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Rhizobium meliloti Lipooligosaccharide Nodulation Factors: Different Structural Requirements for Bacterial Entry into Target Root Hair Cells and Induction of Plant Symbiotic Developmental Responses

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Rhizobium meliloti **produces lipochitooligosaccharide nodulation NodRm factors that are required for nodulation of legume hosts. NodRm factors are O-acetylated and N-acylated by specific C16-unsaturated fatty acids. *nodL* mutants produce non-O-acetylated factors, and *nodFE* mutants produce factors with modified acyl substituents. Both mutants exhibited a significantly reduced capacity to elicit infection thread (IT) formation in alfalfa. However, once initiated, ITs developed and allowed the formation of nitrogen-fixing nodules. In contrast, double *nodF/nodL* mutants were unable to penetrate into legume hosts and to form ITs. Nevertheless, these mutants induced widespread cell wall tip growth in trichoblasts and other epidermal cells and were also able to elicit cortical cell activation at a distance. NodRm factor structural requirements are thus clearly more stringent for bacterial entry than for the elicitation of developmental plant responses.**

INTRODUCTION

Rhizobia (currently classified into three genera, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*) are soil bacteria that can elicit the formation of nitrogen-fixing nodules on the roots of selected species of the legume family. Nodule induction is specific, and a given rhizobial strain can infect a limited number of hosts. For example, *R. meliloti* nodulates *Medicago*, *Melilotus*, and *Trigonella* species, whereas *R. leguminosarum* bv *viciae* nodulates *Lathyrus*, *Pisum*, and *Vicia* species (Young and Johnston, 1989). Symbiotic nodule formation is based on two processes, a controlled infection and the induction and development of a novel organ (Nap and Bisseling, 1990; Brewin, 1991). To penetrate into their hosts via root hairs, rhizobia elicit the stimulation and reorientation of root hair cell wall growth (for a review, see Kijne, 1992). This rhizobia-induced tip growth results first in the entrapment of the bacteria within curled root hairs (shepherd's crooks) and then in the initiation and development of infection threads (ITs), tubular structures through which bacteria pass on their way down the root hair and into the underlying cortical cell layers (Brewin, 1991; Kijne, 1992). Ahead of the advancing threads, cells in the inner cortex are induced to dedifferentiate and divide, and a nodule primordium is formed (Brewin, 1991). As ITs penetrate and ramify within the primordium, a zone of apical meristematic activity directs outward growth of the developing nodule. At maturity, alfalfa nodules have the particular histology of indeterminate-type nodules. Longitudinal sections reveal the presence of an apical meristem, followed by an infection zone containing a network of ramified ITs from which bacteria are released into the plant cytoplasm, where bacteria differentiate into bacteroids that fix nitrogen (Vasse et al., 1990). Thus, rhizobia elicit a series of sequential and interrelated developmental responses in their host plants.

Rhizobium genetic analysis has led to the identification of a set of nodulation (*nod*) genes required for infection, nodule formation, and the control of host specificity. The common *nodABC* genes are present in all rhizobia, and mutations in these genes lead to a complete abolition of all detectable plant responses, including hair curling, IT formation, cortical cell divisions, and nodulation (see Dénarié et al., 1992; Fisher and Long, 1992). In addition, there are *nod* genes that are species specific, such as *nodH*, *nodPQ*, *nodFE*, and *nodL* in *R. meliloti*, and that are involved in the control of host specificity. *nod* genes are involved in the synthesis of lipooligosaccharide extracellular signals, the Nod factors, which elicit early nodulation events on the corresponding host plants (for reviews, see Dénarié et al., 1992; Fisher and Long, 1992; Spaink, 1992;

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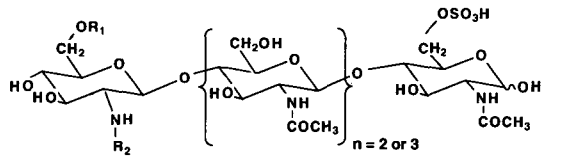
Dénarié and Cullimore, 1993). The Nod factors produced by strains from different species of *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* share a common basic structure: they are β-1,4-linked tetramers or pentamers of D-glucosamine, mono-N-acylated on the terminal nonreducing residue, and N-acetylated on the other residues (Lerouge et al., 1990; Roche et al., 1991a, 1991b; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993; Poupot et al., 1993). Nod factors differ in terms of the substituents linked to the chitin oligomer backbone. For example, the *R. meliloti* nodulation (NodRm) factors are O-sulfated on the C6 of the reducing amino sugar, O-acetylated on the C6 of the terminal nonreducing amino sugar, and mono-N-acylated by unsaturated C16 acyl groups and a series of hydroxylated fatty acids (Lerouge et al., 1990; Roche et al., 1991a, 1991b; Schultze et al., 1992; Demont et al., 1993, 1994) (Figure 1).

The role of the various structural *nod* genes in the control of Nod factor biosynthesis is currently under study. The common *nodABC* genes are involved in the synthesis of the lipooligosaccharide core (Spaink et al., 1991; Atkinson and Long, 1992; Debellé et al., 1992; John et al., 1993). The species-specific NodPQ and NodH proteins, which are homologous to ATP sulfurylase, adenosine 5′ phosphosulfate kinase, and sulfotransferases, have been shown to control the O-sulfation of the reducing glucosamine residue of the *R. meliloti* NodRm factors, a modification that confers alfalfa specificity (Schwedock and Long, 1990; Roche et al., 1991a; Leyh et al., 1992). The species-specific *nodFE* and *nodL* genes are involved in the decoration of the nonreducing end of the chitooligosaccharidic backbone. The NodF and NodE proteins, which are homologous to acyl carrier proteins and β-ketoacyl synthases, are involved in the synthesis of the particular polyunsaturated fatty acids that N-acylate the Nod factors of *R. l. viciae* (with C18:4 acyl chains) and *R. meliloti* (with C16:2 and C16:3) (Spaink et al., 1991; Demont et al., 1993). NodL, which is homologous to acetyl transferases (Downie, 1989; Baev and Kondorosi, 1992), is required for the O-acetylation of the terminal nonreducing glucosamine residue (Spaink et al., 1991; Bloemberg et al., 1994; M. Ardourel, G. Lortet, F. Maillet, J.C. Promé, and C. Rosenberg, manuscript in preparation).



STRAINS	R_1	R_2
2011	Ac or H	C16:1
		C16:2
		C16:3
nodL⁻	H	Idem
nodFE⁻	Ac or H	C18:1
nodFL⁻	H	C18:1

Figure 1. Structures of the Lipooligosaccharide Nod Factors Produced by the Various *R. meliloti* 2011 Derivatives Used in This Study.

For Nod factor structural determination, overproducing strains carrying the pMH682 plasmid were used and carried the following *nod* regions: 2011, *nod* structural genes of the wild-type strain (Roche et al., 1991a); *nodL*⁻, *nodL*::Tn*5* insertion; *nodFE*⁻, *nodFE* deletion (Demont et al., 1993); *nodFL*⁻, *nodF* deletion and *nodL*::Tn*5* insertion. For R_2, only the fatty acids that differ between the strains are represented. The series of (ω-1)-hydroxylated fatty acids (Demont et al., 1993), which exist in Nod factors from all the strains, are not represented. Ac, CH_3CO.

Purified Nod factors elicit a number of responses, in a specific manner, on the roots of legumes that are similar to those induced by rhizobia, such as deformation of root hairs (Lerouge et al., 1990; Roche et al., 1991a; Spaink et al., 1991; Price et al., 1992; Relic et al., 1993), cortical cell divisions and the formation of nodule primordia (Spaink et al., 1991; Truchet et al., 1991; Relic et al., 1993), and in some host plants, the formation of nodules (Truchet et al., 1991; Mergaert et al., 1993; Stokkermans and Peters, 1994). Circumstantial evidence suggests that Nod factors might also be involved in the formation of ITs. Thus, the exogenous supply of *R. l. viciae* Nod factors elicits the formation of radially aligned cytoplasmic structures in the outer and middle cortex of vetch roots; these structures are similar to the preinfection thread structures induced by the infecting rhizobia (van Brussel et al., 1992). In addition, Nod factors induce the transcription of the plant early nodulin genes *ENOD5* and *ENOD12*, which are related to the infection process (Nap and Bisseling, 1990; Horvath et al., 1993; Journet et al., 1994). Thus, Nod factors elicit a number of different symbiotic developmental responses.

The study of the symbiotic behavior of *Rhizobium nod* mutants producing modified Nod factors could help to decipher the role of these factors in the elicitation of this complex set of responses. This study provides a detailed analysis of the symbiotic behavior of *R. meliloti nodFE*, *nodL*, and double *nodFlnodL* mutants, which has led to the identification of different structural requirements for the Nod factors at various steps of the symbiotic process. We show that the synthesis of signal molecules O-acetylated and N-acylated by unsaturated C16 fatty acids at the terminal nonreducing end is required for the initiation of IT formation and alfalfa infection. We also show that NodRm structural requirements are more stringent for bacterial entry into root hairs than for the elicitation of plant developmental responses, such as tip growth in root hair and epidermal cells and the activation of a differentiation program in cortical cells.

RESULTS

Symbiotic Properties of *nodL* Mutants

R. meliloti 2011 produces Nod factors in insufficient quantities for detailed chemical studies. It was thus necessary to construct overproducing strains carrying *nod* gene transcriptional activators cloned on multicopy plasmids. An *R. meliloti* 2011 derivative carrying the pMH682 plasmid (containing the regulatory *nodD3* and *syrM* genes, see Figure 2) produces a range of sulfated NodRm factors that differ according to the following structural features (see Figure 1). First, there are variations in the length of the glucosamine oligosaccharide backbone, with a majority of tetramers and a minority of pentamers (Roche et al., 1991a, 1991b; Schultze et al., 1992). Second, the C6 of the nonreducing terminal glucosamine residue is frequently, but not always, O-acetylated (Roche et al., 1991a, 1991b; Truchet et al., 1991; Schultze et al., 1992). Third, the N-acyl moiety comprises mono-, di-, and triunsaturated C16 fatty acids with the unsaturations in positions Δ9, Δ2,9, and Δ2,4,9, respectively (Lerouge et al., 1990; Schultze et al., 1992; Demont et al., 1993) and a series of C18 to C26 (ω-1)-hydroxylated fatty acids (Demont et al., 1993, 1994) (see Figure 1).

