

A New *Rhizobium meliloti* Symbiotic Mutant Isolated After Introducing *Frankia* DNA Sequence into a *nodA::Tn5* Strain

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Received 24 April 1991. Revised 16 September 1991. Accepted 7 October 1991.

Our goal was to isolate *Frankia* genes by complementation of *Rhizobium meliloti* mutants. By mating a library of *Frankia* genes in pLAFR3 with a *R. meliloti nodA::Tn5* recipient, we isolated a clone that appeared to confer nodulation functions, although the apparently complemented transconjugant (Rm5610 NS6) remained Fix^- on alfalfa. Further experiments have shown that the $\text{Nod}^+ \text{Fix}^-$ phenotype was due to genomic mutations in *R. meliloti* and was independent of the *Frankia* DNA. Rm5610 NS6 had apparently lost Tn5 and simultaneously acquired neomycin resistance. We subsequently isolated a spontaneous neomycin-resistant derivative of *R. meliloti* 1021 that appeared to be

identical to NS6. The nodules elicited by the neomycin-resistant mutants contained infection threads that penetrated the nodule; rhizobia were released from the threads, but they did not differentiate into elongate bacteroids. We examined nodulin gene expression in the ineffective mutant-induced nodules by northern blot and by *in situ* hybridization analyses and found that transcripts for the nodulins MsENOD2, MsENOD12-1, and leg-hemoglobin were detectable in the same cellular location as in wild type-induced nodules. These results confirm that the expression of these nodulin genes is regulated by signals exclusive of bacteroid differentiation and nitrogen fixation.

Nitrogen-fixing bacteria are taxonomically diverse and occupy various ecological niches. Some genera, including *Rhizobium*, *Frankia*, *Anabaena*, and *Nostoc*, live symbiotically with plants and induce the formation of new or altered structures on their plant hosts (Dixon and Wheeler 1986). *Frankia* and *Rhizobium* induce the formation of root nodules, which house the nitrogen-fixing bacteria. Because of the agricultural importance of the host legumes, considerable research has been carried out on the *Rhizobium*-legume symbiosis. The Rhizobiaceae have been well characterized genetically, and many of their symbiotic genes have been identified and sequenced (Long 1989). Similarly, a number of nodule-specific proteins, termed nodulins, have been characterized. Several nodulin cDNA as well as genomic clones have been isolated and sequenced (see Delauney and Verma 1988).

In contrast, the nitrogen-fixing symbiosis between *Frankia* and woody dicotyledons (actinorhizal) host plants has received little attention. Although not as intensively studied as the *Rhizobium*-legume association, this symbiosis is economically very important because actinorhizal trees are widely used in land reclamation and agroforestry. The *Frankia*-actinorhizal symbiosis also contributes greatly to biological nitrogen fixation worldwide (Torrey 1978; National Research Council 1984). Moreover, the broad host range of *Frankia* makes this plant-microbe association a significant one to study in terms of understanding the evolution of nitrogen-fixing symbioses. However, progress in *Frankia* research has been impeded by a number of factors (Simonet *et al.* 1989), including the bacteria's slow growth and filamentous habit. Although several laboratories have

used spores to initiate single cell-derived cultures (J. G. Torrey, Harvard University, personal communication), most *Frankia* cultures are started from inocula consisting of a group of filaments, with potentially varied genetic backgrounds. Furthermore, there are no methods developed to mutagenize *Frankia* or mediate genetic exchange. Alternative strategies to study *Frankia* genetics have not been formulated as of yet.

