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Assignment of Symbiotic Developmental Phenotypes to Common and Specific Nodulation (*nod*) Genetic Loci of *Rhizobium meliloti*

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***Rhizobium meliloti* nodulation (*nod*) genes required for specific infection and nodulation of alfalfa have been cloned. Transposon Tn*5* mutagenesis defined three *nod* regions spanning 16 kilobases of the pSym megaplasmid. Genetic and cytological studies of 62 nodulation-defective mutants allowed the assignment of symbiotic developmental phenotypes to common and specific *nod* loci. Root hair curling was determined by both common (region I) and specific (region III) *nod* transcription units; locus IIIb (*nodH* gene) positively controlled curling on the homologous host alfalfa, whereas loci IIIa (*nodFE*) and IIIb (*nodH*) negatively controlled curling on heterologous hosts. Region I (*nodABC*) was required for bacterial penetration and infection thread initiation in shepherd's crooks, and the *nodFE* transcription unit controlled infection thread development within the alfalfa root hair. In contrast, induction of nodule organogenesis, which can be triggered from a distance, seemed to be controlled by common *nodABC* genes and not to require specific *nod* genes *nodFE* and *nodH*. Region II affected the efficiency of hair curling and infection thread formation.**

Rhizobia are soil bacteria which can invade the roots of leguminous hosts and induce the formation of root nodules in which they fix nitrogen. Nodule induction is specific: a *Rhizobium* strain can generally form nodules with a limited range of plants, e.g., *R. meliloti* strains with species of *Medicago*, *Melilotus*, and *Trigonella* and *R. trifolii* with *Trifolium* species (26). In *Medicago sativa-R. meliloti* symbiosis, the developmental sequence leading to nodule formation can be divided schematically as follows: attachment to root hairs, root hair curling, infection thread formation within root hairs, growth of the thread toward the inner root cortex, initiation of a nodule meristem, and nodule organogenesis (16, 40–43, 46). Thus, a procaryotic organism, amenable to simple genetical analysis, is involved in the control of a eucaryotic developmental process, including cell morphogenesis, cell wall synthesis, induction of cell dedifferentiation and division, and triggering of an organogenesis program (26, 44). Whether host specificity is involved for each step of the infection process is not known.

In *R. meliloti*, nodulation (*nod*) genes are located on a pSym megaplasmid (1, 27, 31). Insertional mutations that simultaneously abolish root hair curling and nodulation have been mapped ca. 25 kilobases (kb) from the nitrogenase genes (19, 24, 27). These *nod* mutations, which can be complemented by nodulation genes from other *Rhizobium* species, define common *nod* genes (14, 24). DNA sequencing has revealed a considerable degree of homology between common *nod* genes from *R. meliloti* and *R. leguminosarum* (33, 36, 37, 39). *R. meliloti* common *nod* genes are organized in two divergent transcription units, one containing *nodABC* and the other *nodD* (12, 19), and their transcription is stimulated by root exudates from homologous and heterologous leguminous hosts (30).

Transfer of *R. meliloti* pSym genes into *Agrobacterium tumefaciens* enables this bacterium to induce nodule formation on alfalfa (17, 23, 47) but not on clover (15, 40, 42), which shows that genes controlling nodule initiation and host range specificity are also located on pSym. Noncommon (specific) *nod* sequences are located between the common *nodABC* genes and the *nifHDK* operon (22, 24, 40).

In this paper we describe the cloning of *R. meliloti* sequences required for the specific infection and nodulation of alfalfa and identify common and specific *nod* loci dispersed over ca. 16 kb of pSym DNA. Use of the methylene blue staining procedure (43) allows a detailed cytological characterization of the mutant infection phenotypes. We can thus assign common and specific *nod* loci to various developmental steps: root hair curling, initiation and growth of the infection thread within the root hair, and initiation of nodule organogenesis. We find that infection via the root hair is determined by both common and specific *nod* loci. Root hair curling, already known to require the two common *nod* transcription units (18, 30), is also determined by at least the two specific transcription units, which we call IIIa (*nodFE*) and IIIb (*nodH*): *nodH* exerts a positive control on curling in the homologous host alfalfa, whereas both *nodFE* and *nodH* exert a negative control on curling in the heterologous host clover. The *nodFE* transcription unit is required for infection thread development within the alfalfa root hair. In contrast, induction of nodule organogenesis seems to be determined by common *nod* genes alone.

(Preliminary results have been presented at the 6th International Symposium on Nitrogen Fixation, Corvallis, Ore., 4–10 August 1985.)

MATERIALS AND METHODS

Genetic methods. Bacterial strains and plasmids used in this study are shown in Table 1. Conditions used for bacterial growth and conjugation experiments have been described elsewhere (40). Selection for Tc^r plasmid transfer (pGMI71, pGMI149, pGMI515, pRmSL26, and pIJ1089) was done at 10 μg/ml for *Escherichia coli* and *Rhizobium* spp. and 4 μg/ml for *A. tumefaciens*. Selection of transposon Tn*5* was done in *E. coli* with kanamycin (30 μg/ml) and in *R.*

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TABLE 1. Bacteria, bacteriophages, and plasmids used in this study

Bacterial strain, bacteriophage, or plasmid	Relevant characteristics	Source or reference
E. coli		
ED8767	*supE supF hsdS met recA56*	21
HB101	*ara gal lac met pro xyl supE rpsL* (Str^r) *recA hsdR hsdM*	28
LC916	*pro recA thy thi* Rif^r	M. Chandler
C600	*thr leu rpsL hsdR*	21
A. tumefaciens		
C58C1	Derivative of C58 cured of its Ti plasmid	31
GMI9050	Spontaneous Str^r Rif^r derivative of C58C1	40
R. meliloti		
RCR2011 = SU47	Wild-type; Nod$^+$ Fix$^+$ on *M. sativa*	31
GMI255	Nod$^-$ Δ(*fix-1074 nod nifHDK*)*7125* (Tn*5*) Nal^r Nm^r	40
GMI766	Nod$^-$ Δ(*nod nifA*)*766* Spc^r	T. Huguet (this laboratory); 40
GMI357	Nod$^-$ Δ(*nodDABC*) *HG.01* Nm^r Rif^r	T. Huguet
Rm6026	Rif^r *recA*::Tn*5-233* (Gm^r Spc^r)	E. Signer
R. trifolii		
ANU843	Nod$^+$ Fix$^+$ on white clover	9
GMI9457	Spontaneous Rif^r derivative of ANU843	This work
R. leguminosarum 3688	*phe trp rif str* Δ(*nod nifHDK fix*)*6007*(pRL1JI), Nod$^+$ Fix$^+$ on pea and *Vicia hirsuta*	11
R. phaseoli 8401(pRL1JI)	Derivative of *R. phaseoli* 8002, cured of pSym and carrying the pRL1JI plasmid from *R. leguminosarum*; Sm^r; Nod$^+$ Fix$^+$ on pea and *Vicia hirsuta*	10
IncP1 plasmids		
RP4	Tc^r Ap^r Km^r Tra$^+$	6
pR751-pMG2	Gm^r Tra$^+$	20
pGMI71	RP4-prime (in vivo); 70-kb insert of pSym2011 with Tn*5* insertion 20 in the *nif* region; Tc^r Ap^r Nm^r	40
pGMI515	RP4-prime (in vitro); Tc^r Ap^r	40
pRK290	*ori*(RK2); Tc^r	8
pGMI149	pRK290-prime; Tc^r	This study
pRmSL26	pLAFR1-prime; Tc^r	27
pIJ1089	pLAFR1-prime with *nod* genes from pRL1JI; Tc^r	10
Other plasmid		
pRK2013	Helper plasmid for mobilization of pRK290 derivatives; *tra*(RK2) *ori*(ColE1); Km^r	8
Bacteriophages		
N3	Transducing phage of *R. meliloti*	29
λ::Tn*5* strain 467	*b*221 *rex*::Tn*5* *c*I857 *O*am29 *P*am80	7

meliloti with neomycin (100 μg/ml) or streptomycin (100 μg/ml) or both. Scoring of Tn*5-233* in *R. meliloti* was done on LB agar with gentamicin (20 μg/ml) and spectinomycin (100 μg/ml). Antibiotics for counterselection of donors were used at the following concentrations: rifampin, 100 μg/ml; spectinomycin, 200 μg/ml; and streptomycin, 100 μg/ml.