The only change in the structure of the Nod factors resulting from mutations in *nodL* is the absence of the O-acetyl decoration. Mutations in the downstream *nolYZ* genes do not result in a detectable change in Nod factor structures, showing that the effect of the *nodL* mutations is not via a polar effect on downstream genes (M. Ardourel, G. Lortet, F. Maillet, J.-C. Promé, and C. Rosenberg, manuscript in preparation) (Figure 2). To facilitate cytological studies of infection, the plasmid pXLGD4 containing a fusion of the constitutive *hemA* promoter fused to the *lacZ* reporter gene was introduced into the strains under study. The presence of the pXLGD4 plasmid did not detectably modify the infection phenotype (data not shown). A *nodL*::Tn*5* mutant (GMI6436) and the isogenic strain carrying the pXLGD4 plasmid (GMI6563) were inoculated on seedlings of alfalfa. Root hair deformations and the infection process were analyzed by light microscopy of the entire root system 5, 7, and 10 days after inoculation. Both GMI6436 and GMI6563 elicited abundant hair deformation, including moderate curling (Figure 3A). In contrast, marked curling with the presence of a refractile spot was very rarely observed and significantly delayed in comparison to the symbiotic phenotype of the wild-type strain (Figure 3B). Quantitative scoring of infection showed that the number of ITs in root hairs was also greatly reduced (Table 1). In numerous plants, no ITs could be seen, but the few ITs that were observed had a normal appearance (Figure 3C) and always originated from curled root hairs. The symbiotic defect was not a result of a polar effect of the *nodL* mutation because mutations in the downstream *nolY* gene did not alter IT formation (Table 1).

Table 1. Induction of IT Formation by Various *R. meliloti nod* Mutants

nod Genotype of Strains	No. of ITs[a]
2011	51.5 ± 12.5
2011(p*nodD3/syrM*)	63.4 ± 14.2
2011*nodL*::Tn*5*	0.8 ± 0.5[b]
2011*nodL*::Tn*5*(p*nodD3/syrM*)	4.5 ± 1.5[b]
2011Δ*nolY*	34.5 ± 10.3
2011Δ*nodFE*	0.5 ± 0.4[b]
2011Δ*nodFE*(p*nodD3/syrM*)	7.8 ± 4.0[b]
2011Δ*nodG*	53.3 ± 7.9
2011Δ*nodF/nodL*::Tn*5*	0[b]
2011Δ*nodF/nodL*::Tn*5*(p*nodD3/syrM*)	4.9 ± 4.3[b]

[a] Each value represents the average number of infection threads (± SE) observed per tube containing two seedlings. Each experiment was performed with 10 tubes. Roots were observed 5 days after inoculation by *Rhizobium*.

[b] Statistically different from the wild-type strain 2011 control at the P = 0.01 level. Analysis of variance with Fisher's test (Snedecor and Cochran, 1980) using Stat View SE+ software (Abacus Concept, Alpha systèmes diffusions, 38240 Meylan, France).

Because the chemical characterization of Nod factors made use of overproducing strains carrying the pMH682 plasmid, it was important to study the infection behavior of such strains. The 2011(pMH682) strain elicited a similar number of ITs as the wild-type strain (Table 1), whereas the *nodL*(pMH682) mutant elicited some marked root hair curling (data not shown)

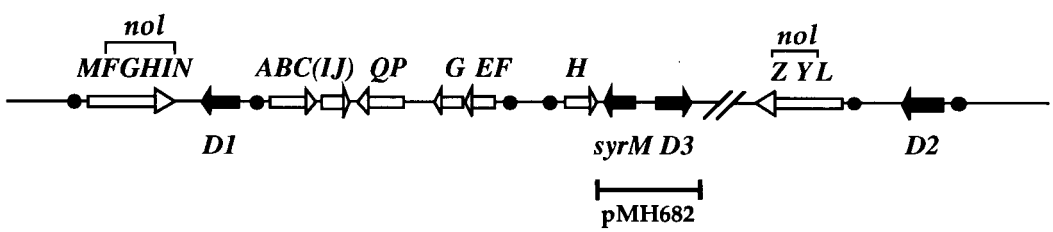


Figure 2. Genetic Map of the *R. meliloti* Region Carrying the Nodulation (*nod* and *nol*) Genes.

The open and filled arrows represent the structural and regulatory *nod* genes, respectively. Capital letters indicate *nod* genes unless otherwise indicated. The black circles indicate the "*nod* box" upstream of *nod* operons.

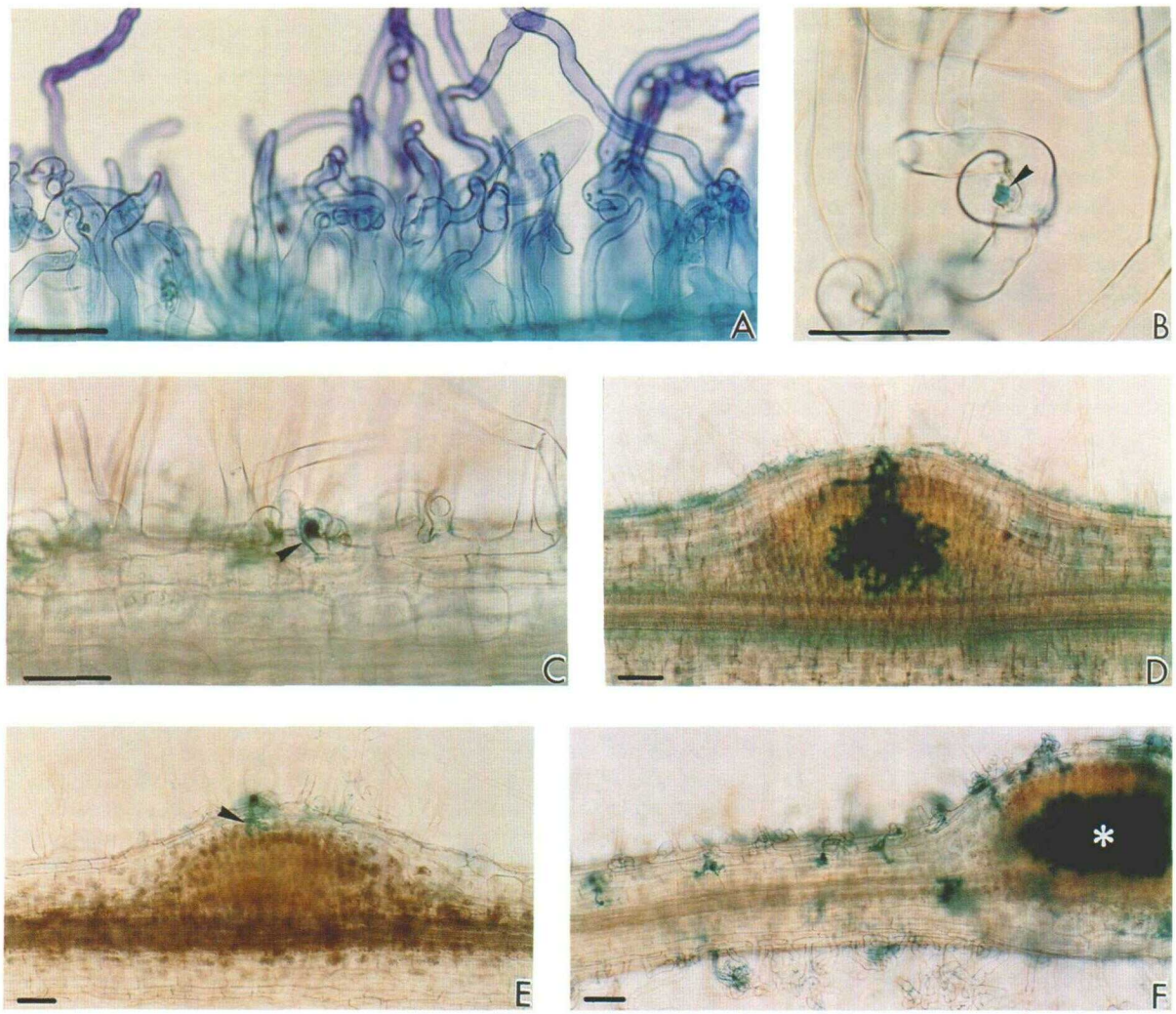


Figure 3. Early Symbiotic Properties of *R. meliloti nodL* and *nodFE* Mutants on Alfalfa.

Plants were inoculated with strains containing a fusion of the constitutive *hemA* gene promoter to the *lacZ* reporter gene. Ten days after inoculation, or 15 days as shown in **(D)**, entire plants were stained either with methylene blue as shown in **(A)** or for β-galactosidase activity as shown in **(B)** to **(F)**, slighty cleared and viewed by light microscopy.

(A) to **(D)** *nodL* mutant. In **(A)**, root hair deformations are shown. In **(B)**, marked root hair curling (Hac phenotype, arrowhead) with bacteria within the curl is evident. The arrowhead in **(C)** indicates IT initiation in a short root hair. The spread of an IT (with bacteria being stained blue) in the central region of a developing nodule is shown in **(D)**.

(E) *nodFE* mutant. A developing nodule with an IT (arrowhead) at top is shown.

(F) *nodG* mutant. Numerous ITs (with bacteria being stained blue) and a developing nodule (asterisk) are evident.

Bars = 50 μm.

with infrequent formation of ITs (Table 1). Because the presence of the pMH682 plasmid increased the production of Nod factors by a factor of at least 100-fold in both the wild-type and *nodL* strains, these results clearly suggest that the symbiotic defect caused by the *nodL* mutation could not be a result of a simple reduction in Nod factor production, but rather to the absence of the O-acetyl decoration. However, even such an increased production of altered Nod factors by the *nodL*(pMH682) strain only slightly increased the frequency of IT formation and was not sufficient to suppress the symbiotic defect (Table 1).

