *DMI2,* and *DMI3* presumably intervene at one or more steps of the pathway downstream of Has and upstream of both Hab and *NSP*. The part of the signaling pathway controlled by *DMI1*, *DMI2*, and *DMI3* would be induced both by Nod factors and by potential mycorrhization signals ("Myc factors") and would play a role in the preparation of the plant for infection by both rhizobia and endomycorrhizal fungi. Ccd, cortical cell division; Hab, root hair branching; Has, root hair swelling.
(B) Model for roles in Nod factor–induced root hair tip growth changes. Nod factors would (1) inhibit endogenous polar root hair growth and (2) initiate Nod factor–dependent growth. *dmi1*, *dmi2* and *dmi3* mutants uncouple these two events, which indicates either that *DMI1*, *DMI2*, and *DMI3* are required for an element or elements of Nod factor signal transduction downstream of (1) and upstream of (2) or that Nod factors trigger (1) and (2) by different pathways. *nsp* mutants are able to initiate polar growth in response to Nod factors, indicating that *NSP* is involved in a component of Nod factor signal transduction downstream of (2). The root hair swelling response is shown as a phenotype associated with the inhibition of endogenous polar root hair growth; the root hair branching response is shown as a phenotype associated with the initiation of Nod factor–dependent root hair growth. Root hair branching is the phenotype observed when purified Nod factors are applied, but in the normal Rhizobium–legume interaction, when Nod factor–producing rhizobia are present, our model predicts that this initiation of Nod factor–dependent root hair growth contributes to marked root hair curling, a key step in the initiation of rhizobial infection.

Dual Role of Nod Factors on Root Hair Tip Growth

Just as in vetch (Heidstra et al., 1994), the Nod factor–induced root hair branching response, or Hab phenotype, in *M. truncatula* starts by transient swelling of root hair tips and is fol-

lowed by the formation of a branch. In contrast to vetch, however, in which only root hairs of zone II have been reported to deform, root hairs throughout the root hair elongation zones I and II in *M. truncatula* respond to Nod factors. Furthermore, the root hair branching phenotype of *M. truncatula* clearly differs from root hair deformation in vetch, being characterized in zones Ia and Ib by a very marked angle of branching and relatively long branches.

dmi mutants apparently perceive Nod factors by way of the same Nod factor perception mechanism that leads to root hair branching in wild-type plants, but they respond by root hair swelling without the production of branches. These mutants thus uncouple two stages in branch formation, the suppression of polar tip growth still being effectively induced by Nod factors but the second stage of reinitiation of tip growth being inhibited. Nod factors, in fact, have a spectacular effect on root hairs of *dmi* mutants, which become extensively swollen, as if the inhibition of polar tip growth is lasting instead of transient, while active, diffuse growth continues. Swollen root hair deformation phenotypes have also been reported for a Nod⁻ mutant of pea, R72 (Markwei and Larue, 1992), and one of *Melilotus alba*, BT62 (Utrup et al., 1993), in response to inoculation by compatible rhizobial strains. Interestingly, both mutants, like *dmi1*, *dmi2,* and *dmi3* mutants, are blocked for root hair infection.

Although our data clearly show that root hair swelling is a response to Nod factors, two alternative explanations can be proposed to explain the subsequent reinitiation of polar growth in *M. truncatula*: (1) the spontaneous restoration of normal, endogenous root hair tip growth after a transient arrest or (2) a novel Nod factor–controlled tip growth. Our data support the second hypothesis for the following reasons. In *dmi* mutants, adding Nod factors produces a lasting inhibition of polar tip growth. This suggests that Nod factors inhibit endogenous polar root hair growth through a component of the Nod factor transduction pathway that is upstream of *DMI1*, *DMI2,* and *DMI3,* whereas the reinitiation of polar growth depends on a component of Nod factor signal transduction that is downstream of the *DMI* genes (Figure 6B). The fact that *nsp* mutants are able to reinitiate polar growth also suggests that a component of the Nod factor transduction pathway required for polar growth reinitiation is upstream of *NSP*. Furthermore, if possibility (1) were correct, we might expect the reinitiation of growth leading to branch formation to be in the same direction as the original direction of root hair growth. Treatments that change the endogenous polarity of root hair growth, for example, do so only temporarily, after which growth returns to the original direction (Bibikova et al., 1997). In the case of Nod factor–induced root hair branching, that is not what was observed, indicating that Nod factors, by controlling the new tip growth, actually modify the polarity of root hair growth. Thus, as previously reported (de Ruijter et al., 1998), Nod factors seem to play two contradictory functions on root hair tip growth: inhibition and reinitiation. Our data allow us to