A number of similarities as well as differences are observed between the *Frankia*-actinorhizal association and the *Rhizobium*-legume symbiosis (see review by Newcomb and Wood 1987). The apparent similarity, especially during the establishment of the symbiosis—root hair curling, penetration of the bacteria into the host cell within a plant cell-derived matrix, induction of mitosis within the root cortex, and differentiation of the nodule—led us to pursue an investigation of the *Frankia* genes involved in the early stages of the interaction with the plant. In *Rhizobium* species, the common *nod* genes, which induce some of the early events, are conserved (see review by Long 1989). Weak hybridization of DNA isolated from some *Frankia* strains to *Rhizobium nod* genes suggested that DNA sequences homologous to *nod* genes may be found in *Frankia* (Drake *et al.* 1985). Because the usual methods for identifying genes (e.g., transposon mutagenesis and cloning of genetically marked DNA) were not available for use in *Frankia*, we attempted to develop an alternate strategy to study nodulation genes in *Frankia*. The strategy was to localize genes on the basis of function, using a method utilized previously by Marvel *et al.* (1985) to isolate *nod* sequences from *Bradyrhizobium*.

Using this strategy of cross-species complementation, we reported the isolation of a cosmid clone of *Frankia* DNA sequences that appeared to complement a *R. meliloti* Dangeard *nodA::Tn5* mutation (Reddy *et al.* 1988). In this report, we describe our subsequent analysis of the strain containing the cosmid clone. We conclude that the *Frankia*

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DNA is not responsible for the Nod⁺ phenotype of the apparently complemented strain, but rather its phenotype is the result of mutations in the *R. meliloti* genome. The mutant *R. meliloti* (Rm5610 NS6), which carries *Frankia* DNA sequences, arrests nodule development at the stage where bacteria are released from infection threads, but no bacteroid differentiation occurs. However, a persistent nodule meristem is established, and the nodule assumes the typical club-shaped form of an alfalfa nodule.

Few well characterized *R. meliloti* mutants block nodule formation at this stage. One is mutant SU47 *fix21*, which induces nodules that contain released, but undifferentiated bacteria (Norris *et al.* 1988). *R. meliloti* *exoH* mutants, which are characterized by exopolysaccharide that lacks succinoglycan residues, elicit the formation of bacteria-free nodules that sometimes contain infection threads within the interior of the nodule. A few bacteria are released from these infection threads, but bacteroids do not differentiate.

In this paper, we describe the nodule phenotype and nodulin gene expression in ineffective nodules elicited by Rm5610 NS6 and also by a spontaneous neomycin-resistant mutant of *R. meliloti* that appears to be phenotypically identical to Rm5610 NS6. Nodules elicited by these mutants express transcripts encoding the nodulins MsENOD2 (Dickstein *et al.* 1988) and MsENOD12-1 (M. Löbner and A. M. Hirsch, unpublished), as well as leghemoglobin (Lb). Moreover, Lb transcripts are found in these Fix⁻ nodules in a location comparable to that in wild type-induced nodules. The fact that these nodulins are expressed in the proper location in these ineffective nodules suggests that the cellular pattern of MsENOD2, MsENOD12-1, and Lb gene expression is independent of bacteroid differentiation and nitrogen fixation.

MATERIALS AND METHODS

Bacterial strains and media. The *Frankia* strain used was isolated at Harvard Forest from nodules of *Casuarina cunninghamiana* Miq. and is designated HFPCc13 (Zhang *et al.* 1984). *Frankia* DNA was ligated to the cosmid vector pLAFR3, which encodes tetracycline resistance (Tc^r) (Staskawicz *et al.* 1987), and the library was maintained in the ampicillin-resistant strain *E. coli* S17-1 (Priefer *et al.* 1985). The helper strain used in triparental matings was *E. coli* MM294A containing the plasmid pRK2013 (Figurski and Helinski 1979; Leigh *et al.* 1985). The *R. meliloti* strains used were: Rm1021, which is a streptomycin-resistant (Str^r) Nod⁺ Fix⁺ derivative of the wild type strain SU47; Nod⁻ strains Rm5610 *nodA::Tn5*, Rm5611 *nodB::Tn5*, and Rm5613 *nodC::Tn5* (Klein *et al.* 1988); rifampicin-resistant (Rif^r) *nod* deletion strains GMI357 Δ HG0.1, and GMI361 Δ HG4.1 (Truchet *et al.* 1985); Rm7055 *exoF::Tn5* Nod⁺ Fix⁻ (Leigh *et al.* 1985); and Phe⁻, Trp⁻, Tyr⁻, and Leu⁻ auxotrophs of Rm1021 generated by Tn5 mutagenesis. Tn5 confers resistance to neomycin sulfate. The neomycin-resistant (Nm^r) *R. meliloti* 1021-derived mutants, Rm5610 NS6 (and its Tc^r derivative NS6-1) and 1021N, were isolated as described in this study. *E. coli* LE392 carrying pRMJ30 was used to mobilize the *nodD1ABC* genes into the mutant strains (Jacobs *et al.* 1985).