A *nodL* mutant exhibits a slight delay in nodulation (Figure 4), and the nodulated seedlings are green and fix nitrogen. The apparent contrast observed between the pronounced reduction in infection and the only limited effect on nodulation and nitrogen fixation prompted us to examine *nodL*-elicited

nodule development in more detail. Observation of plants 15 days after inoculation with a *nodL*(*phemA*::*lacZ*) strain showed that ITs were still rare and mostly restricted to the top of nodules (Figure 3D). ITs, originating from curled root hairs, were present in the inner tissue of developing nodules (Figure 3D), indicating that the rare ITs formed were able to grow and develop a network and were efficient in eliciting nodule development. The proportion of abortive ITs was clearly reduced as compared with the wild-type strain. Longitudinal sections of nodules revealed the presence of a network of ramified ITs in the central tissue and showed that bacteria were released into the host cytoplasm (data not shown). After external sterilization of a sample of nodules, bacteria were reisolated, purified, and inoculated again on sterile alfalfa seedlings. The reisolated bacteria exhibited the same symbiotic phenotype as the *nodL* mutant and showed the same alteration in IT formation (data not shown). This rules out the possibility that the rare infections observed were a result of *nodL* revertants or suppressor mutants. We can thus conclude that the *nodL* mutants, which produce non-O-acetylated Nod factors, are modified in their capacity to initiate IT formation but the *nodL* mutation does not significantly alter IT development once it has been initiated.

Symbiotic Properties of *nodFE* Mutants

The *nodF*, *nodE*, and *nodFE* mutants of *R. meliloti* excrete Nod factors in which the terminal nonreducing glucosamine residue is not N-acylated by C16 unsaturated fatty acids but by vaccenic acid (C18:1 Δ11) (Demont et al., 1993; see Figure 1). It was therefore of interest to study the infection behavior of this other class of mutants altered at the nonreducing end of Nod factors. To eliminate the possibility of reversions of *nodFE* mutants, we used the strain GMI5622 in which the *nodFE* genes have been deleted. In alfalfa, the symbiotic properties of the *nodFE* mutants were very similar to those of *nodL* mutants: (1) a lower frequency of marked root hair curling was noted (data not shown); (2) very low numbers of ITs initiated from root hair cells (Table 1); (3) once initiated, ITs grew and ramified in the root cortex and were associated with nodule development (Figure 3E); and (4) bacteria were released in the central tissue of mature nodules that fixed nitrogen (data not shown). Bacteria reisolated from nodules exhibited the same pattern of IT formation as the original *nodFE* mutant, showing that the rare infections that were initiated were indeed provoked by the *nodFE* mutant and not by a *nodFE* suppressor mutant.

An *R. meliloti* 2011 derivative, GMI5621, having a deletion in *nodG*, elicited the formation of numerous ITs in root hairs (Table 1; Figure 3F), showing that the infection defect of the *nodFE* mutant was not a result of a possible polar effect on the downstream genes (Figure 2). These results show that *R. meliloti nodFE* mutants, which produce Nod factors in which C16 unsaturated fatty acids are replaced by vaccenic acid, have a phenotype similar to that described above for *nodL* mutants.

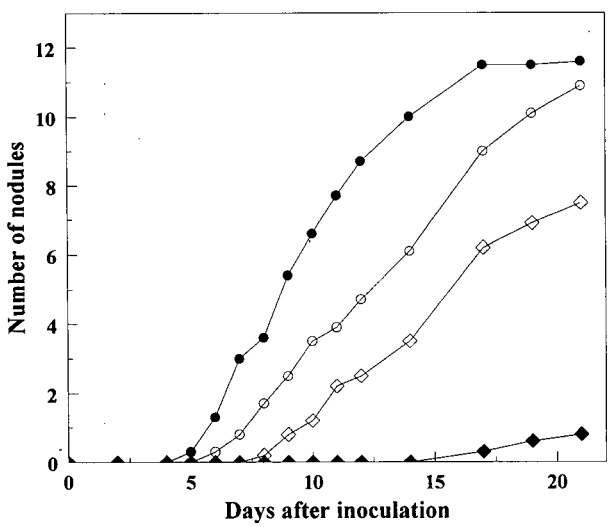


Figure 4. Nodulation Kinetics with *R. meliloti* Mutants on *M. sativa*.

Twenty plants were used for each experiment, with two seedlings per tube. The number of nodules corresponds to the average number of nodules present in a tube. Wild-type strain, black circles; *nodF* mutant, open circles; *nodL* mutant, open diamonds; *nodF/nodL* mutant, black diamonds.

Construction and Nod Factor Characterization of Double *nodF/nodL* Mutants

Because both the *nodFE* and the *nodL* mutants are leaky for the Inf$^-$ phenotype, it was interesting to determine whether mutants producing Nod factors modified both in the acyl moiety and by the absence of the O-acetyl group would still be able to elicit low-frequency infection. To address this question, we constructed double mutants by introducing a *nodL*::Tn*5* mutation in a strain carrying a *nodF* deletion by means of a transducing phage (see Methods). To facilitate structural studies of Nod factors produced by double *nodF/nodL* mutants, an overproducing strain was constructed by introducing the pMH682 plasmid into a representative mutant, strain GMI6628. As expected, we found that the Nod factors were doubly modified at their nonreducing end. Liquid secondary ion mass spectrometry (LSIMS) analysis in the positive ion mode revealed that all the Nod factors exhibited a shift of −42 mass units, corresponding to the absence of the O-acetyl substituent on the terminal nonreducing glucosamine residue (Figure 5A). A combined analysis by LSIMS and by gas chromatography of the fatty acids released after acidic methanolysis (Figure 5B) showed that Nod factors were amidified by the (ω-1)-hydroxylated C18 to C26 fatty acid series and by vaccenic acid (C18:1 Δ11) and a C20:1 fatty acid. The relative ratios were estimated to be 2:1:7 for the C18:1, C20:1, and (ω-1)-hydroxylated fatty acids, respectively. No N-acylation by unsaturated C16 acids could be detected.

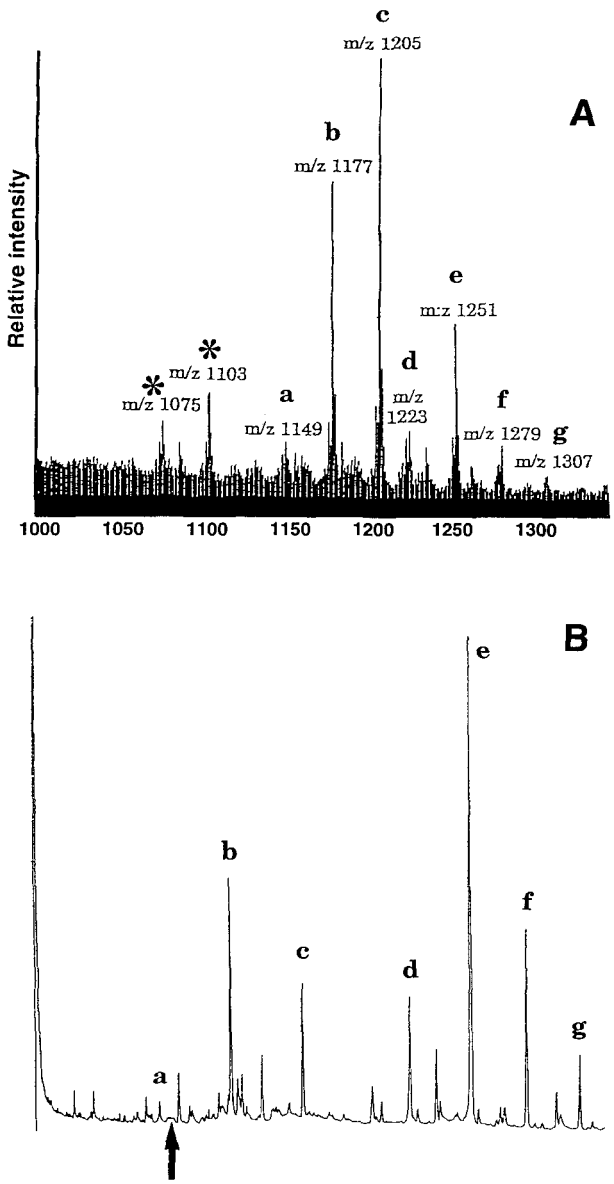


Figure 5. Characterization of Nod Factors from the *nodFInodL* Mutant of *R. meliloti.*

(A) Positive ion mass spectrum using the sodium attachment method. Lettered peaks correspond to the $(M - H + 2Na)^+$ ions of sulfated chitotetramers acylated with different fatty acids: a, NodRm-IV(S,C16:1); b, NodRm-IV(S,C18:1); c, NodRm-IV(S,C20:1); d to g, NodRm-IV(S) acylated by a series of (ω-1)-hydroxylated fatty acids. Peaks labeled with asterisks correspond to MNa^+ ions of desulfated Nod factors. *m/z*, mass-to-charge ratio.

(B) Gas chromatography profile of fatty acids released by acid methanolysis of Nod factors. Hydroxylated fatty acids were trimethylsilylated prior to analysis. Peaks with lowercase letters correspond to fatty acids released from the Nod factors marked with the corresponding letters in **(A)**. a, Δ9 C16:1; b, Δ11 C18:1; c, Δ13 C20:1 and (ω-1)-hydroxylated fatty acids; d, 19-OH C20; e, 21-OH C22; f, 23-OH C24; g, 25-OH C26. The arrow indicates the C16:2 fatty acid retention time.

nodFInodL Mutants Are Hyperactive in Eliciting Tip Growth in Trichoblasts and Other Epidermal Cells But Are Defective in Infection and Nodulation

Seven double *nodFInodL* mutants were inoculated on *Medicago sativa* cv Gemini seedlings. Three weeks later, no nodules could be detected on any of the plants examined. One representative mutant, GMI6628, and its derivative carrying the p*hemA*::*lacZ* plasmid, GMI6628(pXLGD4), were used in further studies. To investigate whether this inability to elicit nodulation was limited to this particular alfalfa cultivar, *nodFE*, *nodL*, and double *nodFInodL* mutants were used to inoculate the following species: other perennial *Medicago* species (*M. falcata* and *M. varia*), annual medics distantly related to alfalfa (*M. littoralis*, *M. lupulina*, and *M. truncatula*), and sweet clover (*Melilotus alba*). Nodules were counted 3 weeks after inoculation (Table 2). Whereas single *nodF* and *nodL* mutations resulted in nodulation delays that varied with the plant species, the double *nodFInodL* mutant was unable to nodulate any of the *Medicago* species tested and only poorly nodulated sweet clover. The very rare nodules that could be observed on sweet clover were pink, and the nodulated seedlings had dark green leaves characteristic of nitrogen-fixing plants.