propose the following interpretation of this apparent contradiction. Nod factors inhibit the endogenous tip growth of root hairs and initiate Nod factor–controlled polar growth. That Nod factors have the intrinsic ability to elicit tip growth is directly supported by the observation that their addition can elicit the formation, on cognate legume hosts, of elongated root hairs in cortical cells, a cell type not ordinarily programmed for tip growth. The production of such root hairs of cortical origin has been observed in vetch (van Brussel et al., 1992; van Spronsen et al., 1994) and alfalfa (G. Truchet, personal communication).

What could be the biological significance of this change in control of root hair tip growth? One key step in the initiation of Rhizobium infection is the formation of a marked curl of root hairs, called shepherd's crooks, which entrap bacterial cells in a pocket, from which they enter into the plant by forming an infection thread. Marked curling involves a modification of root hair growth by rhizobial cells that requires the production of Nod factors, because Rhizobium mutants unable to produce cognate Nod factors are unable to elicit this response. Van Batenburg et al. (1986) proposed that rhizobia induce marked root hair curling by redirection of tip growth. Our data are consistent with the following mechanism for this "hijacking" of root hair growth: by way of Nod factor secretion, rhizobia both inhibit endogenous tip growth and initiate a Nod factor–dependent polar growth (Figure 6B). Interestingly, *nsp* mutants respond to Nod factors by initiating root hair growth but are defective for shepherd's crook formation in the presence of rhizobia, suggesting that one or more additional mechanisms controlled by the Nod factor signaling pathway characterized here are involved in shepherd's crook formation.

Numerous studies of tip growth in root hairs of Arabidopsis, in fungal mycelia, and in pollen tubes, essentially in *Lilium longiflorum* and *Agapanthus umbellatus,* have shown that a tip-focused calcium gradient, the result of a localized calcium influx, controls tip growth and orientation (reviewed in Malhò, 1998; Yang, 1998; Sanders et al., 1999). For example, tip swelling in pollen tubes after treatments that disrupt polar growth is preceded by loss of the tip-focused calcium gradient and coincides with the reestablishment of this gradient (Malhò et al., 1995). Reinitiation of root hair tip growth, after Nod factor treatment, also involves the establishment of a tip-focused calcium gradient (de Ruijter et al., 1998), whereas changes in actin filaments have been associated both with the stage at which Nod factors abolish tip growth and with the reinitiation of growth (Cárdenas et al., 1998; de Ruijter et al., 1999; Miller et al., 1999). Future studies of Nod factor influence on legume root hair polar growth will aim at further dissecting the Nod factor transduction pathway to identify what components are required for both inhibiting endogenous root hair tip growth and initiating a Nod factor–dependent tip growth. For example, cell biology studies of Nod factor responses of wild-type and *dmi* mutants should allow us to identify

the role of calcium and of various cellular components (e.g., the cytoskeleton, Golgi vesicles, and the nucleus). The finding that a lasting inhibition on tip growth is observed in *dmi* mutants in response to Nod factors may also be a useful tool for studying the mechanisms underlying polar growth. For example, in contrast to the transient suppression of tip growth by various chemicals, such as cytochalasin D (Miller et al., 1999), or by mechanical constraints (Bibikova et al., 1997), a lasting inhibition of tip growth is observed in *dmi* mutants by a defined ligand at low, nontoxic concentrations (10^{-8} to 10^{-11} M).

DMI1, *DMI2*, and *DMI3* Control Steps of a Pathway Common to Nodulation and Endomycorrhization

dmi1, *dmi2,* and *dmi3* mutants are not only defective for symbiosis with Rhizobium but are also unable to establish a symbiotic association with arbuscular endomycorrhizal fungi. Furthermore, these mutants are blocked at an early stage of both interactions, with mycorrhizal infection being limited in each case to formation of appressoria. Such Nod⁻Myc⁻ mutants were first identified in pea (*Pisum sativum*) and faba bean (*Vicia faba*), where their ability to interact with soil pathogens was shown to be unaffected (Duc et al., 1989; Gollotte et al., 1993). Four genes essential for both the rhizobial and the endomycorrhizal symbioses have now been identified in pea (*SYM8*, *SYM9*, *SYM19*, and *SYM30*; Albrecht et al., 1999), and Nod⁻Myc⁻ mutants have been characterized in other legume species also, including bean (*Phaseolus vulgaris*) (Shirtliffe and Vessey, 1996), *L. japonicus* (Szczyglowski et al., 1998; Wegel et al., 1998), *M. sativa* (Bradbury et al., 1991), and *M. truncatula* (Sagan et al., 1995, 1998; this study). Also, in pea, mycorrhizal mutants have been identified that both are affected at the stage of arbuscule development and show a delayed nodulation phenotype (Gianinazzi-Pearson, 1996).