Plasmid pR751-pMG2 (20) was used for eliminating IncP1 plasmid derivatives; its transfer was selected with gentamicin (20 μg/ml). Plasmid pR751-pMG2 is relatively unstable in *R. meliloti*; its elimination was obtained by growing bacteria in antibiotic-free LB broth. When the culture reached about 5×10^7 cells/ml, it was diluted 2,000 times in fresh medium. After three cycles of growth, bacteria were spread on LB agar, and Gm^s colonies were detected by replica plating. The frequency of clones having spontaneously lost pRm751-pMG2 was ca. 1%.

Cloning of the nodulation region. Partial *Eco*RI digests of plasmid pGMI71 were cloned into the *Eco*RI site of pRK290 (Tc^r), a broad-host-range cloning vehicle (8). After ligation, plasmids were used to transform *E. coli* C600 by the calcium chloride procedure (28). The pRK290 random insert population was then mobilized by triparental crosses with *E. coli* HB101(pRK2013) into two *R. meliloti* recipients carrying large *nod* deletions of pSym2011, GMI255 (Nm^r Str^r Nal^r), carrying a 280-kb deletion ending at the left of the *nifA* gene, and GMI766 (Spc^r), carrying a deletion of ca. 300 kb extending at least 70 kb to the right of the *nifHDK* operon (40). Tetracycline-resistant *R. meliloti* transconjugants were pooled in batches of about 1,000 colonies, suspended in sterile water, and inoculated on alfalfa seedlings to select for *R. meliloti* Nod$^+$ transconjugants by the procedure of Long et al. (27). Bacteria were reisolated from nodules as follows. Nodules were detached from roots and surface sterilized by immersion in 6% calcium hypochlorite for 5 to 15 min according to nodule size. Nodules were rinsed five times in sterile water and crushed; after suitable dilution, a 0.1-ml

sample was then spread on TY agar with and without the appropriate antibiotics. Tc^r bacteria isolated from nodules were purified, and their plasmid profile was determined by agarose electrophoresis (32). Plasmids were then extracted by the alkaline lysis procedure described by Kondorosi et al. (24).

Tn*5* mutagenesis of nodulation region. Plasmid pGMI149 was mutagenized in *E. coli* with Tn*5* insertion in two different ways: (i) pGM149 was introduced into strain ED8767, carrying a Tn*5* insert in the chromosome, and (ii) by infecting strain LC916(pGMI149) with the λ::Tn*5* phage 467 (3). Phage λ::Tn*5* stock preparation and infection were performed as described by de Bruijn and Lupski (7). After mutagenesis the *E. coli* strains carrying a population of various pGMI149::Tn*5* insertions were mated by a triparental cross involving *E. coli* HB101 carrying the mobilizing plasmid pRK2013 with an *R. meliloti* GMI766 Nod^- recipient. GMI766(pGMI149::Tn*5*) transconjugants (Tc^r Nm^r Str^r Spc^r) were individually tested for nodulation on alfalfa seedlings.

Each *nod*::Tn*5* insert was then introduced into the pSym of the wild-type RCR2011 strain by a modification of the method of Ruvkun and Ausubel (35). The transducing phage N3 was grown on each of the GMI766(pGMI49 *nod*::Tn*5*) clones as already described (29); lysates were used to transduce an RCR2011 recipient carrying the IncP1 plasmid pR751-pMG2, selecting for transductants containing Tn*5* (Nm^r Str^r) and pR751-pMG2 (Gm^r); loss of the pGMI149 vector was checked by loss of Tc^r; and curing of the chasing plasmid pR751-pMG2 was then done as described above.

That the *nod*::Tn*5* insert was introduced in the corresponding region of pSym2011 was verified by hybridization experiments. A radioactive Tn*5* probe was hybridized with *Hin*dIII-digested total DNA of transductants and with *Hin*dIII-digested DNA of the corresponding pGMI149 *nod*::Tn5 plasmids.

Complementation studies. For complementation tests, representative mutants in regions I and III, carrying a *nod*::Tn*5* insert (Nm^r Str^r) in pSym, were made Rec^- by introduction of a *rec*::Tn*5-233* mutation (Gm^r Spc^r) with the transducing phage N3. Phage N3 was grown on *R. meliloti* Rm6026, a Rif^r derivative of RCR2011 carrying a Tn*5-233* insert in the *recA* gene (G. DeVos, G. C. Walker, E. R. Signer, Mol. Gen. Genet., in press). Selection of transductants was done on LB agar with gentamicin (20 μg/ml); transductants were then checked for the Tn*5* (Nm^r) and Tn*5-233* (Spc^r) unselected markers, and their Rec^- phenotype was checked by UV sensitivity. The second *nod*::Tn*5* mutation was then introduced by mating with a suitable pGMI149 *nod*::Tn*5* plasmid; selection was done on L agar with tetracycline (10 μg/ml) and gentamicin (20 μg/ml). The number of nodules was scored on 20 seedlings of alfalfa for each treatment 7 days after inoculation: complementation was considered positive when more than 10 nodules were detectable, and negative when no nodule could be seen. Mutations which do not complement define a complementation group.

General DNA techniques. Purification, restriction enzyme digestion, gel electrophoresis, and hybridization of DNA were performed as described previously (40). Bacterial trasformations were done by the calcium chloride method (28).

Plant assays. For alfalfa (cv. Gemini) and white clover (cv. Ladino), the source of seeds and growth conditions for nodulation tests (macroscopic scoring) and for microscopic observation of the root system have been described (40). The whole root system of a minimum of 12 plants was observed. Observations of methylene blue-stained specimens were made at 5, 7, and 10 days after inoculation, as described previously (43). Light and electron microscopy of root deformations were performed as described previously (42), with the following modifications: roots were fixed in a solution of 25% glutaraldehyde and 0.15 M sodium cacodylate (1:8, vol/vol) for 1.5 h, washed in 0.3 M sodium cacodylate, and postfixed in 4% osmium tetroxide in 0.6 M sodium cacodylate (vol/vol) for 2 h at room temperature. After dehydration with acetone, specimens were embedded in Spurr medium.

Seeds of *Neptunia oleracea* were a gift from Bernard Dreyfus (ORSTOM, Senegal). For nodulation tests, seeds were surface sterilized by concentrated sulfuric acid for 30 min, washed twice with sterile water, immersed in 6% calcium hypochlorite for 20 min, and rinsed five times with sterile water. Seeds were germinated in soft C agar medium (4) for 24 h at 30°C. Seedlings were aseptically grown in test tubes on Jensen nitrogen-deficient agar slants (45) in a controlled environment cabinet at 27°C, with 16 h of lighting per 24-h cycle. Each seedling was inoculated with 5 ml of bacterial suspension in Jensen liquid medium diluted four times.