Thorough cytological observation of seedlings of the various hosts 5 and 7 days after inoculation with GMI6628(pXLGD4) did not reveal ITs either in the root hairs or within the cortex. However, some differences could be observed in root hair cell (trichoblast) and other epidermal cell responses between the species. In the case of *M. sativa* and *M. varia*, root hairs were longer than usual and exhibited very striking root hair deformations. In contrast to seedlings inoculated with the wild-type strain, in which ~10% of the root hairs were deformed (Figure 6A), deformations affected a very high proportion (more than 80%) of the root hairs of plants inoculated with the double mutant (Figure 6B). Shepherd's crooks could not be observed, but root hairs were highly deformed and individual cells exhibited multiple deformations, suggesting that each trichoblast was the site of multiple abortive tip growth events (Figure 6B). Surprisingly, deformations were not restricted to root hairs but also affected unhaired epidermal cells, particularly on secondary roots. Two main types of epidermal cell deformations were observed. The first type corresponded to a rounded convex swelling of the outer periclinal cell wall, which was probably a result of a loosening of the wall, giving an undulated aspect to the root surface (Figure 6C). The second type was characterized by multiple budding of short root hairlike structures, which could correspond to abundant induction of abortive tip growth (Figures 6C and 6D).

Light microscopy of *M. alba* and *M. falcata* inoculated with GMI6628(pXLGD4) confirmed that the *nodFInodL* double mutant was unable to infect these host plants. Root hairs were highly deformed, and very exceptionally curled root hairs (Hac) could be seen 10 days following inoculation (data not shown). Finally, in the case of *M. truncatula* with which the wild-type *R. meliloti* strain elicited only a limited number of ITs (Figure 6E), the *nodFInodL* mutant induced root hair swellings and the formation of shepherd's crooks. Bacteria accumulated within

Table 2. Nodulation Phenotype of *nodF*, *nodL*, and Double *nodF*/*nodL* Mutants on Various Host Plants

	Numbers of Nodules per Tube[a]						
Bacterial Strains	*Medicago sativa*	*Medicago varia*	*Medicago falcata*	*Medicago lupulina*	*Medicago littoralis*	*Medicago truncatula*	*Melilotus alba*
2011	11.4	16	19.4	4.6	8.8	11.5	15.6
2011Δ*nodF*	7.7	7.6	20.2	3.9	10.0	13.2	9.9
2011*nodL*::Tn*5*	6.7	13	0.5	NT	NT	7.2	9.5
2011Δ*nodF*/*nodL*::Tn*5*	0.4	0.2	0	0	0	0.1	1.1

[a] Scored on 20 plants (with two seedlings per tube) 21 days after inoculation. NT, not tested.

the enlarged center of the curls, but no elongated infection threads could be seen developing from these enlarged infection centers (Figures 6F and 6G).

These results show that *R. meliloti* cells producing Nod factors that are neither O-acetylated nor N-acylated by the appropriate C16 unsaturated fatty acids are able to stimulate cell wall growth and induce tip growth in trichoblasts and other epidermal cells, but are unable to initiate IT formation and penetrate into their hosts.

It has previously been found that certain mutant *Rhizobium* strains that either fail to induce nodules or induce empty nodules when inoculated alone onto alfalfa seedlings can complement each other extracellularly when coinoculated (Kapp et al., 1990). We thus inoculated *M. sativa* seedlings with a mixture (ratio of ~1:1) of two Nod$^-$ mutants producing Nod factors modified at either the nonreducing end (GMI6628, the *nodF*/*nodL* mutant used in the above-mentioned experiments) or the reducing end (GMI5431, a *nodH* mutant producing nonsulfated factors). After 3 weeks, no nodules were formed. This absence of complementation of the *nodF*/*nodL* mutant by a strain that produces Nod factors having the correct decorations at the nonreducing end but being nonsulfated suggests that the presence of the O-sulfate group at the reducing end is also required for IT initiation.

nodF/*nodL* Mutants Elicit Activation of Remote Root Cortical Cells

In the course of infection, rhizobia elicit a differentiation program within cortical cells. This program is characterized cytologically by a decrease of vacuole volume, an increase of nucleus volume, and the formation of cytoplasmic bridges (Kijne, 1992; van Brussel et al., 1992). We have tried to establish a simple bioassay for what we propose to call cortical cell activation (CCA). This bioassay was designed to test the ability of different mutant strains and Nod factors to elicit these cortical responses. Previous observations had shown that after the addition of Nod factors, numerous starch granules can be seen in certain areas of the cortex and in developing nodules (Truchet et al., 1991). We therefore checked whether starch granule accumulation, which can easily be detected using potassium iodide, could serve as a cytological marker of CCA. Seven days after inoculation of alfalfa seedlings with the *R. meliloti* wild-type strain, amyloplasts were found to accumulate in dividing inner cortical cells observed in the front of growing ITs (Figure 7A); 10 days after inoculation, amyloplasts were abundant in the developing nodules (Figure 7B). No accumulation of amyloplasts was detected in the roots of uninoculated alfalfa seedlings (Figure 7C) or in roots of plants inoculated with a non-nodulating *R. meliloti nodA*::Tn*5* mutant (Figure 7D).

The *R. meliloti nodF*/*nodL* mutant elicited a very marked starch granule accumulation in the cortex of secondary roots. Seven days after inoculation, this accumulation was evenly distributed along the rootlets (Figure 7E). Light microscopy at a higher magnification revealed that starch granules accumulated in the center of large-sized cortical cells (Figure 7F) and that starch accumulation was correlated with a decrease of vacuole volume, an increase of nucleus size, and the development of cytoplasmic strands. Fifteen days after inoculation, clusters of cortical cells having a reduced volume and separated by periclinal septa were observed (Figures 7G and 7H). Thus, the *nodF*/*nodL* mutant elicits a few cortical cell mitoses and induces, on the same rootlet, starch accumulation in dividing as well as in nondividing cortical cells. Ultrastructural studies confirmed that (1) starch accumulates in plastids; (2) amyloplasts surround the nucleus located in a central position in the cortical cell; and (3) both the nucleus and the surrounding amyloplasts lie on an anticlinal cytoplasmic strand (Figure 7I). We also noticed that at the contact zones between the cytoplasmic bridge and the distal (outer) periclinal wall of the cortical cells, the cell wall was often undulated with a lower electron density (Figure 7I). It is worth noting that the location of amyloplasts surrounding the nucleus in the *nodF*/*nodL* mutant is different from that described by Bakhuizen (1988) for normal infection, in which there is a polarization of cortical cells with amyloplasts mostly present at one side of the nucleus. The distribution of the amyloplasts around the nucleus in the *nodF*/*nodL* mutant may suggest that the mutant signals are unable to polarize plant cells properly.

nodF/*nodL* mutants are unable to penetrate into alfalfa cells, and bacteria remain bound to the outer surface of root hairs and epidermal cells. These mutants, however, are able to elicit a pronounced activation of cells located at a distance in all cortical cell layers. It seems reasonable to hypothesize that the modified lipooligosaccharides excreted by the mutants at the root surface are able to signal at a distance to plant cells

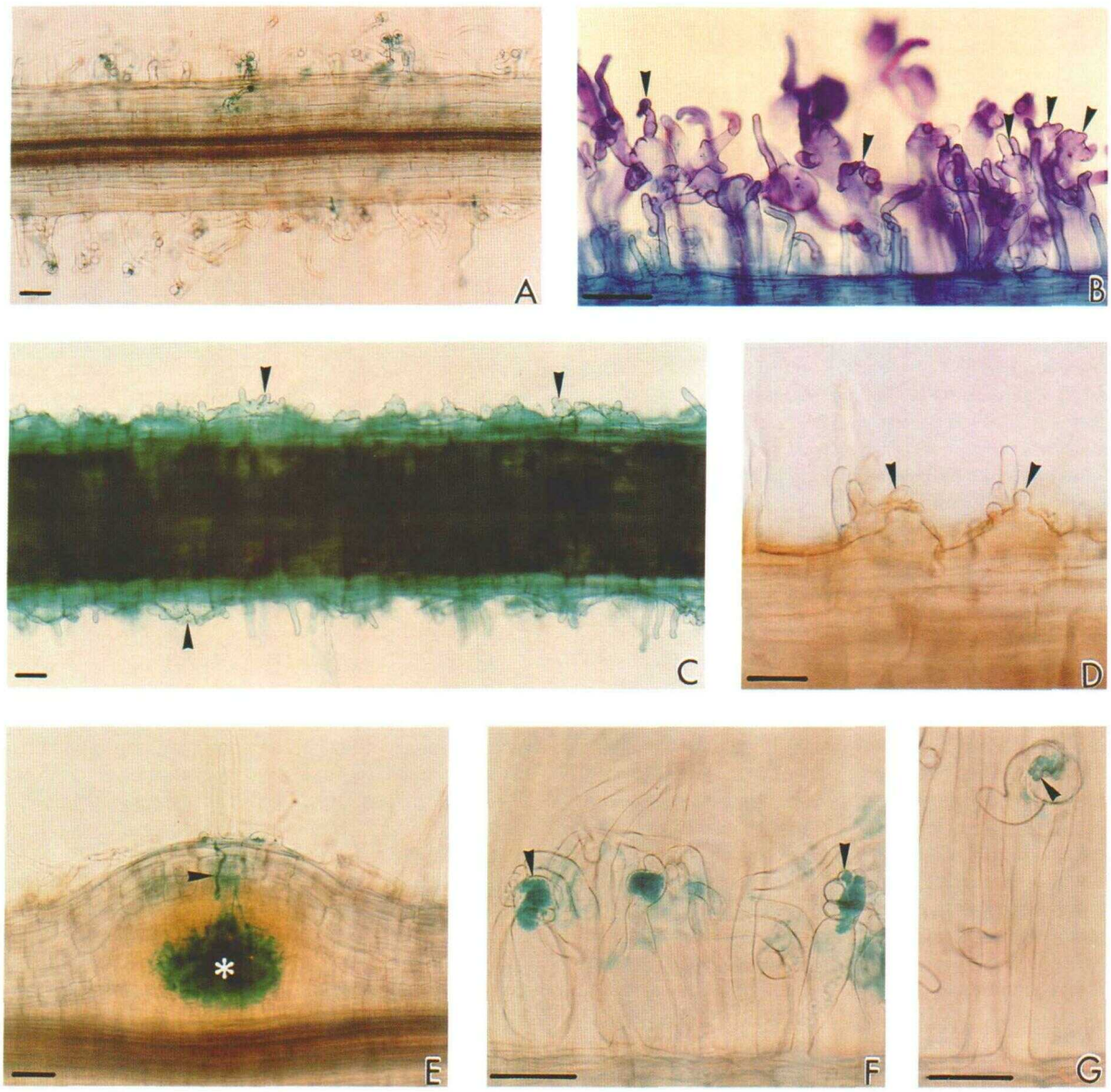


Figure 6. Early Interactions of the Wild-Type *R. meliloti* 2011 Strain and Its *nodFInodL* Derivative on Different Hosts.