Bacterial matings and phage transductions in which the recipient was a *R. meliloti* auxotrophic mutant were plated on a *Rhizobium*-defined minimal medium (RDM, Vincent 1970) containing the appropriate antibiotics. The rich medium used in various experiments was Luria-Bertani (LB) broth or agar (Maniatis *et al.* 1982). Antibiotics were used at the following final concentrations (μ g/ml): streptomycin, 250; neomycin, 35 in initial selection medium and 50 for subsequent colony purification; tetracycline, 5 for *R. meliloti* and 10 for *E. coli*.

Construction of *Frankia* HFPCc13 library. DNA was isolated from *Frankia* HFPCc13 by using a modification of the Marmur procedure (1961), in which cell lysis was enhanced by using the enzyme achromopeptidase (Simonet *et al.* 1984). The vector pLAFR3 was prepared following previously described procedures (Staskawicz *et al.* 1987), ligated to dephosphorylated HFPCc13 chromosomal DNA, and the packaged ligation mixture was used to transduce *E. coli* S17-1. From the initial number of Tc^r transductants selected, the library was calculated to be completely representational of the HFPCc13 genome, with an average insert size of 25 kb.

Bacterial matings and phage transductions. Triparental matings were performed essentially as described previously (Ruvkun and Ausubel 1981). As controls, each strain alone, or pairwise combinations of the strains were carried through the mating procedure in parallel with the triparental matings every time. Transconjugant colonies were plate-purified before they were used in further experiments.

The generalized transducing phage M12 was used to transduce genetic markers among *R. meliloti* strains using established procedures for propagating the phage and for transduction (Finan *et al.* 1984).

Screening for Nod⁺ *R. meliloti* transconjugants. *Medicago sativa* L. 'Iroquois' seeds were surface-sterilized as previously described (Hirsch *et al.* 1983) and planted aseptically in test tubes containing 10 ml of nitrogen-free Jensen's agar (Vincent 1970). About 500–1,000 cfu of the Tc^r Nm^r transconjugants from matings with *R. meliloti* Nod⁻ mutants were pooled in 3–5 ml of distilled water and used to inoculate 3-day-old alfalfa plants. Plants were inoculated with either 100, 200, or 500 μ l of the transconjugant mixture. For each mixture at least 10 tubes of plants were inoculated. Plants were observed for the formation of root nodules or hypertrophies every week until they were about 7 wk old. With each screen of transconjugants, controls were included of 5–10 plants, inoculated either with *R. meliloti* 5610 or 1021, or left uninoculated.

Nodule squashes. Nodules were surface-sterilized in sterile petri dishes by rinsing them in 95% ethanol for 30 s, 10% commercial bleach for 2 min, and 3 \times 5 min in sterile distilled water. Each nodule was squashed in 200 μ l of distilled water, and the suspension and dilutions were plated on LB medium. The plates were incubated for 3–5 days at 30 $^{\circ}$ C. The growth was then replica-plated to the appropriate selective LB medium to identify the bacteria.