RESULTS AND DISCUSSION

Cloning of alfalfa nodulation genes. pGMI71 is a derivative of plasmid RP4 that contains a 70-kb insert of the pSym megaplasmid of *R. meliloti* RCR2011. This plasmid restores the Nod^+ phenotype of *R. meliloti* Nod^- mutants carrying large deletions of pSym extending over more than 360 kb (40). Moreover, *A. tumefaciens* GMI9050(pGMI71) transconjugants induced the formation on alfalfa, although not on clover, of genuine nodules having a cortical origin, an apical meristem, and peripheral vascular bundles and endodermis. Thus, pGMI71 carries the genetic information for triggering nodule formation on the homologous host. pGMI71 DNA was used for cloning host-specific *nod* genes. Partial *Eco*RI digests were cloned in *E. coli* into the *Eco*RI site of pRK290, a broad-host-range cloning vehicle. The pRK290 random insert population was then mobilized into two *R. meliloti* Nod^- recipients (GMI255 and GMI766) carrying large deletions of pSym. *R. meliloti* exconjugant populations were inoculated en masse on alfalfa seedlings to select for *R. meliloti* Nod^+ transconjugants by the procedure of Long et al. (27). Bacteria isolated from nodules carried pRK290 inserts of various sizes, but these plasmids always included *Eco*RI fragments of 8.7, 1.8, 1.2, 2.1, and 15.3 kb, regardless of whether the recipient was GMI255 or GMI766. These fragments were adjacent on the *Eco*RI map of the corresponding pSym region (Fig. 1) (2, 27). One of the smallest insert clones, pGMI149 (29.1 kb; Fig. 1), was used in further experiments.

A. tumefaciens GMI9050(pGMI149) transconjugants induced the formation on alfalfa roots of empty nodules of cortical origin, which showed that pGMI149 contained the sequences required for alfalfa nodule initiation. Plasmid pGMI1149 was then transferred into strains of three *Rhizobium* species. *R. leguminosarum* 3688 and *R. phaseoli* 8401(pRL1JI) nodulate pea and *R. trifolii* ANU843 nodulates clover, but none of the three has ever been observed to nodulate alfalfa. However, in each case the pGMI149 transconjugants induced abundant root deformations on alfalfa (Table 2). Cytological studies showed that these were genuine alfalfa nodules with an apical meristem, peripheral vascular bundles, and endodermis (Fig. 2A). Marked root hair

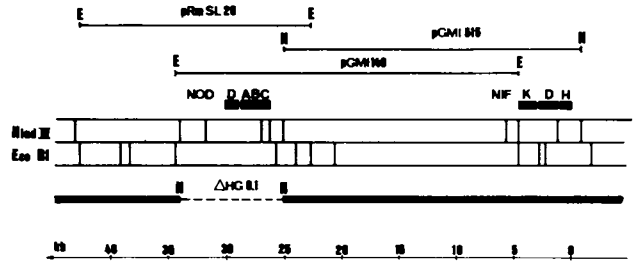

FIG. 1. Map of nodulation region of pSym2011: *Hin*dIII (H) and *Eco*RI (E) physical maps. Above the map are shown the recombinant plasmids pRmSL26, pGMI149, and pGMI515. Below the map, the ΔHG.01 site-directed deletion of the common *nod* region is represented by an interrupted line. On the scale the coordinates (in kilobases) are given from the promoter of the *nifHDK* operon as localized by Sundaresan et al. (38). The *nodDABC* genes are located according to Jacobs et al. (19) and Egelhoff et al. (12).

TABLE 2. Symbiotic behavior on alfalfa of *Rhizobium* species carrying plasmid pGMI149

Strain	Phenotype[a]		No. of nodules[b]
	Hac	Inf	
R. meliloti RCR2011	+	+	93
R. trifolii GMI9457	−	−	0
R. trifolii GMI9457(pGMI149)	+	+	95
R. leguminosarum 3688	−	−	0
R. leguminosarum 3688(pGMI149)	+	+	16
R. phaseoli 8401(pRL1JI)	−	−	0
R. phaseoli 8401(pRL1JI, pGMI149)	+	+	61

[a] Symbiosis phenotypes according to Vincent (46): Hac, marked hair curling (presence of shepherd's crooks); Inf, infection thread formation.
[b] Ten tubes counted; nodules were scored 3 weeks after inoculation.

curling and formation of infection threads within root hairs were normal (Fig. 2B), but the central tissue of nodules was free of bacteria (Fig. 2C). Therefore, these *Rhizobium* spp. carrying pGMI149 seemed to induce plant cell dedifferentiation, proliferation, and nodule organogenesis from a distance. This has already been reported for *A. tumefaciens* hybrids carrying *R. meliloti* pSym genes as well as for certain *R. meliloti* mutants, which either cannot be released from infection threads into the host cytoplasm (41) or cannot form infection threads and consequently remain intercellular in the superficial cortex (13, 15, 17, 25, 42, 47). Plasmid pGMI149 thus carries not only the genes required for nodulation, but also genes that determine specificity for alfalfa infection; both types of genes can be expressed in other *Rhizobium* species.

Tn*5* mutagenesis of the nodulation region. To define nodulation regions present in the 29.1-kb pSym fragment cloned in pGMI149, we first mutagenized this plasmid with transposon Tn*5* in *E. coli*, using either a Tn*5* insertion in the *E. coli* chromosome or a λ::Tn*5* bacteriophage. We then mobilized the resulting pGMI149::Tn*5* insertions en masse by a triparental cross into the Nod⁻ deletion strain *R. meliloti* GMI766. Individual GMI766(pGMI149::Tn*5*) transconjugants were tested for nodulation of alfalfa. Among 1,000 transconjugants isolated from seven independent experiments, 61 were found to be altered in the nodulation process, either completely defective (Nod⁻) or delayed (Nod^d).

Each putative *nod*::Tn*5* insert was then introduced to the corresponding site on the pSym of the wild-type RCR2011 strain by a modification of the method of Ruvkun and Ausubel (see Materials and Methods). In particular, we cured each resulting strain of the IncP1 plasmid used to remove the pGMI149 vector, to avoid any possible complication from presumptive bacterial surface changes.

Figure 3 shows a restriction map of the nodulation region contained in plasmid pGMI149, including *Eco*RI and *Hin*dIII sites already reported (2, 14, 19, 40) and *Bam*HI and *Pst*I sites determined in this work. Localization of Tn*5* inserts was achieved by digesting the mutated pGMI149 *nod*::Tn*5* plasmids with various combinations of *Pst*I, *Eco*RI, *Bam*HI, and *Hin*dIII. The Tn*5* inserts associated with nodulation defects were all located within approximately 16 kb of the pSym DNA cloned in pGMI149 and clustered in three regions. Region I (3.5 kb) included the common *nodDABC* region distal to *nifHDK* already reported (12, 14, 19, 27); region II, including inserts in two clusters (IIa and IIb) over about 3.5 kb, was in the center of the cloned region; region III, also including inserts in two clusters (IIIa and IIIb) over about 3 to 3.5 kb, was proximal to *nifHDK* (Fig. 1 and 3). In *R. meliloti* RCR2011, as in strain 41 (22, 24), *nod* genes are distributed over about 16 kb, whereas in *R. leguminosarum* (10) and *R. trifolii* (9, 18) they are clustered within about 10 kb.

The mutants fell into two distinct phenotypic classes, Nod⁻ (no nodules even at 45 days after inoculation) and Nod^d (no nodules at 8 days but nodules at 15 days). At 20°C, region I mutants were all Nod⁻, region II mutants were all Nod^d, and region III mutants included both Nod^d (IIIa) and Nod⁻ (IIIb) phenotypes. But at a higher temperature (27°C), whereas region I mutants were Nod⁻, some region IIIb mutants elicited the formation of a small number of delayed nodules (for example, with insertion 2313, 2219, or 148). Figure 4 shows the nodulation kinetics of the Nod^d mutants. Region IIIa mutants had a longer delay than region II mutants. In general, delay was more pronounced when the *nod*::Tn*5* insert was in plasmid pGMI149, in the pSym-deleted strain GMI766, than when it was located in wild-type pSym. This suggests that the pSym deletion of GMI766 has caused the loss of a gene(s) playing a role in the kinetics of nodulation. For both region II and region IIIa Nod^d mutants, the nodules had unambiguous nitrogenase activity (Nod^d Fix⁺).