Plants were inoculated with strains containing the chimeric *hemA::lacZ* gene. Seven days after inoculation, entire plants were processed for β-galactosidase activity, stained with methylene blue if necessary (**[B]** and **[C]**), and slightly cleared before observation by light microscopy.
(A) and **(B)** *Medicago varia*. In **(A)**, ITs (with bacteria being stained blue) induced by wild-type *R. meliloti* 2011 are observed within root hairs and in the cortex. In **(B)**, root hair deformations elicited by the *nodFInodL* double mutant are evident. Note the multiple deformations on single root hairs (arrowheads).
(C) and **(D)** The *nodFInodL* double mutant induces swelling of epidermal cells that gives an undulated aspect to the root surface of *M. sativa* in **(C)** and *M. varia* in **(D)**. Multiple budlike structures (arrowheads) are observed on the epidermal cells of *M. varia* in **(D)**.
(E) to **(G)** *M. truncatula*. In **(E)**, ITs induced by *R. meliloti* 2011 are located on the top (arrowhead) and in the central region (asterisk) of a developing nodule. **(F)** and **(G)** show that root hair curling is elicited by the *nodFInodL* double mutant. The arrowheads point to the unusual accumulation of bacteria inside curled root hairs.
Bars = 50 μm.

separated from the bacteria by many cell layers and trigger a differentiation program. Whereas the presence of the O-acetyl group and the appropriate unsaturated C16 acyl groups is not required to elicit CCA, the presence of the O-sulfate group seems required because *nodH* mutants, which are unable to invade alfalfa and produce nonsulfated Nod factors, do not elicit a significant accumulation of amyloplasts or cell divisions in root cortex (data not shown).

Biological Activity of Mutant Nod Factors

R. meliloti Nod factors are able to elicit a number of responses on axenic alfalfa seedlings. These responses are similar to the responses induced by living rhizobial cells and include root hair deformations, induction of mitosis in the inner cortex, and nodule formation (Lerouge et al., 1990; Roche et al., 1991a; Truchet et al., 1991; Schultze et al., 1992). It was therefore important to address the question of the biological activity of the NodRm factors produced by the *nodFE*, *nodL*, and double *nodFlnodL* mutants.

In the different assays, *M. sativa* seedlings were treated with Nod factors prepared from the following strains: (1) 2011(p*nodD3/syrM*) control; (2) a 2011Δ*nodFE*(p*nodD3/syrM*) mutant; (3) a 2011*nodL*(p*nodD3/syrM*) mutant; and (4) a 2011Δ*nodFlnodL*(p*nodD3/syrM*) double mutant. The differences between Nod factors isolated from the four strains are given in Figure 1. All four Nod factor preparations had similar hair-deforming activities and were active in this root surface bioassay at concentrations down to 10^{-12} M (data not shown). The presence of the O-acetyl group had no detectable influence. This contrasts with the striking decrease in Had (root hair deformation) activity observed when the sulfate moiety is removed from the reducing glucosamine (Roche et al., 1991a).

Experiments were then performed to assess Nod factor CCA activity. The *nodFlnodL* factors exhibited mitotic activities in the same range (10^{-7}/10^{-9} M) as the Nod factors from the wild-type strain. Interestingly, after 15 days of growth, the purified *nodFlnodL* factors were more efficient in eliciting starch accumulation than the "wild-type" factors at concentrations as low as 10^{-10} M. In the presence of "*nodFlnodL* factors," starch accumulation was more evenly distributed along the secondary roots and mimicked that observed after inoculation with the corresponding bacteria (see Figures 7B and 7E). In comparison, the Nod factors produced by *nodFE* and *nodL* mutants were poorer elicitors of starch accumulation. Finally, the Nod factors produced by *nodFE*, *nodL*, and double *nodFlnodL* mutants were clearly less active than those produced by the wild-type strain in inducing nodule formation (Figure 8).

DISCUSSION

The Nod factors of *R. meliloti* possess three substitutions on the chitooligosaccharide backbone, O-sulfation at the reducing end, and O-acetylation and N-acylation with specific fatty acids at the nonreducing end. We had previously shown that the O-sulfation, specified by the *nodH* and *nodPQ* genes, is essential for alfalfa infection and nodulation (Roche et al., 1991a). We show here that the other two substitutions, O-acetylation and the N-acylation with C16 unsaturated fatty acids, are also important for alfalfa infection. *R. meliloti nodL*, *nodFE*, and double *nodFlnodL* mutants produce Nod factors with modifications in the substitutions at the nonreducing end of the chitooligosaccharide backbone. *nodL* mutants produce non-O-acetylated Nod factors (M.Y. Ardourel, G. Lortet, F. Maillet, J.C. Promé, and C. Rosenberg, manuscript in preparation), and *nodFE* mutants produce factors that differ from those secreted by the wild-type strain in being N-acylated by vaccenic acid instead of unsaturated C16 fatty acids (Demont et al., 1993). Double *nodFlnodL* mutants produce non-O-acetylated Nod factors in which unsaturated C16 fatty acids are replaced by vaccenic acid and a C20:1 fatty acid.

To study the symbiotic behavior of these mutants, we have used bacteria containing a constitutive *lacZ* fusion. This method appears to be extremely sensitive, allowing easy detection of individual bacterial cells and ITs within the plant roots. Both *nodL* and *nodFE* mutants have a reduced capacity to elicit IT initiation in *M. sativa* root hairs. Nevertheless, rare infections do take place, and once initiated, ITs develop into an IT network, leading to the development of nitrogen-fixing nodules. Double *nodFlnodL* mutants are unable to elicit IT formation and nodulation of their legume hosts. These results clearly show that specific decorations at the nonreducing end of the Nod factors are also required for IT initiation in alfalfa root hairs and support the model that the "decoration" of the core chitin oligomers confers to the Nod factors their plant specificity (Roche et al., 1991a; Dénarié and Roche, 1992).

The study of the symbiotic behavior of the *nodFE*, *nodL*, and double *nodFlnodL* mutants has also revealed that bacteria producing Nod factors with modified structures can provoke an uncoupling between different plant responses, such as root hair deformation, IT initiation, IT growth, cortical cell activation, and nodulation. We now discuss how this can help to dissect the plant responses to bacterial signaling during different steps of the infection process.

Uncoupling of Root Hair Deformation, Marked Curling, and Infection

In alfalfa, *R. meliloti nodFE* and *nodL* mutants are able to cause root hair deformations (Had) but have a very reduced capacity to initiate both marked curling (Hac) and IT formation (Inf). The rare ITs observed originated from curled root hairs. More strikingly, the double *nodFlnodL* mutants elicited multiple deformations of root hairs but were completely unable to elicit Hac and Inf. Thus, among root hair responses, these various mutants exhibited an uncoupling of Had from Hac/Inf. This uncoupling, revealed by mutants producing modified Nod factors, indicates that Had and Hac/Inf responses have different structural requirements for Nod factors. Only bacteria producing sulfated Nod factors substituted at the nonreducing end

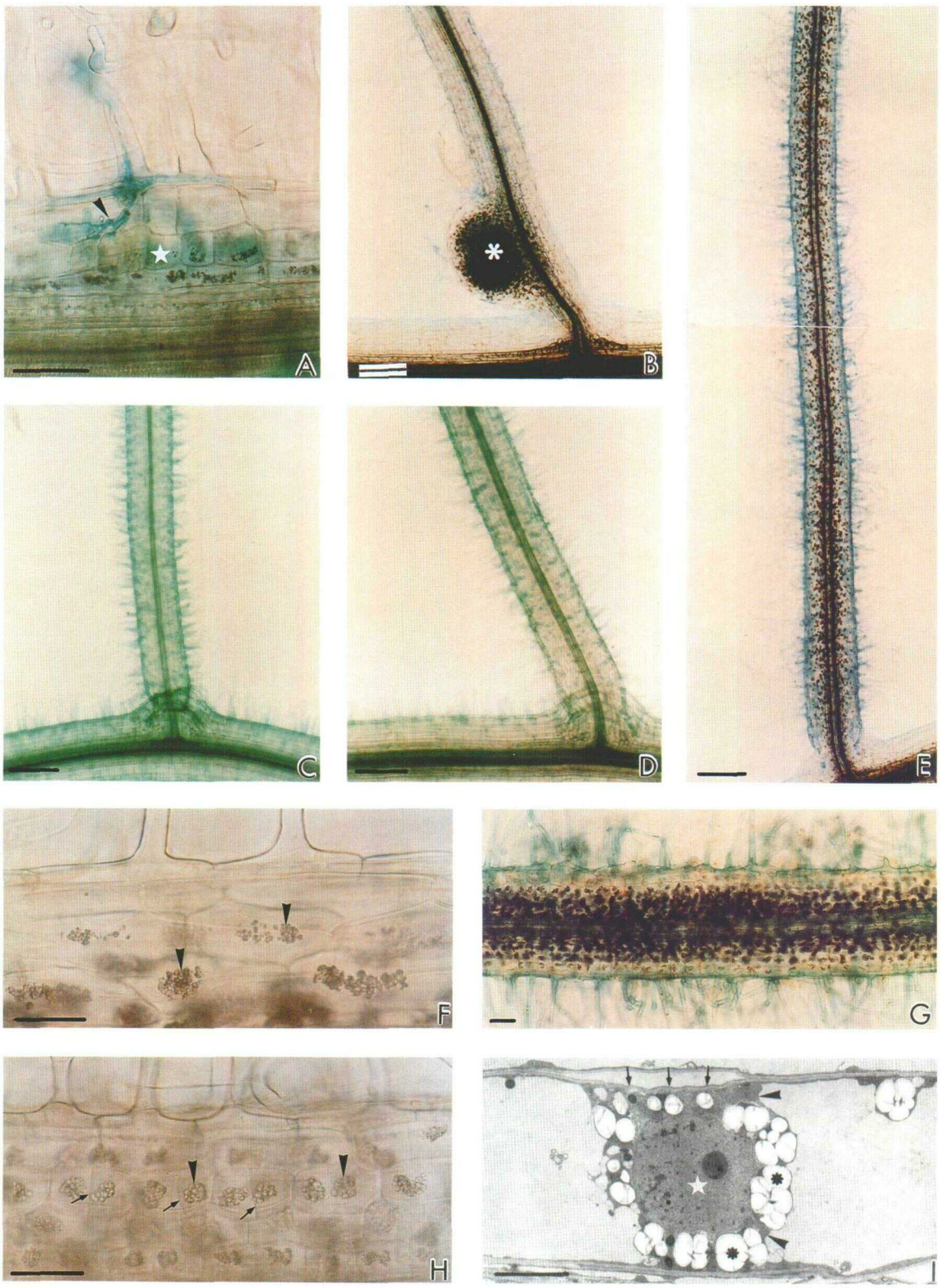


Figure 7. Starch Granule Accumulation in Cortical Cells of *M. sativa* Inoculated with Various *R. meliloti* Derivatives.