Besides controlling both the Rhizobium–legume and the endomycorrhizal symbioses, the gene *SYM8* of pea controls a step of a signal transduction pathway, induced both by Nod factors and by endomycorrhizal fungi and leading to *PsENOD5* and *PsENOD12A* expression (Albrecht et al., 1998). Like *SYM8* of pea, *DMI1*, *DMI2*, and *DMI3* control both Nod factor signal transduction (leading to nodulin gene expression) and endomycorrhizae formation, and possibly one of the *M. truncatula* genes described in this work is the ortholog of *SYM8* of pea. Nevertheless, we can say that *M. truncatula* has at least three common steps to the establishment of nodulation and endomycorrhization, all of which are involved in a Nod factor–activated signal transduction pathway leading to the induction of symbiotic responses. Previous data have suggested a possible conservation of signal transduction pathways between these two symbioses (van Rhijn et al., 1997; Albrecht et al., 1998), and the activation by both symbiotic partners of a genetic program that paves the way for accepted infection has been proposed as the welcoming "red carpet" (Gianinazzi-Pearson and Dénarié, 1997).

Unlike mutations in *DMI1*, *DMI2,* and *DMI3*, mutations in *NSP* do not affect the ability of plants to establish a symbiotic association with arbuscular endomycorrhizal fungi. Our data therefore indicate that we have identified a component required for Nod factor–activated signal transduction that is specific to the Rhizobium–legume symbiosis. In contrast to the Rhizobium–legume association, arbuscular endomycorrhizal symbioses show very little host specificity (for reviews on endomycorrhizae, see Gianinazzi-Pearson, 1996; Smith and Read, 1997; Harrison, 1999). Given this difference, it is perhaps surprising that genes intervening at early steps of the Nod factor–activated pathway should be common to both endosymbiotic associations. However, by analogy to the role of Nod factors in activating this pathway, it has been proposed that mycorrhizal signals also exist (Figure 6A, "Myc factors") and activate the pathway during the endomycorrhizal symbiosis (Albrecht et al., 1998, 1999). As a next step toward understanding the mechanisms involved in the establishment of rhizobial and endomycorrhizal symbioses, we have initiated map-based cloning projects for *DMI1*, *DMI2*, and *DMI3* in collaboration with the group of T. Huguet (INRA-CNRS, Toulouse, France).

METHODS

Bacterial Stains

Bacterial strains and plasmids are described in Table 1. Conditions used for bacterial growth and conjugation experiments have been described previously (Truchet et al., 1985). The strain GMI3198 was constructed by introducing the plasmid pGMI1394, carrying the regulatory gene *nodD1,* into the strain *Sinorhizobium meliloti* 2011(pXLDG4) by triparental mating using the helper plasmid pRK2013. The plasmid pGMI1394 confers the ability to overproduce nodulation (Nod) factors in various genetic backgrounds (Demont et al., 1994). The strain GMI3125 was constructed by marker exchange between the plasmid pRmM57, which carries a spectinomycin resistance (Sp) cassette inserted in the *S. meliloti nodC* gene, and the megaplasmid pSymA of *S. meliloti* GMI6628, which carries mutations in the *nodF* and *nodL* genes. pRmM57 was introduced into GMI6628 by triparental mating using the helper plasmid pRK2013, and phleomycin- (Pm; 20 μg/mL) and tetracycline- (Tc; 10 μg/mL) resistant colonies were selected. pRmM57 was then excluded from this strain by introducing the incompatible plasmid pR751-pMG2. Gentamicin- (Gm; 50 μg/mL) and Sp- (100 μg/mL) resistant colonies from this conjugation were found to be Tc-sensitive, indicating loss of pRm57. Homologous recombination in *nodC* was confirmed by DNA gel blot analysis (data not shown).