Common and specific nodulation regions. Plasmid pIJ1089, a pRK290 derivative carrying the *nod* genes of *R. leguminosarum* required for nodulation of *Pisum sativum* (pea) and *Vicia hirsuta*, was introduced into several of the mutants to test whether heterologous *nod* genes could complement the mutant lesions. Complementation was scored from 5 to 25 days after inoculation by comparison of nodulation kinetics (Table 3). Normal nodulation of alfalfa was restored for region I mutants, as well as for the Nod⁻ strain GMI361 carrying a deletion (ΔHG.01; see Fig. 1) of region I, confirming the previous finding by Fischer et al. (14) of a functional complementation between the *nodABC* genes of strain RCR2011 and those of another *Rhizobium* species (*R. trifolii*). In contrast, normal nodulation of alfalfa could not be restored for any region III mutants (Table 3). Hence, in strain RCR2011, as proposed by Kondorosi et al. (22, 24) for *R. meliloti* 41, a cluster of host-specific nodulation (*hsn*) genes lies between *nodDABC* and the *nifHDK* operon.

Because this nodulation delay of region II mutants (when *nod*::Tn*5* inserts were located in a wild-type pSym) was very limited (Fig. 3), the results of complementation experiments

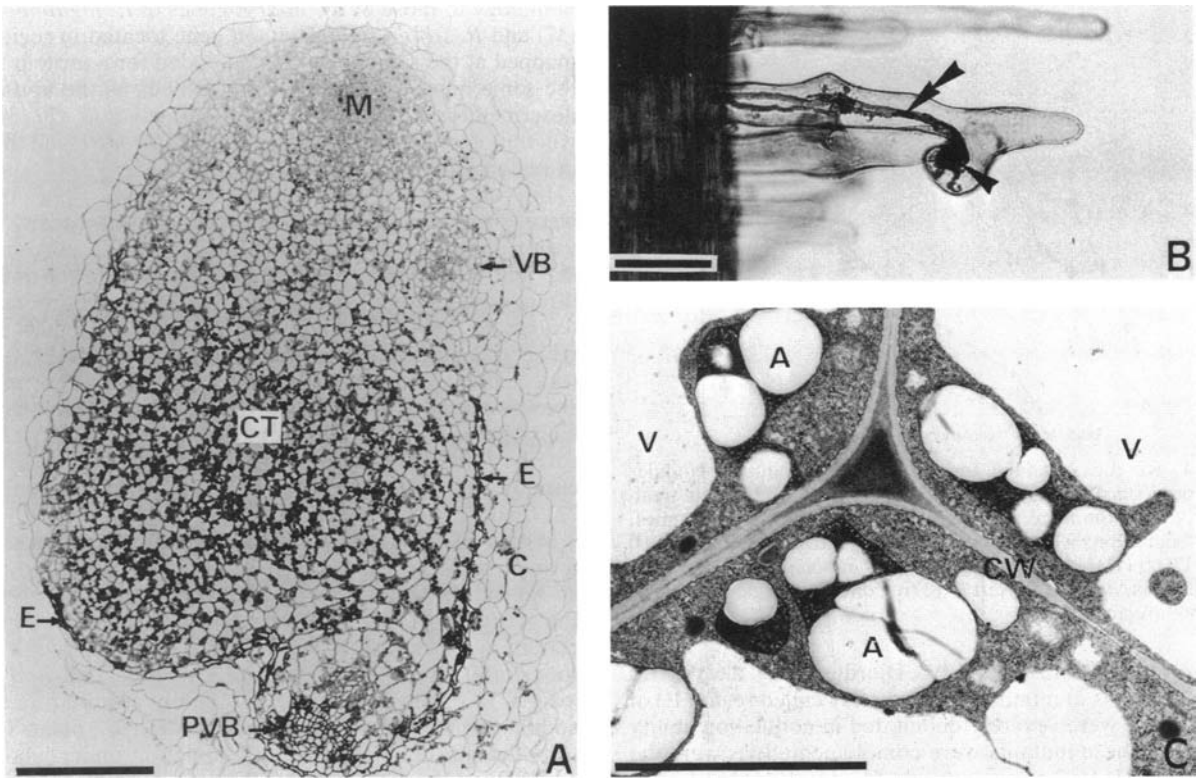

FIG. 2. Nodulation of alfalfa by *R. trifolii* transconjugant GMI9457(pGMI149). (A) Longitudinal section of a nodule prepared for microscopy 16 days after inoculation. An apical meristem (M), a peripheral endodermis (E), and peripheral vascular bundles (VB) are seen. The bacterium-free central tissue (CT) is filled with amyloplasts. C, Cortex; PVB, plant vascular bundles. Bar, 500 μm. (B) Infection process on agar-grown seedlings observed 5 days after inoculation and stained with methylene blue. The infection thread is seen within a root hair. Note the continuity between the hyaline spot (arrow) and the thread itself (double arrow). Bar, 50 μm. (C) Transmission electron microscopy of three adjacent nodular cells of the central tissue. The bacterium-free cytoplasm is reduced to a thin layer against the cell wall (CW). A, Amyloplast with starch granules; V, vacuole. Bar, 5 μm.

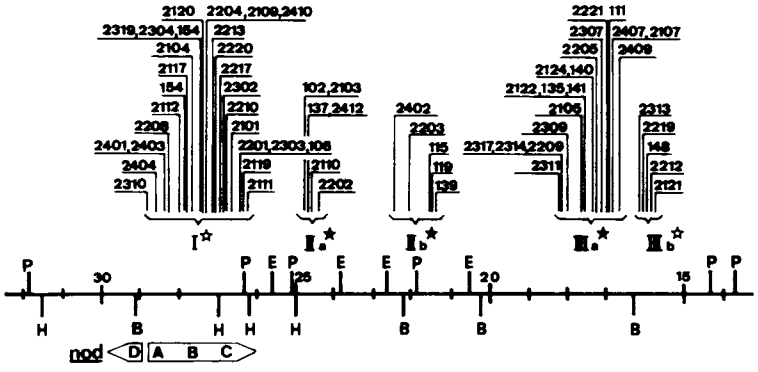

FIG. 3. Physical and genetic map of the *R. meliloti* RCR2011 nodulation region. Map of Tn*5* inserts in the nodulation region contained in pGMI149. Position of insert is shown by a verticle line; the number of the mutation is listed horizontally at the top of the line. Symbols: open stars, Nod$^-$ phenotype; solid stars, Nod^d phenotype (nodulation delay). Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. See the legend to Fig. 1.