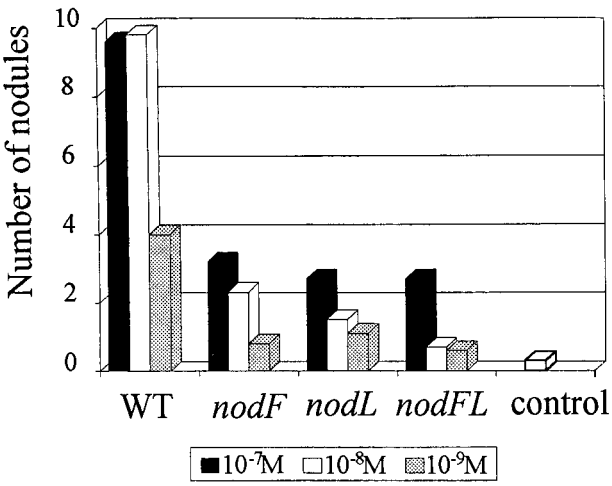


Figure 8. Effect of Purified Nod Factors from *R. meliloti* Mutants on the Nodulation of *M. sativa*.

Twenty plants were used for each dilution with two seedlings per tube. Nodules were scored (nodule number per tube) 38 days after the addition of the Nod factors. Nod factors were purified from the wild-type strain (WT), *nodF*⁻ strain (*nodF*), *nodL*⁻ strain (*nodL*), and *nodFlnodL*⁻ strain (*nodFL*).

with *O*-acetate, and the specific unsaturated C16 fatty acids were able to elicit efficient curling and IT initiation. For Had, the presence of sulfate is essential (Debellé et al., 1986; Roche et al., 1991a); however, the presence of the specific two substitutions at the nonreducing end is not required.

In a recent review, Kijne (1992) has proposed a coherent interpretation of the *Rhizobium* infection process in legumes, such as pea and alfalfa. We will make use of this interpretation to discuss the possible role of Nod factors at various steps of the infection process. Young developing root hairs have an endogenously controlled tip growth. A computer simulation study of root hair curling predicted that marked curling would result from the superimposition of a second pole of tip growth elicited by attached rhizobia, creating a reorientation of the root hair growth (Van Batenburg et al., 1986). We can hypothesize that Nod factors with the three correct substitutions are required to allow the bacteria attached to the root hairs to establish intimate interactions with the target cell and form a proper infection site (Inf site). When an Inf site is established, an efficient second pole of tip growth allows marked curling and IT initiation to occur. *nodFE*, *nodL*, and double *nodFlnodL* mutants, producing modified Nod factors, are inefficient or unable to establish this infection site. Nevertheless, they remain able to elicit new tip growth in root hairs (Had). The computer simulation showed that Had may result from the inability of the rhizobia to initiate a proper Inf site (Van Batenburg et al., 1986). Thus, the structural requirements to form an Inf site are more stringent than those to simply elicit tip growth in root hairs.

nodFlnodL mutants exhibit an unusual phenotype. They elicit multiple deformations of single root hair cells and a very extensive budding of the other epidermal cells. In contrast, the *R. meliloti* wild-type strain induces the formation of a single IT in a root hair and does not elicit detectable tip growth in epidermal cells. The following hypothesis can be proposed to explain these results. Once an Inf site has been initiated, for example by the wild-type strain, cellular modifications suppress the possibility of further tip growth and Inf site formation in root hairs and surrounding epidermal cells. On the other hand, *nodFlnodL* mutants, being unable to elicit Inf site formation, would not activate the negative autoregulatory control over tip growth deformations. Finally, the *nodFlnodL* mutant phenotype indicates also that not only trichoblasts but other epidermal cells can recognize and respond to Nod factors.

Uncoupling of Infection Thread Initiation and Development

After marked curling, rhizobia entrapped in a root hair curl may be ingested by root hair cells. This ingestion process most

Figure 7. (continued).

Whole plants inoculated with strains containing the chimeric *hemA::lacZ* gene were observed by light microscopy, and starch accumulation is shown in **(A)** to **(H)**. The plants were processed for detection of β-galactosidase activity and then doubly stained with potassium iodide and methylene blue.

(A) and **(B)** Starch accumulation following inoculation with wild-type *R. meliloti* 2011 is shown. In **(A)**, the starch granules (darkly stained spots) are observed in the cells of a nodule primordium (star) located in front of an IT (arrowhead). In **(B)**, the starch granules are observed in a young nodule (asterisk) developing at the axil of a lateral root.

(C) and **(D)** No starch accumulation is detected in a noninoculated control plant **(C)**, nor in a plant inoculated with a *nodA*::Tn*5* mutant strain **(D)**.

(E) to **(H)** Starch granule formation induced by the *nodFlnodL* double mutant is shown. In **(E)**, starch deposits (black dots) are detected in the cortical cells of a secondary root 7 days after inoculation. In **(F)**, starch granules (arrowheads) are observed in the center of large cortical cells. In **(G)**, a heavy deposition of starch is evident in cortical cells 15 days postinoculation. In **(H)**, starch granules (arrowheads) are seen packed in the center of each daughter cell derived from dividing cortical cells. The arrows point to anticlinal divisions.

(I) Electron microscopy of a cortical cell after inoculation with the *nodFlnodL* double mutant. Amyloplasts (asterisks) surrounding the nucleus (star) are seen in a cytoplasmic bridge (arrowheads). Note the low electron density and the undulation of the cell wall in contact with the cytoplasm (arrows).

In **(A)** and **(F)** to **(H)**, bars = 50 μm; in **(B)** to **(E)**, bars = 200 μm; in **(I)**, bar = 5 μm.

probably includes host cell wall degradation and invagination of the host plasma membrane (Callaham and Torrey, 1981). Initial uptake of rhizobia is followed by the formation of a growing tubular invagination of the host plasma membrane and is accompanied by deposition of cell wall material on subterminal regions of the invaginating membrane, giving rise to an IT (reviewed in Brewin, 1991 and Kijne, 1992). IT growth, similar to root hair growth and root hair curling, requires the induction of a tip growth process. IT growth in the root cortex involves the crossing of host cell walls. As in the case of the initial rhizobial ingestion during the initiation of IT formation, adjacent cell walls gradually disappear and a pore is formed. After penetration of bacterial cells through the cell wall pore, a tip growth area is formed again (van Spronsen et al., 1994).

Once IT formation has been initiated, Nod factors may also be required for IT growth and ramification. The following circumstantial evidence is in favor of such a continuous role for Nod factor signaling: (1) *nod* genes are expressed in ITs during the entire infection process, in the root hairs, in the root cortex, and in the nodule infection zone (Sharma and Signer, 1990; Schlaman et al., 1991; G. Truchet, F. Maillet, S. Camut, and J. Dénarié, unpublished results); (2) Nod factors induce root hair formation and branching and thus elicit cell wall tip growth (Lerouge et al., 1990; Roche et al., 1991a; Spaink et al., 1991; Relic et al., 1993); (3) Nod factors elicit the formation of cytoplasmic bridges in cortical cells similar to the preinfection threads induced by infecting rhizobia (van Brussel et al., 1992); and (4) the transcription of plant infection–related early nodulin genes, *ENOD5* and *ENOD12*, is induced by Nod factors (Nap and Bisseling, 1990; Scheres et al., 1990; Horvath et al., 1993; Pichon et al., 1993; Journet et al., 1994). Interestingly, the *nodFE* and *nodL* mutants show a clear uncoupling between IT initiation and IT growth. They are inefficient in eliciting IT initiation at the root hair surface and seem to be competent in eliciting IT growth and development once the initial ingestion is complete. In cytological terms, the initial crossing of the host cell wall seems to be similar to those which follow within the cortex (Kijne, 1992); this poses intriguing questions concerning the different Nod factor structural requirements for IT initiation and IT growth. These results suggest that there is a particular mechanism of selective entry control at the root hair surface with a stringent structural requirement for Nod factors at their nonreducing end.

Very recently it has been reported that the exogenous addition of purified Nod factors can restore the ability of *nodABC* mutants of *Rhizobium* sp strain NGR234 and *B. japonicum* to infect and form nitrogen-fixing nodules on their legume hosts *Vigna unguiculata* and *Glycine max*, respectively, suggesting that Nod factors are the signals that permit rhizobia to penetrate legume roots via infection threads (Relic et al., 1994). Such complementation has not been observed, however, with legumes forming indeterminate nodules (with both a persistent IT network and a persistent meristem), such as alfalfa, pea, and vetch (van Brussel et al., 1993; G. Truchet, F. Maillet, and J. Dénarié, manuscript in preparation). Two hypotheses can be proposed to explain this lack of complementation. First, the Nod enzymes involved in Nod factor synthesis might also be involved in the synthesis of other bacterial surface components required for infection. However, it has been shown in *R. leguminosarum* that the substitution of exopolysaccharides is not influenced by *nod* genes (Canter Cremers et al., 1991; O'Neill et al., 1991). Further, in *R. meliloti* 2011, mutants defective in the synthesis of lipopolysaccharides are not altered for infection and nodulation (Clover et al., 1989), and *nod* genes do not provoke detectable changes in the fatty acid composition of lipid A (Demont et al., 1994). Moreover, the strongly additive effect of mutations in *nodFE* and *nodL* suggests that the putative alternate substrate for NodFE and NodL enzymes should be an *N*-acetylglucosamine residue (substrate of NodL) with closely linked substitutions of an O-acetyl group and an unsaturated C16 acyl chain. Because lipid A is not modified by *nod* genes, the nature of the alternate substrate is not obvious. The second hypothesis to explain the lack of complementation has been proposed by Hirsch (1992) and is based on the idea that bacterial penetration of root hair cells might require a very localized effect of Nod factors. For example, Nod factors associated to bacterial membranes could provoke a capping of Nod factor receptors, which might be required to trigger bacterial ingestion. The addition of a solution of exogenous Nod factors would not allow receptor capping and would thus not allow complementation of nod mutants.