Plant Growth Conditions

Freshly collected seeds of *Medicago truncatula* enter dormancy within ~1 week after pod falling; the germination rate is then very low

for 3 to 4 months. To avoid the entry into dormancy, the majority of freshly collected seeds were stored at 4°C. To germinate them, the seeds were removed from their pods, dried overnight at 30°C, scarified with sandpaper, and then surface-sterilized with 12% sodium hypochlorite for 2 min. Seeds that had been stored at room temperature for >3 months were scarified directly by treatment with concentrated sulfuric acid for 7 min and then sterilized as described above. Both types of seeds were germinated on 1% deionized water agar plates at 14°C for 16 to 24 hr; plates with freshly collected seeds were first stored for at least 5 days at 4°C. When seeds were germinated to grow plants for seed production, sterilized seeds were vernalized for 10 days at 4°C before germinating them at 14°C.

Plants were all axenically grown on Fahraeus medium (0.132 g/L $CaCl_2$, 0.12 g/L $MgSO_4.7H_2O$, 0.1 g/L KH_2PO_4, 0.075 g/L $Na_2HPO_4.2H_2O$, 5 mg/L Fe-citrate, and 0.07 mg/L each of $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnCl_2$, H_3BO_3, and $Na_2MoO_4.2H_2O$, adjusted to pH 7.5 before autoclaving), except in aeroponic culture. The composition of the low-nitrogen aeroponic medium was 1 mM $CaCl_2$, 0.25 mM $MgSO_4.7H_2O$, 1.7 mM KH_2PO_4, 3.8 mM K_2HPO_4, 0.5 mM K_2SO_4, 0.05 mM $FeSO_4.7H_2O$, 0.05 mM Na_2EDTA, 30 μM H_3BO_3, 10 μM $MnSO_4$, 0.7 μM $ZnSO_4$, 1 μM $Na_2MoO_4.2H_2O$, 0.04 μM $CoCl$, and 0.2 μM $CuSO_4.5H_2O$ (Lullien et al., 1987). Certain plants were grown on Fahraeus agar (1.5%), either as slopes (Truchet et al., 1985) for nodulation tests or in Petri dishes for histological and cytological studies, root hair deformation tests, and *MtENOD40* expression studies. Others were grown on liquid medium, either in aeroponic culture in large plastic trash bins—modified by the addition of a motor to vaporize the growth medium and a lid adapted with 1000 small holes to enable the roots of seedlings to be pushed through for nodulation tests and *MtENOD11–GUS* transgenic studies—or in growth pouches (Journet et al., 1994) for nodulin gene expression studies. Plants were grown in the absence of added nitrate, except for studies on the effect of nitrate on root hair deformation and for cosegregation studies, in which the plants were first grown for 7 days in high-nitrogen aeroponic medium containing 5 mM NH_4NO_3 and then starved of nitrogen for 2 days before inoculation. Plants grown in aeroponic culture were placed in a growth chamber at 20°C with a 14-hr photoperiod at 70 μE sec^{-1} m^{-2}. All other axenic plants were placed in a growth chamber at 20°C with a 16-hr photoperiod at 65 μE sec^{-1} m^{-2}. For allelism tests and seed production, plants were grown in pots in a mixture of sterile sand/compost (1:2) in a growth room at 25/20°C, a 16-hr photoperiod, and 230 μE sec^{-1} m^{-2}. Plants for allelism tests were grown in 9 × 9 × 8-cm pots and allowed to spread horizontally. Plants for seed production were grown in 8 × 8 × 7-cm pots and trained to grow vertically inside inverted 1.5-liter plastic, mineral water bottles. This system was essentially a large Aracon system (Beta-Tech, Gent, Belgium), as used for *Arabidopsis thaliana*. Initially, a single bottle was inserted into an Aracon, and young plants were trained to grow into the bottle; subsequently, as plants grew, a second and a third neckless bottle were added. In this way, as many as 100 plants could be grown per square meter, thus maximizing the use of growth room space. These vertically grown plants started flowering within 10 days, producing at least 300 seeds per plant over two to three months.