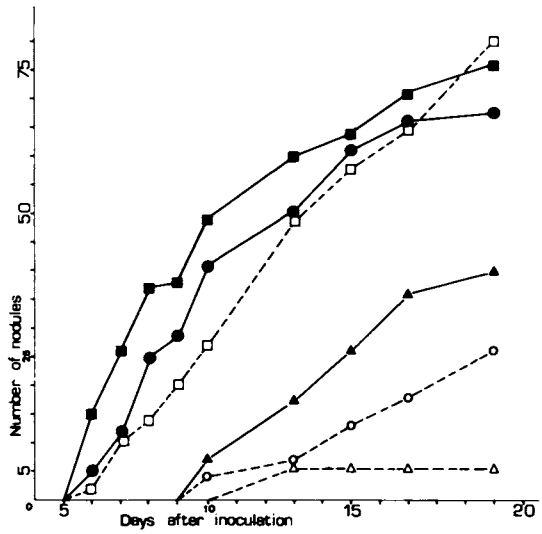

FIG. 4. Kinetics of nodule formation of Nod^d mutants. Nodules were scored on *Medicago sativa* cv. Gemini (10 tubes per treatment). Nodule number represents the number per 10 tubes. *nod*::Tn*5* mutations were tested in strains RCR2011 (solid symbols) and GMI766(pGMI149) (open symbols). Symbols: squares, control strains; circles, *nod*::Tn*5* insert 2412 (region II); triangles, *nod*::Tn*5* insert 2309 (region IIIa).

were not significant. Recently, Djordjevic et al. (9, 18) described a class of mutants (in what they called region II) of *R. trifolii* that were severely debilitated in nodulation ability on clovers. These mutants were complemented, as were the *R. trifolii nodDABC* mutants (region I), by the recombinant *R. meliloti* plasmid pRmSL26, suggesting the existence of common *R. meliloti nod* genes other than *nodDABC* in the pSym *Eco*RI fragments cloned in pRmSL26 (Fig. 1). Plasmid pRmSL26 carried region IIa (Fig. 1 and 3), but whether the *R. meliloti* genes that complement *R. trifolii* region II are the ones we have identified remains to be determined.

Complementation studies. In an attempt to define *nod* transcription units, we constructed strains merodiploid for the nodulation region and containing pairs of *nod*::Tn*5* inserts. First, mutants carrying a *nod*::Tn*5* insert (Nm^r Str^r) in pSym were made Rec$^-$ by introduction of a *rec*::Tn*5-233* (Gm^r Spc^r) insert; cytological studies showed that the introduction of the *rec*::Tn*5-233* insert into various mutants did not detectably change their symbiotic properties (data not shown). Then, a second *nod*::Tn*5* insert was introduced on plasmid pGMI149 (Tc^r). Nodulation phenotype was scored early (between 5 and 10 days after inoculation) to avoid the background of nodule formation by Nod^d mutants. Within region I (common *nod*), two mutations in genes *nodA* and *B* or *nodB* and *C* did not complement, confirming genetically that *nodABC* genes belong to the same transcription unit (12, 19). Mutations from regions I and III did complement. Within region III, mutations in region IIIa clearly complemented mutations in region IIIb; in contrast, no clear complementation could be detected between two mutations in region IIIa or between two mutations in region IIIb, suggesting that regions IIIa and IIIb correspond to two different transcription units.

In a separate work (6a), the complete nucleotide sequence of region III (6 kb) was determined. All the *nod*::Tn*5* inserts were located within three open reading frames (ORFs). The sequence data were in agreement with complementation studies. The two *nod* ORFs located in region IIIa probably belong to the same transcription unit, whereas the ORF located in region IIIb is transcribed divergently. The DNA sequences of the two ORFs of region IIIa showed strong homology to those of the *nodFE* genes of *R. leguminosarum* (37) and *R. trifolii* (36). The *nod* gene located in region IIIb mapped at the same location and coded for a protein having the same number of amino acid residues as the *nodH* gene described for *R. meliloti* 41 by Kondorosi et al. (22, 34). We will thus call the two genes in region IIIa *nodFE* and the gene in region IIIb *nodH* (6a).

Infection phenotypes on alfalfa. Twenty-eight mutants were characterized cytologically by light microscopy observation of the root system of alfalfa seedlings. Methylene blue staining was used to facilitate observation of infection threads (43) (Table 4). All the Nod$^-$ mutants in region I (common *nodABC*) showed no marked root hair curling (Hac$^-$), in agreement with previous studies (16, 19, 27); the root hairs were straight (Fig. 5A). *nodH*::Tn*5* mutants showed patterns of hair distortions ranging from slight root hair deformations (Had$^+$) for insertions 2212 and 2121 located in the central part of *nodH* (6a) to the presence of curled hairs without bright spots (Hac^s) for insertions 2313, 2219, and 148 (Fig. 3 and 5B).

With Nod^d Fix$^+$ mutants in region II, shepherd's crook formation was slightly delayed; this delay was followed by an unusually large number of shepherd's crooks (Hac^{++}; Fig. 5C) and of infection threads which could produce, in certain mutants such as GMI5518, a kind of network in the root cortex (Inf^{++}; Fig. 5C and D). Note that *nod* genes located in region II were not absolutely required for alfalfa nodule formation since their inactivation by polar Tn*5* inserts did not drastically affect nodulation under laboratory conditions. Nevertheless, by their control of infection efficiency they might play an important role in competition with other rhizobia in natural and agricultural environments, a possibility we are currently exploring. Nod^d mutants of *R. trifolii* (region II), which had a similar Hac^{++} phenotype but formed short and truncated infection threads, were comple-

TABLE 3. Interspecific complementation of *nod*::Tn*5* mutations for alfalfa nodulation

Strain	*nod*::Tn*5* mutation[a]	*nod* region	No. of nodules on alfalfa[b]	
			No plasmid	pIJ1089[c]
GMI5386	2310	I	0	30
GMI5382	2208		0	21
GMI5384	154		0	23
GMI5383	2304		0	30
GMI5387	2217		0	24
GMI5390	2412	II	27	41
GMI5392	2402		26	28
GMI5388	115		36	31
GMI5381	2309	IIIa	20	20
GMI5393	135		23	22
GMI5380	2205		19	17
GMI5378	2407		24	28
GMI5375	2121	IIIb	0	0
GMI5376	2212		0	0
RCR2011			30	30

[a] *nod*::Tn*5* mutations were present in the *R. meliloti* RCR2011 wild-type strain.
[b] Scored in 10 tubes 25 days after inoculation.
[c] Plasmid pIJ1089 contains the nodulation genes of *R. leguminosarum*.

TABLE 4. Infection phenotypes on alfalfa and clover

Strain	*nod*::Tn*5* mutation[a]	*nod* region	Alfalfa[b]				Clover[b]		
			Had	Hac	Inf	Nod	Had	Hac	Inf
RCR2011			+	+	+	+	+	−	−
GMI5386	2310	I (*nodABC*)	−	−	−	−	−	−	−
GMI5382	2208		−	−	−	−	−	−	−
GMI5384	154		−	−	−	−	−	−	−
GMI5383	2304		−	−	−	−	−	−	−
GMI5387	2217		−	−	−	−	−	−	−
GMI5389	2303		−	−	−	−	−	−	−
GMI5390	2412	II	+	+	+	+d	+	−	−
GMI5514	2110		+	+	+	+d	+	−	−
GMI5517	2202		+	++	++	+d	+	−	−
GMI5392	2402		+	++	++	+d	+	−	−
GMI5518	2203		+	++	++	+d	+	−	−
GMI5388	115		+	+	+	+d	+	−	−
GMI5513	119		+	++	++	+d	+	−	−
GMI5515	2122	IIIa (*nodFE*)	+	+	−	d	+	+	−
GMI5394	2311		+	+	−	d	+	+	−
GMI5519	2209		+	+	−	d	+	+	−
GMI5381	2309		+	+	−	d	+	+	−
GMI5393	135		+	+	−	d	+	+	−
GMI5380	2205		+	+	−	d	+	+	−
GMI5521	2307		+	+	−	d	+	+	−
GMI5520	2221		+	+	−	d	+	+	−
GMI5512	111		+	+	−	d	+	+	−
GMI5378	2407		+	+	−	d	+	+	−
GMI5431	2313	IIIb (*nodH*)	+	s	−	−	+	s	−
GMI5429	2219		+	s	−	−	+	s	−
GMI5430	148		+	s	−	−	+	s	−
GMI5376	2212		+	−	−	−	+	s	−
GMI5375	2121		+	−	−	−	+	s	−

[a] *nod*::Tn*5* mutations were present in the *R. meliloti* RCR2011 wild-type strain.