DNA isolation and DNA:DNA hybridization. Total genomic DNA from *R. meliloti* strains was prepared using the Marmur procedure (1961). Plasmid extracts were made by the alkaline lysis procedure (Maniatis *et al.* 1982). Geno-

mic DNA for hybridization experiments was digested with *EcoRI* and blotted to Genescreen Plus membranes (DuPont-New England Nuclear, Boston, MA) using the method of Southern (1975). To detect *nod* and *nif* sequences, blots were probed with a 3.5-kb *BamHI-EcoRI* fragment of plasmid pEK12 containing *nodABC* (Kondorosi *et al.* 1984), and a 2.2-kb *BglII-EcoRI* fragment of plasmid pRmR2 (Ruvkun *et al.* 1982b) with *nifHD* genes. To detect Tn5 sequences, blots were probed with the 3.35-kb internal *HindIII* fragment of Tn5 isolated from the plasmid pGS220 (De Vos *et al.* 1986), and also with the DNA fragment containing the IS50L and IS50R sequences. The presence of the neomycin phosphotransferase type II (NPTII) gene from Tn5 was probed for by using an approximately 1-kb *BglII-SmaI* NPTII-containing fragment isolated from the plasmid pHP45 Ω (Fellay *et al.* 1987). Probe DNAs were labeled with ³²P-dCTP using a Pharmacia random primer labeling kit (Pharmacia Inc., Piscataway, NJ). Hybridizations were done using solutions prescribed by the manufacturer, and blots were washed at 60° C in 2× SSC with 1% sodium dodecyl sulfate (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate).

RNA isolation and northern blots. Root nodules were harvested 3–4 wk after inoculation, and total RNA was extracted from them as previously described (Goldberg *et al.* 1981). The poly(A)⁺ RNA fraction was isolated on oligo(dT)-cellulose columns (Maniatis *et al.* 1982). RNAs were subjected to electrophoresis on a formaldehyde gel and blotted to Schleicher & Schuell (Keene, NH) Nytran membranes using the manufacturer's instructions. The northern blots were probed with a ³²P-dCTP-labeled fragment of approximately 300 bp containing MsENOD2 isolated from the cDNA clone pA2ENOD2 (Dickstein *et al.* 1988), and a 509-bp cDNA fragment from the clone pMsENOD12-1 (M. Löbner and A. M. Hirsch, unpublished). Hybridization buffers containing formamide and dextran sulfate were used as recommended by Schleicher and Schuell. Hybridizations were performed at 37° C, and the blots were washed in 0.2× SSC with 1% sodium dodecyl sulfate at 50° C.

Microscopy and *in situ* hybridizations. Nodules were harvested from alfalfa roots 3–4 wk after inoculation with *R. meliloti* wild type or mutant bacteria. The nodules were fixed in formaldehyde-acetic acid-alcohol (FAA), dehydrated through a *tert*-butyl alcohol series, embedded in Paraplast, and prepared for *in situ* hybridization using ³⁵S-labeled UTP probes as described by van de Wiel *et al.* (1990). To detect *Lb* transcripts, the alfalfa *Lb* clone isolated by Dunn *et al.* (1988) was used. For the *in situ* experiments, the nodulin cDNA fragments were recloned into the pBS+ vector (Stratagene, La Jolla, CA) at the *SmaI* site.

RESULTS

Cross-species complementation analysis. We constructed a genomic library of *Frankia* strain HFPCc13 in pLAFR3. The cosmid library was mobilized by a RP4-mediated triparental mating system into various *R. meliloti* Tn5-generated mutants that were either auxotrophs or defective in nodulation. Preliminary results from matings with *R. meliloti* Phe[−], Trp[−], Tyr[−], and Leu[−] recipients indicated that it was possible to complement *R. meliloti* with *Frankia*

DNA sequences, because in all cases we obtained Tc^r Nm^r transconjugants that appeared to be prototrophic. However, restriction digests of plasmids isolated from the *R. meliloti* Phe[−] Nm^r recipients showed that there was no single *Frankia* DNA fragment that was common to all of the plasmids (data not shown).