Bacterial Inoculation and Nod Factor Treatment of Plants

Plants were inoculated with 0.5 mL of a suspension of 10^8 bacteria/mL in water covering the root system, except for in situ hybridizations and cytology studies, when Petri dish–grown plants were spot-inoculated with 0.2 μL of a bacterial suspension in the region of root hair emergence. Inoculation was performed at the following times after germination: 3 days for nodulation tests in agar slopes, 5 days for nodulation tests in aeroponic culture, 7 days for histological and cytological characterization and in situ hybridizations, and 9 days for cosegregation studies in aeroponic culture. For Nod factor tests on root hair deformation, plants were treated with Nod factors (prepared as previously described by Roche et al. [1991a]) diluted in water at 24 hr after germination. When studying the effect of nitrate on root hair deformation, however, we treated plants 7 days after germination, and for nodulin gene expression studies we treated them 5 days after germination (transgenic plants) or 7 to 11 days after germination (for reverse transcription–polymerase chain reaction [RT-PCR] and RNA gel blot analysis). Nod factors were added directly to plants growing on agar for root hair deformation studies, directly to plants growing in growth pouches for RT-PCR, and as described by Pingret et al. (1998) and Cook et al. (1995) for transgenic plants and RNA gel blot analysis, respectively. Root hair deformation tests were compared between the seedlings grown first on agar and then transferred to Fahraeus slides, as described by Heidstra et al. (1994), and the seedlings growing on agar. Because this comparison gave similar results (data not shown), all reported results are those for studies performed directly on seedlings grown on agar.

Isolation of Nod⁻ Mutants and Genetic Analysis

Seedlings from ~50,000 ethyl methanesulfonate (EMS)–mutagenized M_2 seeds of *M. truncatula* Jemalong (Penmetsa and Cook, 2000) were grown in aeroponic culture conditions (~700 seedlings per container) and scored for absence of nodules 10 days after inoculation with *S. meliloti* ABS7 (Table 1). Putative Nod⁻ mutants were planted and left to set seed. Nodulation tests were performed on 20 M_3 plants from each mutant inoculated with *S. meliloti* GMI6526 (Table 1). Mutants for which all 20 plants were Nod⁻ 3 weeks after inoculation were considered as confirmed Nod⁻ mutants. Three γ-ray Nod⁻ mutants of *M. truncatula* were included in this study (kindly provided by G. Duc, INRA, Dijon, France). All mutants were crossed manually (by using method 3 of Pathipanawat et al. [1994], except that female flowers were emasculated by removing anther sacs with forceps and crosses were performed at any time of the day) to transgenic Nod⁺ *M. truncatula* plants expressing a fusion between the *MtENOD11* promoter and the reporter gene encoding β-glucuronidase (*GUS*) (D.G. Barker, J.-L. Pingret, M. Chabaud, and E.-P. Journet, unpublished results). In these crosses, transgenic plants were used as the source of pollen, which was transferred to flowers of emasculated mutant plants. The presence of the transgene in the F_1 progeny (detected by nonsymbiotic expression of *MtENOD11–GUS* in root caps, lateral root primordia, and aerial parts of plants) confirmed that these plants were true hybrids. Nodulation tests (with *S. meliloti* GMI6526) were performed on F_1 plants to determine the recessive/dominant nature of the mutations. F_1 plants were then left to set seed, and nodulation tests (with *S. meliloti* GMI6526) were performed on F_2 plants to determine whether the mutations were inherited monogenically. Phenotypic characterization was performed on F_3 mutant plants (i.e., backcrossed once). In general, several different F_3 families (each derived from a single Nod⁻ F_2 plant) were checked for the Nod⁻ phenotype, and then a single F_3 family was selected for detailed characterization. To be able to study Nod factor induction of the *MtENOD11–GUS* fusion, we checked nonsymbiotic expression of *MtENOD11–GUS* (in root caps, lateral root primordia, and aerial

parts of plants) in 16 to 20 F_3 plants to confirm that they were derived from F_2 plants homozygous for the transgene. For allelism tests, mutants were crossed to each other. Whenever possible, each mutant was used as both a male and a female partner in each pairwise cross, with the male partner carrying the transgene *MtENOD11–GUS* as a marker (see above). A wild-type Nod$^+$ phenotype of F_1 plants was scored as indicating complementation. Mutants were considered allelic when three or more independent crosses, all confirmed by the presence of the marker, yielded only F_1 plants with the mutant Nod$^-$ phenotype.