[b] Abbreviations are used to describe the infection phenotypes (9, 40, 46). Had, All root hair distortions (branching, bulging, moderate curling, swelling, waviness) except marked hair curling. Hac, Marked root hair curling (shepherd's crook), with three mutant phenotypes: Hac$^-$, no marked hair curling; Hac^{++}, proportion of hair curling unusually high; Hac^s, marked root hair curling but bright refractile spot only rarely present in the middle of the crook. Inf, Infection thread formation within root hairs, with two mutant phenotypes: Inf$^-$, no infection thread; Inf^{++}, proportion of root hairs containing threads higher than that of wild-type control. Nod, Nodule formation, with three mutant phenotypes: Nod$^-$, no nodules; Nod^d, delayed nodulation, Nod^{+d}, slightly delayed nodulation (see Fig. 4).

mented by plasmid pRmSL26, carrying *nod* regions I and IIa of *R. meliloti* (9).

The Nod^d region IIIa (*nodFE*) mutants were Hac$^+$, forming genuine shepherd's crooks with bright refractile spots. However, in contrast to the wild type, at 5 days after inoculation no infection threads could be detected (Inf$^-$; Fig. 5E); after 10 days rare infection threads were observed in root hairs, and they could be seen at day 15 at the top of the rare and delayed nodules (Fig. 5F). With these Nod^d Fix$^+$ mutants, the nodules formed seemed to depend on the very rare infection threads. Thus, regions I (*nodABC*) and IIIb (*nodH*) appear to determine root hair curling, region IIIa (*nodFE*) to determine infection thread formation, and region II to determine the intensity and kinetics of root hair curling and infection thread formation.

Positive control of root hair curling on homologous host. Root hair curling of the homologous host alfalfa was determined by both regions I and IIIb. Region I has been shown by others to include two divergent transcription units, one containing *nodD* and the other *nodABC* (12, 19). Inserts in *nodABC* genes are Hac$^-$ and can be complemented for hair curling of the homologous host alfalfa by genes from heterologous rhizobia (14). Thus, common *nodABC* genes are absolutely required to trigger root hair curling.

Inserts in region IIIb (*nodH*) were Hac$^-$ or Hac^s on alfalfa, but they could not be complemented for hair curling of alfalfa by *R. leguminosarum* nodulation genes (data not shown). *nodH* thus probably controls specifically root hair curling of the homologous host. This was supported by the introduction of plasmid pGMI515, which carries *nod* regions II and III, into an *R. trifolii* ANU843 recipient. Whereas ANU843 was Hac$^-$ on alfalfa, ANU843(pGMI515) transconjugants formed typical shepherd's crooks on alfalfa (data not shown). Plasmids pGMI515 carrying Tn*5* insertions in regions II or III were introduced into ANU843; only ANU843(pGMI515::Tn*5*) transconjugants carrying Tn*5* inserts in *nodH* were Hac$^-$ on alfalfa. Thus, whereas *nodABC* are required absolutely for hair curling (19, 27), *nodH* acts as a positive determinant to control its specificity on the homologous host alfalfa.

Other *R. meliloti* genes are also involved in alfalfa root hair curling. *R. meliloti* mutants deficient in a wild-type exopolysaccharide were recently shown to be Hac$^-$ (13, 25). These *exo* mutations, which are not located on the pSym megaplasmid, could define common symbiotic loci having their functional equivalent in other *Rhizobium* species, since *R. leguminosarum* and *R. trifolii* carrying pGMI149 induced genuine shepherd's crooks and infection threads within alfalfa root hairs (Table 2, Fig. 2B).

Negative control of root hair curling on heterologous host. In a previous paper (40) we described the transfer of pGMI149 (*nod* regions I, II, and III), pRmSL26 (regions I and IIa), and pGMI515 (regions II and III) into *R. meliloti* GMI766 and *A. tumefaciens* GMI9050. Results showed that sequences located in region II or III negatively controlled the formation of shepherd's crooks on the heterologous host white clover. The infection phenotypes on white clover of

TABLE 5. Nodulation of *Neptunia oleracea*

Strain	*nod*::Tn*5* mutation[a]	*nod* region	No. of nodules[b]
GMI766			0
GMI766(pGMI149)			25
GMI5116	2310	I (*nodABC*)	0
GMI5136	2208		0
GMI5101	154		0
GMI5111	2304		0
GMI5145	2217		0
GMI5110	2303		0
GMI5182	2412	II	24
GMI5089	115		16
GMI5178	2402		27
GMI5117	2311	IIIa (*nodFE*)	19
GMI5175	2309		31
GMI5092	135		25
GMI5133	2205		19
GMI5179	2407		20
GMI5180	2409		30
GMI5140	2212	IIIb (*nodH*)	22
GMI5170	2121		19

[a] *nod*::Tn*5* mutations were studied in *R. meliloti* GMI766(pGMI149).

[b] For five plants 32 days after inoculation.