Uncoupling of Infection, Cortical Cell Activation, and Nodulation

nodF/nodL mutants, in spite of their inability to infect the plant, elicit activation of cortical cells at a distance, as shown by a strong accumulation of starch granules. These results confirm that structural requirements for Nod factors are more stringent for Inf site formation and bacterial entry than for the triggering of further plant symbiotic responses.

When added exogenously, purified NodRm factors from *nodFE* and *nodL R. meliloti* mutants have a reduced capacity to induce nodule formation, suggesting that, in contrast to CCA, nodule induction has stringent structural requirements. However, *nodFE* and *nodL R. meliloti* mutant strains efficiently elicit nodulation once ITs are formed. Similar discrepancies have been observed with *R. l. viciae nodFE*, and *nodL* mutants exhibit only a slight delay in nodulation, whereas purified factors from these mutants seem unable to elicit nodule meristem formation (Spaink et al., 1991; van Brussel et al., 1992; Sutton et al., 1993). In the case of *R. leguminosarum*, it has been proposed that the NodO extracellular bacterial protein could compensate for the reduced activity of the modified Nod factors by interacting with plant membranes (Sutton et al., 1993). Another possible interpretation of the reduced stringency in the case of bacterial nodule induction could be that *nodFE* and *nodL* mutants are able to deliver the modified factors from ITs at the appropriate site, time, and concentrations in the root cortex and hence induce nodule formation in spite of the reduced intrinsic biological activity of these factors. In the case

of the *nodF/nodL* mutants in the absence of infection, the concentrations of the modified Nod factors that are at least 100-fold less active than the wild-type Nod factors for nodule induction would not reach the required threshold in the target cortical cells to trigger nodule meristem formation.

Uncoupled Plant Responses and Nod Factor Receptors

How are the Nod factors perceived by the plant to initiate the various responses involved in infection? The elicitation of some of these responses at extremely low concentrations by factors with very specific molecular structures suggests that high-affinity Nod factor receptors are involved. The uncoupling of different responses described in this study suggests that more than one mechanism of Nod factor recognition is involved. Previous work has already shown that not all plant reactions have the same Nod factor structural requirement. For example, nodule primordium and cytoplasmic bridge formation in vetch have a stringent requirement for *R. l. viciae* Nod factors amidified by polyunsaturated fatty acid (C18:4), whereas root hair initiation and deformation can be induced by factors acylated by vaccenic acid (Spaink et al., 1991; van Brussel et al., 1992). These responses, however, involved different tissues or cell types (i.e., epidermal, outer or inner cortical cells), and the differences in structural requirements could also be attributed to differences in diffusion or transport of the different Nod factors through the root tissues.

In this study, some of the observed differences concern the same cell type (growing root hairs) and cells that were in direct contact with Nod factors; therefore, it is reasonable to hypothesize that the differences in responses are a result of the existence of more than one type of Nod factor recognition mechanism at the root hair surface. This could be a result of either different interactions of various ligands with a single type of receptor or the presence of different types of receptors, with both mechanisms leading to the activation of different signal transduction pathways. A number of observations support a model involving two types of receptors. First, the differences observed in the induction of plant responses by the wild-type

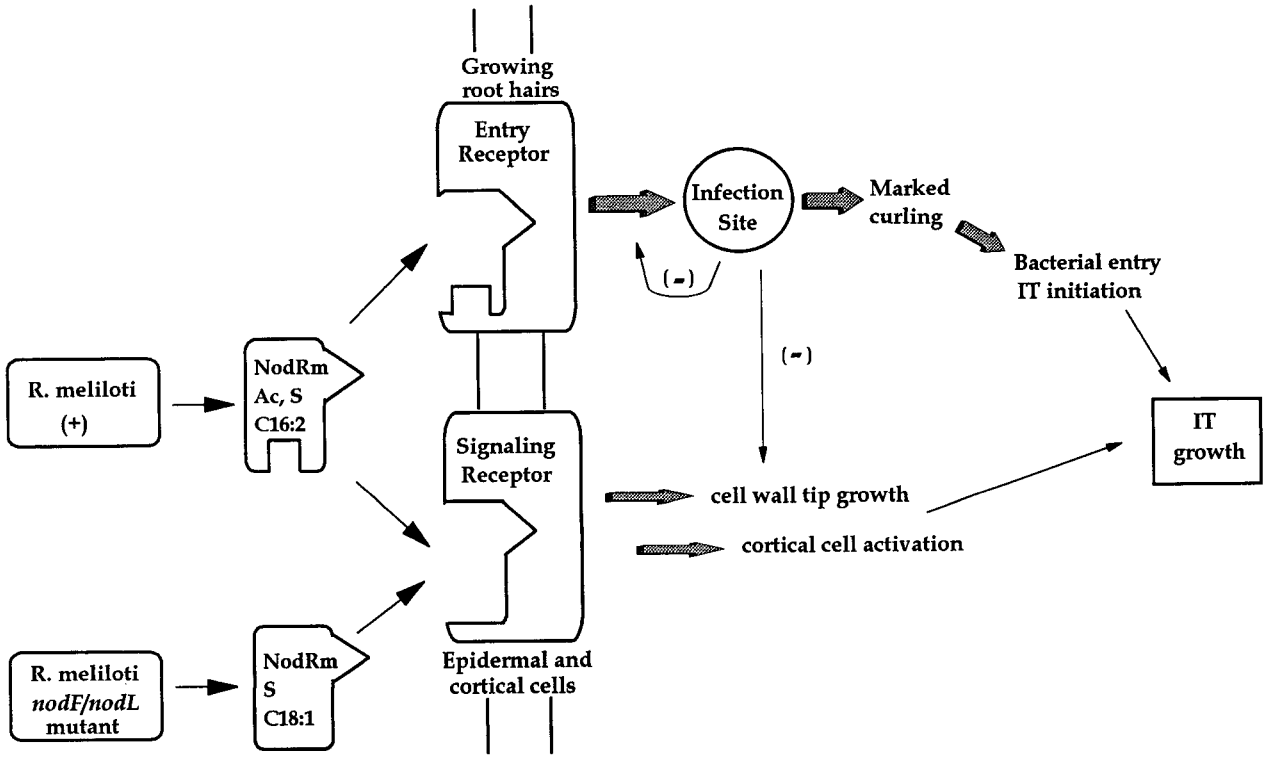


Figure 9. A Model for the Role of *R. meliloti* Nod Factors and Their Putative Plant Receptors in IT Formation in *M. sativa.*

At least two types of receptors would be involved in Nod factor recognition. Entry receptors having stringent requirements for the nonreducing end substitutions of NodRm factors would operate during the initial bacterial ingestion in growing root hairs. Signaling receptors, having less stringent requirements, would be involved in signaling to plants in the course of the infection process in epidermal and cortical cells. For a description of the infection process, see Kijne (1992). The (−) signs illustrate the negative control exerted by one infection site that, when formed in a root hair, represses the formation of other infection sites in the trichoblast and surrounding cells. Ac, acetylated; S, sulfated; *R. meliloti* (+), wild-type strain.

strain and the *nodF/nodL* mutant are very striking. In contrast to the wild type, the mutant strongly activates multiple tip growth in epidermal cells and starch granule accumulation in cortical cells, but is completely unable to penetrate into root hairs. We might expect that interactions of different ligands with the same receptor would not lead to such all-or-nothing differences. Second, the sites of the perception of Nod factors, with different degrees of stringency, are different. Third, as we will discuss below, Nod factor recognition at different sites and with different stringencies could correspond to different cellular functions.

A first type of receptor (signaling receptor), recognizing Nod factors even when their nonreducing end is modified, would be involved in signaling to the plant to pave the way for infection. This signaling would set into motion the cellular mechanisms that would permit the tip growth process, hair deformation, and IT growth (Figure 9). The symbiotic function of this type of receptor would be to allow the bacteria to signal to the plant to coordinate their functioning to achieve the symbiotic infection. A second type of receptor, having stringent structural requirements for substitutions at the nonreducing end of the molecules, would be involved in the specific recognition of rhizobial cells to allow the formation of the Inf sites and the initial bacterial ingestion, a kind of entry lock. This "entry receptor" would operate at the surface of root hairs and "open the door" specifically to bacteria producing factors with the appropriate alfalfa-specific decorations at both the reducing end (O-sulfate) and the nonreducing end (C16 fatty acids and O-acetyl) (see Figure 9). If only one substitution at the nonreducing end is incorrect, some bacterial entry could be possible, allowing the initiation of rare ITs. The symbiotic functions of the entry receptor would be (1) to recognize specifically the appropriate rhizobial cells (cell sorting) among numerous other soil microorganisms (including nonspecific rhizobia or pathogens) present at densities of approximately a thousand million per gram of rhizosphere soil, and (2) to trigger the rhizobial ingestion process.

The study of the various mutants also indicates that Nod factor receptors are likely to be present in different plant cell types. The "budding" phenotype of *nodF/nodL* double mutants indicates that nonstringent "signaling receptors" are located not only at the root hair surface but also at the surface of other epidermal cells. This is consistent with the previous observations that Nod factors induce the differentiation of epidermal cells into trichoblasts (Roche et al., 1991a; van Brussel et al., 1992) and the transcription of the *ENOD12* early nodulin gene in epidermal cells before the differentiation of root hairs (Pichon et al., 1992; Journet et al., 1994). We have described above a series of evidence that supports a continuous role of Nod factor signaling during the development of the IT network in the root cortex (and later in the apical region of the nodule). Corresponding signaling receptors would thus be present in these tissues. It is worth noting that in contrast to *nodFE* and *nodL* mutants, *nodH* mutants also lose the ability to deform and to infect alfalfa root hairs, and the nonsulfated Nod factors that *nodH* mutants secrete do not elicit root hair deformations, *ENOD12* gene transcription, and nodulation in alfalfa at physiological concentrations (Roche et al., 1991a; Journet et al., 1994). These results suggest that the putative alfalfa receptors of both entry or signaling types are located either in epidermal or cortical cells and require sulfated NodRm signals for their activation.