Microscopic Methods

Plants were observed for infection events by histochemical staining for β-galactosidase activity expressed by the plasmid pXLGD4, as previously described (Ardourel et al., 1994). Plants were observed for root hair deformations after staining with methylene blue as previously described (Vasse and Truchet, 1984). Transgenic *MtENOD11–GUS* plants were stained and observed for GUS activity as described by Pingret et al. (1998), except that plants were stained at 5 days after germination. To look for cortical cell divisions, we embedded spot-inoculated root sections in polyethylene glycol as described by Timmers et al. (1999) and prepared semithin sections (15 to 20 μm thick) by using a microtome (model 2040; Reichert-Jung, Vienna, Austria). One mutant was studied for each locus (B129, TR25, TRV25, and B85), and between 20 and 30 individuals were scored for each mutant. For bright- and dark-field microscopy, plants were viewed with Zeiss (Jena, Germany) Axiophot I and II microscopes.

RT-PCR Assay

At 0, 12, and 48 hr after Nod factor addition, reactive root zones (3 cm from the primary root tip) of ∼30 seedlings that were 7 to 11 days old were frozen in liquid nitrogen, and the total RNA was extracted as described by Jackson and Larkins (1976). cDNA synthesis and RT-PCR were performed as described by Pingret et al. (1998), except that cDNA samples were purified by passage through Sephadex G-50 columns (Amersham Pharmacia Biotech, Saclay, France), precipitated, and resuspended to ∼5 ng/μL, of which 10-μL aliquots were used for PCR amplification (20 or 25 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min).

   MtENOD11 cDNA was amplified (25 cycles) between positions 82 and 519 by using the forward primer 5′-CTCCATCCCACAATATGC-CTCCA-3′ and the reverse primer 5′-ATCGATGCTAGGTGGAGGCT-3′. To control for equivalent cDNA contents, we also performed PCR amplifications for *MtPR10-1*, a constitutively and strongly expressed gene of roots (Gamas et al., 1998). The *MtPR10-1* cDNA was amplified (20 cycles) between positions 149 and 433 by using the forward primer 5′-CTCCATCCCACAATATGCCTCCA-3′ and the reverse primer 5′-ATCGATGCTAGGTGGAGGCT-3′. To control that the amount of amplified product was proportional to the input of cDNA, we also performed PCR with cDNA dilutions, and the results were analyzed with a PhosphoImager (model Storm 840; Molecular Dynamics, Sunnyvale, CA). PCR products were analyzed by DNA gel blotting with the 450-bp *MaeI/BstEII* fragment of *MtENOD11* (E.P. Journet, C. Calantzis, V. Vernoud, F. de Billy, G. Truchet, M. Pichon, A. Dedieu, D. Morandi, V. Gianinazzi-Pearson, and D. Barker, unpub-

lished results) and with the complete cDNA of *MtPR10-1* (Gamas et al., 1998).

RNA Gel Blot Analysis

At 0, 12, 24, and 48 hr after Nod factor addition, reactive root zones (3 cm from the primary root tip) of ∼30 seedlings 7 to 11 days old were frozen in liquid nitrogen. Total RNA and RNA gel blot analysis for *rip1* expression was performed as described in Cook et al. (1995).

In Situ Hybridization Assay

In situ hybridizations were performed on 7-μm-thick sections essentially as described by Gamas et al. (1998). Approximately 10 plants were analyzed per mutant. An *MtENOD40* cDNA clone (Gamas et al., 1996) was used to make *MtENOD40* RNA probes with T3 (sense) or T7 (antisense) RNA polymerase (Promega) and α-^{35}S-UTP (DuPont–New England Nuclear). In vitro transcription was performed at 37°C for 1 hr, followed by treatment with 1 unit of RQ1 RNase-free DNase (Promega) for 15 min and extraction with phenol-chloroform. Unincorporated ribonucleotides were removed by passage of the probes through MicroSpin S-200 columns (Pharmacia Biotech); the probes were then partially hydrolyzed by incubation in 60 mM Na_2CO_3, 40 mM $NaHCO_3$, and 7.5 mM DTT in the presence of 80 units of a ribonuclease inhibitor (RNasin; Promega) at 60°C for 40 min. This period was calculated to reduce the probes from full length (700 bp) to ∼150 bp. Hydrolysis was stopped by adding acetic acid to a final concentration of 0.1 M, after which the probes were ethanol-precipitated in the presence of 2 μg of yeast tRNA. Probe sizes were determined by analyzing aliquots taken before and after hydrolysis on formaldehyde gels with an RNA ladder (Gibco BRL). Incorporation of radiolabel was measured with a Packard Tri-Carb 2100TR liquid scintillation analyzer (Meriden, CT), and each slide was hybridized with 6×10^6 count/min.

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Four Genes of *Medicago truncatula* Controlling Components of a Nod Factor Transduction Pathway

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