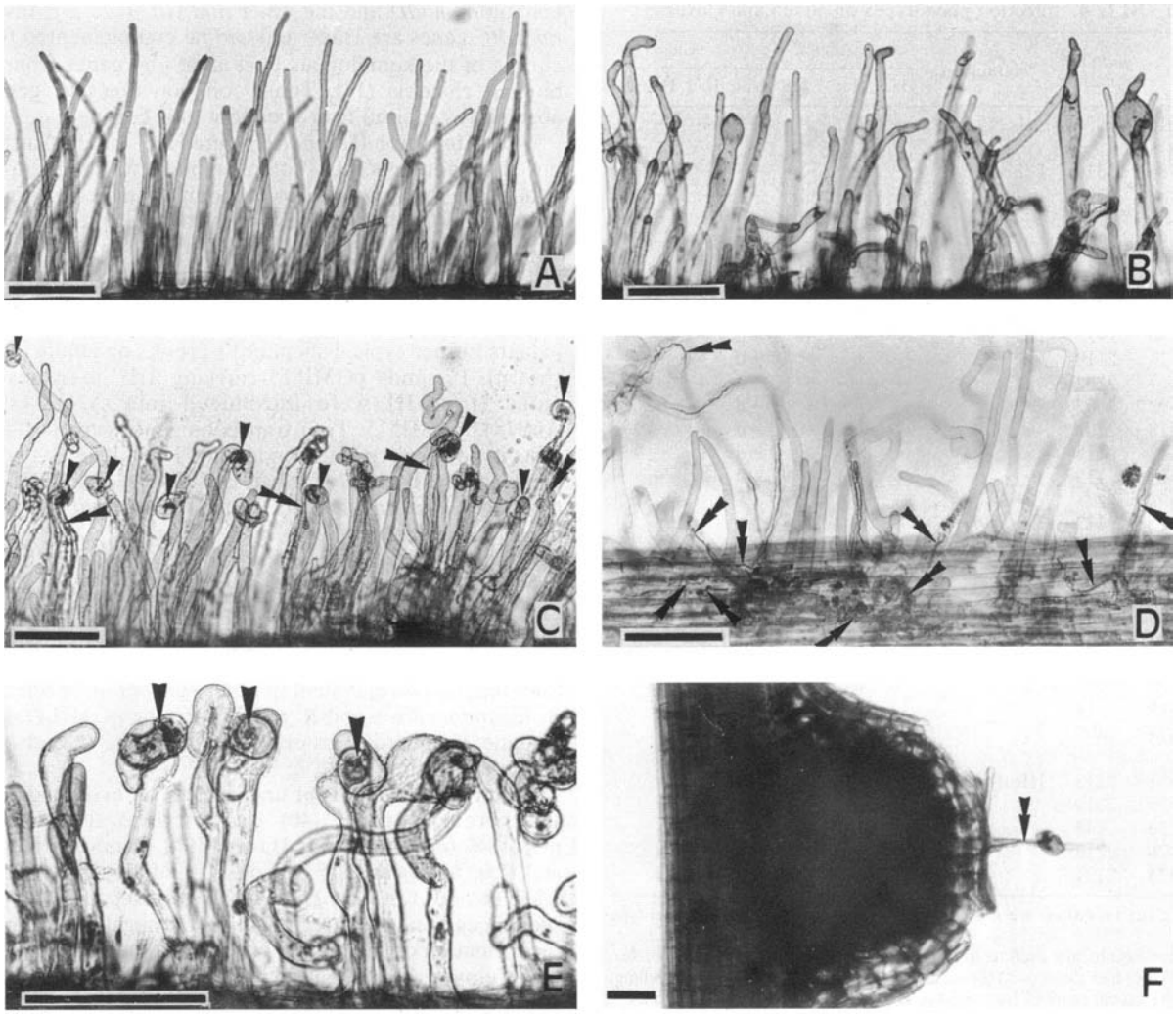

FIG. 5. Infection phenotypes on alfalfa root hairs of *R. meliloti* RCR2011 with various *nod*::Tn*5* inserts. (A) *nod*::Tn*5* insert 2208 (region I). Had$^-$ phenotype: 10 days after inoculation the root hairs remain straight. (B) *nod*::Tn*5* insert 2121 (region IIIb). Had$^+$ phenotype: no Hac$^+$ phenotype detected 10 days after inoculation. (C and D) *nod*::Tn*5* inserts in region II (2202 and 2203, respectively). Hac^{++} Inf^{++} phenotype. (C) Many shepherd's crooks (Hac^{++}, arrows) and infected root hairs (Inf^{++}, double arrows) observed 7 days after inoculation. (D) Overexposed micrograph showing the Inf^{++} phenotype. Numerous infection threads (double arrows) are seen through the root hairs or inside the root cortex (10 days after inoculation). (E and F) *nod*::Tn*5* inserts in region IIIa (2311 and 2205, respectively). (E) Hac$^+$ Inf$^-$ phenotype 5 days after inoculation: arrows point to shepherd's crooks. (F) Infection thread (double arrow) in a root hair at the top of a late-developing nodule, 15 days after inoculation. In all micrographs, the bar equals 100 μm.

the mutants described here are illustrated in Fig. 6 and summarized in Table 4. Region I and II mutants behaved like the wild-type: root hairs were straight or only slightly distorted (data not shown). In contrast, Tn*5* inserts in region III resulted in marked hair curling on the heterologous host: mutants carrying inserts in *nodFE* provoked the formation of genuine shepherd's crooks with a bright refractile spot (Hac$^+$; Fig. 6A and B), whereas mutants carrying inserts in *nodH* provoked abundant marked hair curling, with a bright spot only rarely present (Hac^s; Fig. 6C to E). This implies that at least two transcription units that negatively control root hair curling on the heterologous host are located in region III, which was shown above to include determinants of host specificity (see the model proposed in Fig. 7). *R. meliloti* GMI766, having a large deletion including the whole *nod* region and carrying the pRmLS26 plasmid (regions I and IIa), induced the formation of shepherd's crooks on white clover much more abundantly than region III *nod*::Tn*5* mutants (40). This suggests either that (i) regions IIIa and IIIb have a cumulative negative effect or (ii) other genes negatively controlling root hair curling on the heterologous host have been removed by the deletion.

Recently, a negative control of host range specificity was also reported for *R. trifolii*. Djordjevic et al. (9) described a class of mutants (called region III) that have extended host range, inducing pronounced root hair distortion, infection threads, and abundant nodules on the heterologous host *P. sativum* (pea). However, the region III mutants of *R. meliloti* reported here, although showing an extended host range for root hair curling, were nevertheless unable to elicit infection thread formation and nodulation on the heterologous host white clover.

Control of infection thread formation. The first visible sign of infection via root hairs is reported to be the formation of

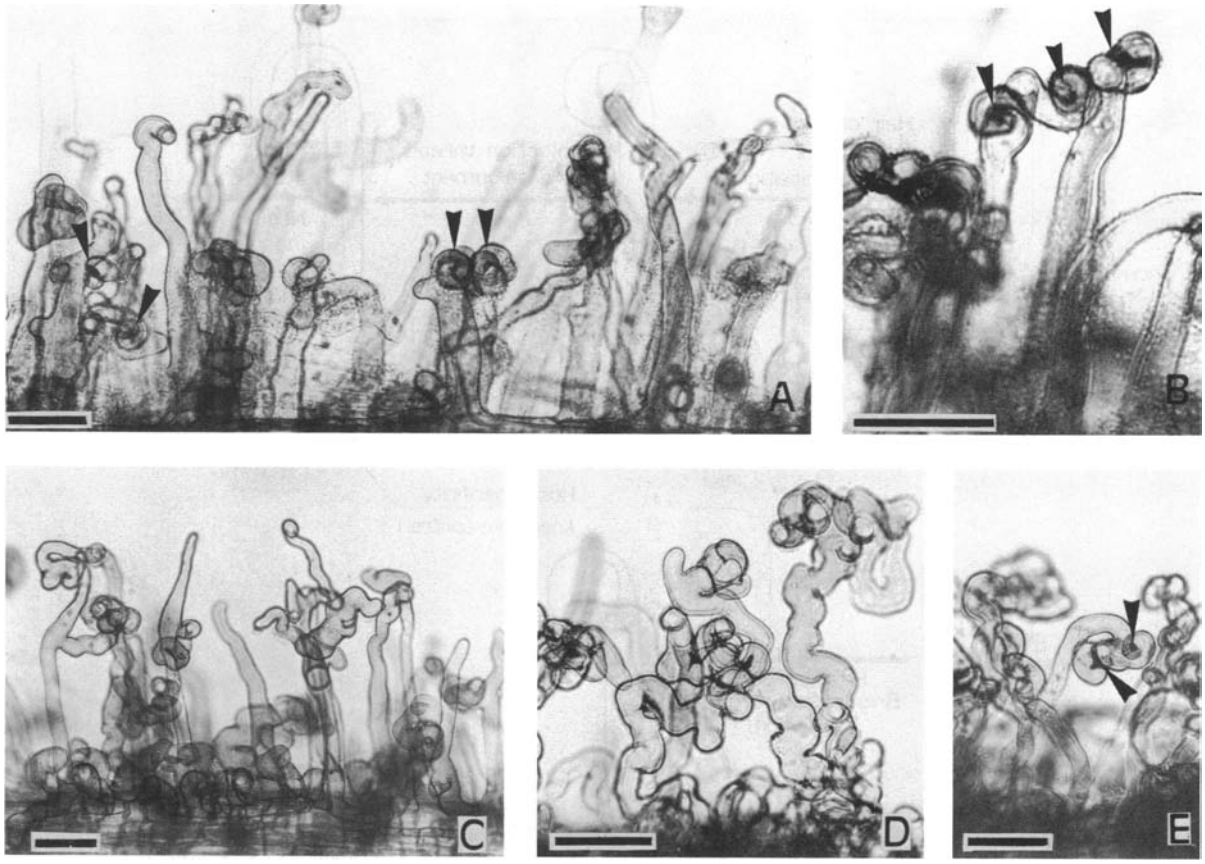

FIG. 6. Infection phenotypes on clover root hairs of *R. meliloti* RCR2011 with *nod*::Tn*5* inserts. (A and B). Hac$^+$ Inf$^-$ phenotype induced by mutations into *nod* region IIIa (insertions 2309 and 2311, respectively). (A) General view 5 days after inoculation; (B) detailed area of root hairs observed 10 days after inoculation. Arrows point to the Hac^{++} deformation (shepherd's crooks). (C and D). Hac^s phenotype incited by Tn*5* inserts into *nod* region IIIb (insertions 2219 and 2313, respectively). (C) General view 5 days afer inoculation; (D) detailed view 10 days after inoculation. Note that root hairs are more markedly deformed with the Tn*5* insert in region IIIb than in region IIIa; the sides of root hairs are twisted and the tips are highly curled (>>360°). (E) *nod*::Tn*5* insertion 2313 in region IIIb, more than 10 days after inoculation. By this time, a few genuine shepherd's crooks can also be detected (arrow). In all micrographs, the bar equals 50 μm.