The *Frankia* genomic library was mated into Nod[−] Rm5610 (*nodA::Tn5*); Tc^r Nm^r transconjugants were selected and screened on alfalfa seedlings for restoration of nodulation ability. After 4–6 wk, the majority of seedlings were not nodulated. However, 15% of the test tubes contained alfalfa plants that developed root nodules or hypertrophies. Tc^r Nm^r *R. meliloti* were recovered from nodules of plants from approximately half of these tubes. Each of these Tc^r Nm^r *R. meliloti* strains isolated from nodules was re-tested for its ability to induce nodules on alfalfa plants. One of the isolates reproducibly formed small, white, Fix[−] nodules on alfalfa plants at high frequency (Fig. 1). The isolate was named *R. meliloti* 5610 NS6 (Nodulating Strain 6) and was found to contain a cosmid clone with a 24-kb insert of *Frankia* DNA. This clone was named pAR1, and a partial restriction map was made (Reddy *et al.* 1988).

Tc^r Nm^r transconjugants obtained from matings to *R. meliloti* *nodB::Tn5* and *nodC::Tn5* mutants as well as from matings to two *nod* deletion mutants (GMI357 and GMI361) elicited nodules on alfalfa plants at a low frequency (2–4%). These frequencies were not higher than those reported for spontaneous nodule formation in alfalfa (Truchet *et al.* 1989), and so were not investigated further.

Genetic linkage. The cosmid clone carrying the *Frankia* sequences, which were presumed to have genetically complemented the nodulation defective phenotype of Rm5610, was transferred either via triparental matings or by transduction to a fresh Nod[−] *R. meliloti* *nodA::Tn5* recipient. Although 10% of the alfalfa seedlings formed nodules after inoculation with these transconjugants or transductants, Tc^r Nm^r bacteria were recovered from only three of the 10 plants that had developed nodules.

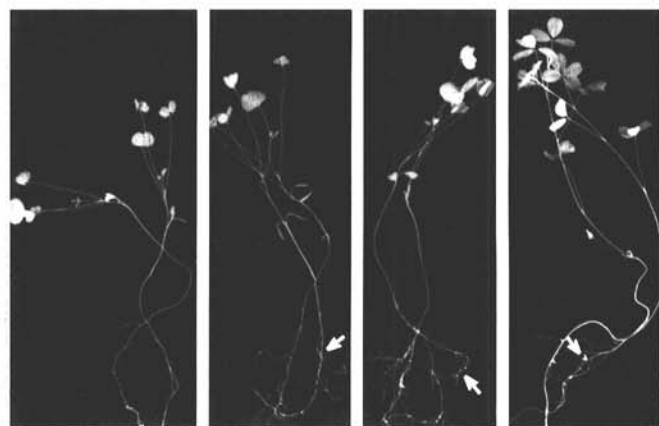


Fig. 1. Alfalfa plants inoculated with various *Rhizobium meliloti* strains. The plants shown are from left to right: uninoculated; and inoculated with Rm1021N; Rm5610 NS6; and wild type *R. meliloti* 1021. The two neomycin-resistant mutants induce numerous, small, white nodules, whereas the wild type-induced nodules are large, pink in color, and often bilobed. The nodules are indicated by arrows.

When total DNA isolated from Rm5610 NS6 was probed with Tn5, we discovered that Tn5 was not detectable (data not shown). Positive hybridizing bands were not evident when Rm5610 NS6 DNA was probed with the internal fragment of Tn5, the IS50 elements, or a NPTII gene fragment. Although Tn5 appeared to be no longer present in *nodA*, Rm5610 NS6 had not reverted to Nod⁺ Fix⁺, but remained Fix⁻. Moreover, the Tc^r Nm^r transconjugant had acquired spontaneous neomycin resistance while simultaneously losing Tn5. To establish unequivocally that the *Frankia* DNA sequences were not involved in the apparent complementation of nodulation, we isolated a Tc^s derivative of the transconjugant (Rm5610 NS6-1), which was cured of the cosmid clone. When Rm5610 NS6-1 was tested for nodulation ability on alfalfa, its phenotype was indistinguishable from its Tc^r parent, indicating that mutations in the *Rhizobium* genetic background were responsible for the Nod⁺ Fix⁻ phenotype.