METHODS

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids are described in Table 3 and Figure 2. Conditions used for bacterial growth and conjugation experiments have been described previously (Truchet et al., 1985). The transfer of the IncP plasmids to *Rhizobium meliloti* was performed by triparental mating using the helper plasmid pRK2013 (Ditta et al., 1980). Transconjugants were selected with 10 μg/mL tetracycline. *Escherichia coli* donors were auxotrophic and counterselected on saccharose V minimal plates (Vincent, 1970). Transduction of *R. meliloti* was performed with the N3 bacteriophage as previously described (Roche et al., 1991a). To construct double *nodF/nodL* mutants, we used the N3 transducing phage to introduce a *nodL*::Tn*5* insertion into a strain carrying a *nodF* deletion (Demont et al., 1993). To eliminate the transductants in which the wild-type *nodF* gene would have been cotransduced with *nodL*::Tn*5*, the presence of the *nodF* deletion was checked by hybridization with appropriate probes. For nodulation (Nod) factor production, *R. meliloti* cultures were grown in 5-L Erlenmeyer flasks filled with 2 L of culture medium containing luteolin (10 μM) as a *nod* gene inducer (Lerouge et al., 1990). The flasks were agitated on a rotary shaker at 50 rpm for ~24 hr at 30°C. When the optical density (600 nm) reached 0.8 to 1.0, the cultures were filtered through a 0.45-μm Millipore filter membrane, and the cell-free culture medium was immediately extracted as described below.

Nod Factor Purification

Nod factors were extracted from the culture medium by butanol and ethyl acetate washing as described previously (Roche et al., 1991b). HPLC was conducted as previously described (Demont et al., 1993). For biological assays, two successive purifications were performed, the first one on a semipreparative C_{18} reverse phase column (Demont et al., 1993) and the second one on an analytical C_{18} reverse phase column (4,6 × 250 mm, Spherisorb ODS1, 5 μm; ChromatoSud, Bordeaux, France). In both cases, the sample was purified first for 3 min in isocratic solvent A (water/acetonitrile 80:20 [v/v]) and then on a 30-min linear gradient from solvent A to solvent B (pure acetonitrile) at a flow rate of 1 mL/min (Demont et al., 1993).

Nod Factor Structural Studies

Mass spectra of Nod factors were recorded on an Autospec instrument (Fisons, VG-analytical, Manchester, UK) equipped with a fast cesium ion bombardment source (liquid secondary ion mass spectrometry [LSIMS]). The cesium gun was operated at 20 kV, and the secondary ion accelerating voltage was 8 kV. One microliter of Nod

Table 3. Bacterial Strains, Plasmids, and Bacteriophage Used in This Study

Designation	Relevant Characteristics[a]	Reference/ Source
R. meliloti		
RCR2011	SU47, wild type, Nod$^+$ Fix$^+$ on *M. sativa*	Rosenberg et al. (1981)
GMI6390	2011(pMH682)	Roche et al. (1991a)
GMI6526	2011(pXLGD4)	This study
GMI5887	2011Δ(*nodF*)13	Debellé et al. (1988)
GMI6488	GMI5887(pMH682)	Demont et al. (1993)
GMI5621	2011Δ(*nodG*)7	Debellé et al. (1988)
GMI5622	2011Δ(*nodFE*)4	Debellé et al. (1988)
GMI6365	GMI5622(pMH682)	Demont et al. (1993)
GMI6528	GMI5622(pXLGD4)	This study
GMI6436	2011*nodL*::Tn*5*	M. Ardourel
GMI6506	2011*nodL*::Tn*5*(pMH682)	M. Ardourel
GMI6563	2011*nodL*::Tn*5*(pXLGD4)	This study
GMI6438	2011Δ(*ORF2*)	M. Ardourel
GMI6564	2011Δ(*ORF2*)(pMH682)	M. Ardourel
GMI6562	2011Δ(*ORF2*)(pXLGD4)	This study
GMI6628	2011Δ(*nodF*)13*nodL*::Tn*5*	This study
GMI6629	GMI6628(pMH682)	This study
GMI6630	GMI6628(pXLGD4)	This study
E. coli		
GMI10726	HB101(pMH682)	Honma et al. (1990)
GMI3686	K12(pRK2013)	Ditta et al. (1980)
GMI10504	HB101(pXLGD4)^r	Ditta et al. (1980)
Plasmids		
pMH682	pWB5a prime (IncP), 8-kb insert from *R. meliloti* p*Syma* carrying *nodD3* and *syrM*, Tc^r	Honma et al. (1990)
pRK2013	Helper plasmid for mobilization of IncP and IncQ plasmids, Km^r	Ditta et al. (1980)
pXLGD4	pGD499 prime (IncP) carrying a *hemA*::*lacZ* fusion	Leong et al. (1985)
Bacteriophage		
N3	Transducing phage of *R. meliloti*	Martin and Long (1984)

[a] Tc^r, tetracycline resistant; Km^r, kanamycin resistant; ORF, open reading frame.

factor solution in methanol (concentration 1 μg/μL) was deposited on the stainless steel target loaded with 1 μL of matrix. The matrix used in the positive ion mode was a 1:1 metanitrobenzyl alcohol/glycerol mixture spiked with an equal volume of 1% sodium iodide solution. In the negative ion mode, the same matrix was used without sodium iodide addition.

Gas chromatograms were performed on a Girdel 30 apparatus (Altech-France, Templeuve, France) equipped with a flame ionization detector and a Ross type injector (Spiral, Dijon, France). Helium was the carrier gas. Separations were achieved on an OV1-coated capillary column (0.32 mm i.d. × 12 m, film thickness 0.1 μm; Altech-France, Templeuve, France) using a linear program from 100 to 280°C at 3°C/min.

Fatty acid release from Nod factors was achieved by acid methanolysis. Approximately 100 μg of Nod factors were dissolved into 0.5 mL of 1 N HCl solution in methanol. After 18 hr at 80°C, the mixture was dried under nitrogen and partitioned between diethyl ether and water. The ether phase was washed with water, dried, and then dissolved into 20 μL of bis-trimethylsilyl-trifluoroacetamide containing 1% trimethylchlorosilane. One microliter of this solution, containing both methyl esters of nonhydroxylated fatty acids and *O*-trimethylsilyl ether methyl esters of hydroxylated fatty acids, was analyzed by gas chromatography. The nature of the fatty acids had previously been established by gas chromatography–mass spectroscopy of the O-trimethylsilyl ether methyl ester derivatives (Demont et al., 1993). In this study, we used characterized fatty acids from previous experiments as reference compounds for the identification of the fatty acids by capillary gas chromatography.

Plant Assays

Seed of *Medicago sativa* cv AS-13 (Ferry-Morse Co., Modesto, CA) were kindly provided by S. Long (Stanford University, Stanford, CA). Seed of *M. sativa* cv Gemini and *Melilotus alba* were provided by P. Guy (Station d'Amélioration des Plantes Fourragères INRA, F86600 Lusignan, France). Seed of *Medicago truncatula* cv Jemalong 2828 were obtained from G. Duc (Station d'Amélioration des Plantes INRA, F21110 Genlis, France) and *Medicago falcata*, *Medicago littoralis*, *Medicago lupulina*, and *Medicago varia* were from J.M. Prospéri (Station d'Amélioration des Plantes INRA, F34130 Mauguio, France). For sterilization, seeds were first treated with concentrated sulfuric acid (10 min for *M. sativa*, 8 min for *M. alba*, and 2 min for the other species) and then with 12% sodium hypochlorite (20 min for *M. sativa* and *M. alba* and 2.5 min for the other species). *M. truncatula* seed were germinated for 48 hr at 14°C. For the other species, seed were first stored overnight at 4°C and germinated for 16 hr at 24°C (or 32 hr for *M. falcata*). Seedlings were transferred to test tubes on Fahraeus agar slants (two seedlings per tube) as described by Truchet et al. (1985) and placed in a growth chamber (20°C, 80% of moisture, and 16 hr under 300 μE m^{-2} sec^{-1} light). When required, rhizobia were reisolated from nodules as previously described (Debellé et al., 1988). The alfalfa nodulation assays with Nod factors were performed as given in Roche et al. (1991a), except that *M. sativa* cv AS-13, which exhibits a reduced level of spontaneous nodulation, was used. Nod factors were added in two stages: first in the melted agar Fahraeus medium immediately before pouring in test tubes and then 1 week after sowing the sterile germinated seeds on the agar slope by adding 1 mL of Nod factor liquid solution on the root system. The alfalfa root hair deformation assays with Nod factors were performed as already described using alfalfa cultivar Gemini (Roche et al., 1991a).

Microscopic Methods

All light microscopic assays described in this study have been performed on whole plants. Hair deformations were observed 5, 7, or 10 days postinoculation on plants stained with methylene blue according to the method described by Vasse and Truchet (1984). Infection and nodulation were examined on plants inoculated with bacterial strains carrying a fusion of the reporter gene *lacZ* of *E. coli* to the *hemA* gene promoter of *R. meliloti*. The *hemA* gene is known not to be under general nitrogen control and is constitutively expressed in free-living *R. meliloti* (Leong et al., 1985) as well as in symbiotic conditions (David et al., 1988). β-galactosidase activity was assayed as described in Boivin et al. (1990) using X-gal as substrate. Histochemical staining of starch was performed on plants that were successively fixed with glutaraldehyde (2.5% in sodium cacodylate 0.15 M, pH 7.2), cleared with sodium hypochlorite (Truchet et al., 1989), and stained with potassium iodide (Vasse et al., 1990). For light microscopy, processed plants were viewed with an Olympus Vanox microscope (Olympus Optical Co. Ltd., Tokyo, Japan) using bright-field optics. For electron microscopy, plants were fixed in 2.5% glutaraldehyde (as described above), dehydrated in an ethanol series, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Hitachi EM600 electron microscope (Hitachi Ltd., Tokyo, Japan).

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