a bright refractile spot in the middle of the shepherd's crook (5, 40, 46). Cytological observations of root hair sections showed the presence of bacteria within the bright spots, suggesting that the spot is the point at which rhizobia enter the root (40). In addition, a continuity between the bright spot and the infection thread can be observed, suggesting that the spot is the site of initiation of the infection thread (9, 43).

A. tumefaciens GMI9050(pRmSL26) and *R. meliloti* GMI766(pRmSL26) transconjugants elicit the formation, on white clover root hairs, of shepherd's crooks with a bright spot (40). Thus, bacterial penetration into the root hair and initiation of infection thread formation may be determined by *nod* region I and possibly IIa. On the other hand, Tn*5* inserts in region IIIa resulted in the formation of shepherd's crooks with a bright spot, but no infection thread could be observed in the root hairs (Hac$^+$ Inf$^-$): thus, the development of an infection thread from the bright spot, in alfalfa root hair, is determined by region IIIa (*nodFE*). This same transcription unit (IIIa) seems to positively control infection thread formation on the homologous host and also to negatively control root hair curling on the heterologous host (Fig. 7). Whether these two phenotypes are due to the same or different genes is not yet clear.

Control of induction of nodule organogenesis. *A. tumefaciens* GMI9050, cured of the Ti plasmid and carrying pGMI149, induced root nodules on alfalfa, presumably due to rhizobial sequences cloned in pGMI149. To identify the *nod* loci on pGMI149 that are directly involved in nodule induction, we used a system in which the first steps of infection are naturally short-circuited.

In a separate study (J. Vasse, B. Dreyfus, C. Rosenberg, S. Camut, J. Denarie, and G. Truchet, in preparation) it was shown that *R. meliloti* strains can induce nodule formation on the aquatic tropical legume *Neptunia oleracea*. The infection process seems to require neither shepherd's crooks nor infection threads in root hairs, but rather intercellular penetration of bacteria. The mutants described above were tested for nodulation of *N. oleracea*. Because *R. meliloti* GMI766(pGMI149), which carries a large *nod* deletion, induced more nodules than the wild type, the mutations were studied in this genetic background (Table 5). Mutants altered in region I (*nodABC*) were Nod$^-$ on *N. oleracea*. However, mutants of regions II and III, which were Nod^d or Nod$^-$ on alfalfa, were all Nod$^+$ on *N. oleracea*. Thus, whereas the *nodABC* genes are essential for nodule induction in *N. oleracea*, the *nod* loci in regions II and III, which above were implicated in the control of infection thread develop-

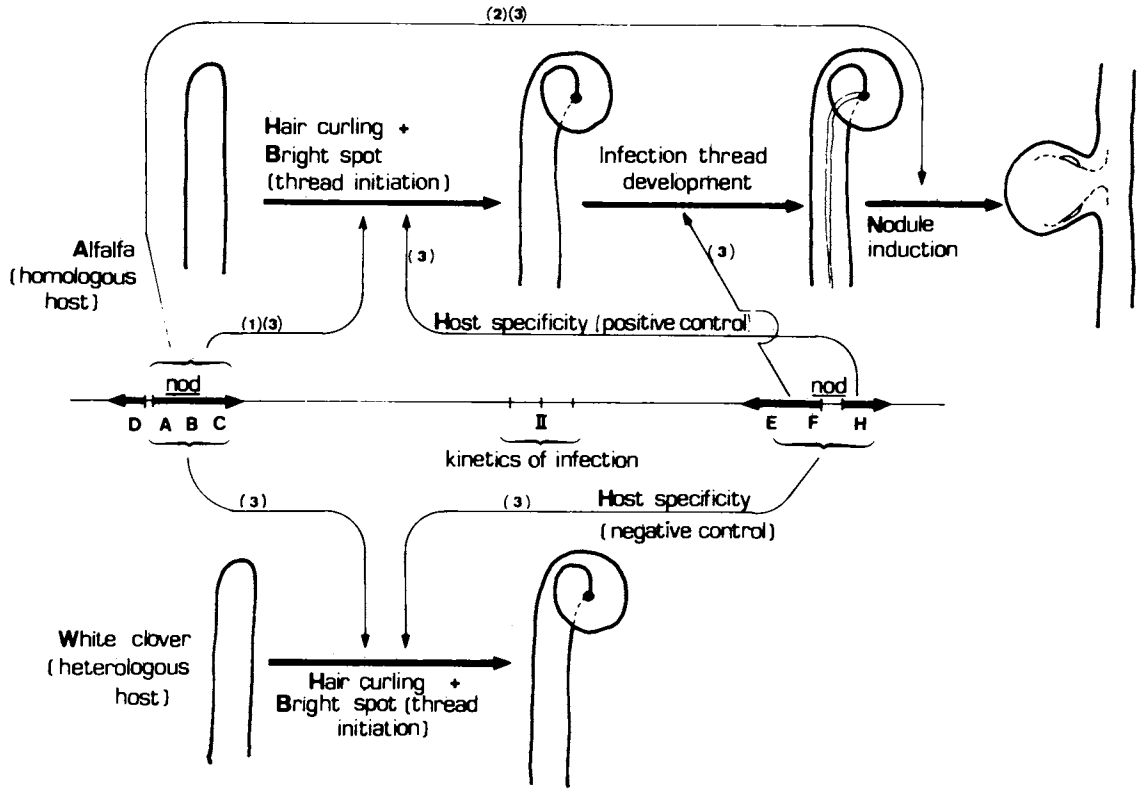

FIG. 7. Alfalfa infection and nodulation: assignment of development step control to common and specific *nod* loci of *R. meliloti*. Thick arrows represent development steps. Thin arrows represent genetic control. Interpretations from: 1, Jacobs et al. (19); 2, Hirsch et al. (15); 3, this work. Mapping and direction of transcription of *nodH* and *nodFE* genes are from F. Debellé and S. Sharma (6a).

ment in root hairs, are not required. This suggests that among the various *nod* loci cloned in pGMI149, the common *nodABC* genes are probably those directly involved in triggering nodule organogenesis (Fig. 7). This hypothesis is supported by the data on alfalfa nodulation described above. The only Tn*5* insertions which completely abolished alfalfa nodulation, at both low (20°C) and high (27°C) temperature, were those located in *nodABC*. If this hypothesis is true, then nodule induction would not be specific, since *R. meliloti* GMI357 and GMI361, deleted of the common *nod* genes (the 8.7-kb *Eco*RI fragment) and carrying instead the common *nod* genes of *R. leguminosarum*, induce the formation of genuine nodules on alfalfa (40).

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