We re-tested some of the transconjugants from the mating into the Phe⁻ auxotrophic mutant to determine if the strategy of genetic complementation of *Rhizobium* mutants as a means of isolating *Frankia* sequences was applicable to the case of amino acid biosynthetic genes. Although Southern hybridization experiments of DNA showed that six of seven prototrophic Nm^r Tc^r transconjugants con-

tained Tn5 in the same site as the original Phe⁻ recipient (data not shown), the Tc^r cosmid clones were unable to restore prototrophy to the Phe⁻ mutant after they were transduced into a fresh Phe⁻ (Nm^r) *R. meliloti*. These results indicate that the change from Phe⁻ to prototrophy was not linked to *Frankia* DNA. They also show that introduction of *Frankia* DNA sequences into *R. meliloti* Tn5 mutants does not always lead to the excision of the transposon.

Characterization of NS6. Rm5610 NS6 curled alfalfa root hairs with kinetics similar to wild type *R. meliloti* (data not shown). The nodules were sectioned and found to exhibit a typical root nodule histology: nodule cortex, endodermis, nodule parenchyma, peripheral vascular tissue, and a central tissue (Fig. 2). In the majority of nodules sectioned, the central tissue was devoid of infected cells, but infection threads were numerous. In the zone proximal to the nodule meristem, the host cells expanded in response to rhizobial infection. Rhizobia were released from the infection threads, but did not elongate and form bacteroids (Fig. 3). Based on tests with 150 alfalfa plants grown aseptically in test tubes inoculated with Rm5610 NS6, we determined that nodules formed on 86% of the plants. Eighty-three nodules were squashed, and 51% of these contained Nm^r Tc^r bacteria.

Our inability to detect Tn5 in Rm5610 NS6 indicated that Tn5 had not transposed to another site, causing a Fix⁻ phenotype. We examined total DNA isolated from strain NS6 and probed it with either *nif* or *nod* genes to determine if the Fix⁻ phenotype of the rhizobia was caused by any obvious deletions or rearrangements of these symbiotic genes. The hybridizing bands in the transconjugant were identical in size to those present in DNA of the wild type strain, indicating that there were no gross changes in the *nodABC* or *nifHDK* operons (data not shown). The plasmid pRmJ30, which carries *R. meliloti* common *nod*

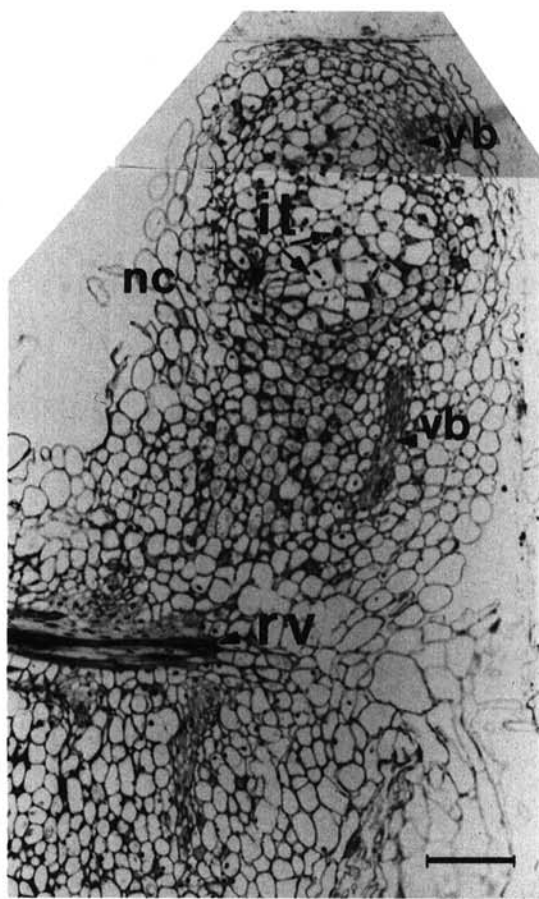


Fig. 2. Alfalfa nodule elicited by Rm5610 NS6. Light micrograph montage of a longitudinal section of a nodule harvested 4 wk after inoculation. The root vasculature (rv), nodule vascular bundle (vb), and nodule cortex (nc) are evident. There are numerous infection threads (it) in the central region of the nodule. Bar = 100 μ m

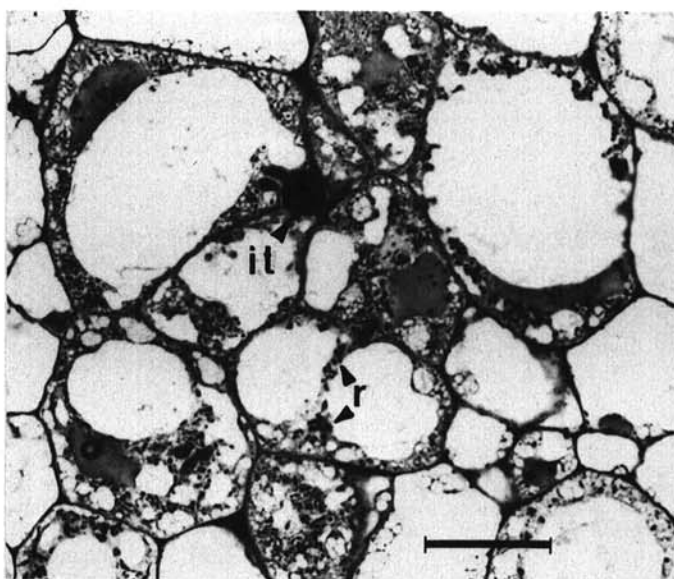


Fig. 3. Light micrograph of infected host cells of a nodule elicited by Rm5610 NS6. Bacteria are present within an infection thread (it), and the host cells contain rhizobia (r) that have been released but have not elongated. Bar = 25 μ m

genes (Jacobs *et al.* 1985), was introduced into the Tc^r derivative of NS6, and the resultant transconjugants were inoculated onto alfalfa plants. The nodule phenotype was not restored to Fix⁺, indicating that cryptic rearrangements or insertions within the common *nod* genes that could have a later effect on nitrogen-fixing ability were not the primary cause of the NS6 phenotype (data not shown).

Several analyses were carried out to determine the nature of the symbiotic defect. Rm5610 NS6 grew on both liquid and solid minimal medium, indicating that it was prototrophic. NS6 fluoresced under ultraviolet light when grown on plates containing Calcofluor. The transconjugant did not have a rough colony morphology, nor did it autoagglutinate in LB medium. NS6 also grew in the presence of deoxycholate. Motility of NS6 was comparable to that of the wild type strain. These characteristics and their comparison to wild type *R. meliloti* are summarized in Table 1.

Neomycin-resistant *R. meliloti*. The results from Table 1 and the lack of *trans* complementation by pRmJ30 suggested that NS6's inability to fix nitrogen *in planta* may have been due to its acquisition of neomycin resistance. We attempted to transduce neomycin resistance to Rm1021 using a lysate of the general transducing phage M12 grown on NS6. However, in six different transduction experiments, we were unable to obtain any Nm^r Rm1021 transductants. In addition to using the standard transduction protocol (Finan *et al.* 1984), we also tested whether increasing the expression time for the resistance made any difference to our transduction results. However, even after growing the transduction mixtures in nonselective LB broth for 24 hr, no Nm^r transductants were obtained when the cultures were subsequently plated on selective medium. The same phage lysates were active in transducing other genetic markers to wild type *R. meliloti*. These results suggest that the Nm^r phenotype of Rm5610 NS6 was caused by more than a single gene mutation.

Spontaneous mutants resistant to 50 µg/ml of neomycin appeared to arise at a low frequency in *R. meliloti* 1021 cultures. We determined the frequency to be approximately 1×10^{-9} . We isolated three spontaneous Rm1021 Nm^r mutants in separate isolation attempts. The mutants were inoculated onto alfalfa plants and after several weeks, 44 of 46 plants developed numerous small, white, ineffective nodules (Fig. 1). The nodule histology was identical to that of Rm5610 NS6. We found that Rm1021N was similar to Rm5610 NS6 in all other characteristics examined (Table 1). In addition, neomycin resistance was not transducible from Rm1021N to wild type Rm1021.

Nodulin gene expression. Poly(A)⁺ RNA was isolated from alfalfa roots and nodules induced either by wild type Rm1021, Rm5610 NS6, Rm1021N, or Rm7055 (*exoF::Tn5*), run on gels, and transferred to Nytran membranes. The membranes were probed with the radioactively labeled cDNAs of the nodulins MsENOD2 and MsENOD12-1. Using the MsENOD2 probe, a major transcript of 1.4 kb was detected in the RNA isolated from the different nodule types (data not shown). A major transcript of approximately 600 bp and minor ones of higher molecular weight hybridized to the MsENOD12-1 probe in lanes containing poly(A)⁺ RNA isolated from nodules induced by wild type *R. meliloti* strain 1021, Rm5610 NS6, or Rm1021N (Fig. 4). No transcript hybridizing to MsENOD12-1 was detected in RNA isolated from nodules elicited by the *exoF* mutant. mRNAs hybridizing to the early nodulin probes were not detected in root RNA.

In situ hybridization. We carried out *in situ* localizations of MsENOD2 and Lb mRNAs in nodules elicited by NS6 and Rm1021N to determine the site of nodulin gene expression in these white, ineffective nodules. Sections of nodules were hybridized to ³⁵S-UTP-labeled antisense RNA probes. MsENOD2 transcripts were detected in nodule parenchyma cells in both NS6- and 1021N-induced nodules (Fig. 5A,B).

Table 1. Relevant characteristics of *Rhizobium meliloti* mutants isolated in this study

<i>R. meliloti</i> strain	Genotype/phenotype ^a	Antibiotic markers	Growth in RDM ^b	Calcofluor staining ^c	Growth in DOC ^d	Autoagglutination in LB	Motility ^e
1021	Wild type Nod ⁺ Fix ⁺	Str ^r	Yes	Bright	Yes	No	Motile
5610	<i>nodA::Tn5</i> Nod ⁻	Str ^r Nm ^r	Yes	Bright	Yes	No	Motile
NS6	Contains pAR1 Nod ⁺ Fix ⁻	Str ^r Nm ^r Tc ^r	Yes	Bright	Yes	No	Motile
NS6-1	Cured pAR1 Nod ⁺ Fix ⁻	Str ^r Nm ^r Tc ^s	Yes	Bright	Yes	No	Motile
1021N	Spontaneous mutant of 1021 Nod ⁺ Fix ⁻	Str ^r Nm ^r	Yes	Bright	Yes	No	Motile
7055	<i>exoF::Tn5</i> Nod ⁺ Fix ⁻	Str ^r Nm ^r	Yes	Dark	Yes	No	ND ^f

^a The nodulation and nitrogen-fixing phenotype on alfalfa was determined.

^b *Rhizobium*-defined minimal medium.

^c Strains were streaked out on Luria-Bertani (LB) agar containing 0.02% calcofluor white M2R (Cellufluor, Polyscience, Warrington, PA), and screened for fluorescence using a handheld long wave UV lamp.

^d Strains were inoculated to solid or liquid LB medium containing 500 µg/ml of sodium deoxycholate (DOC).

^e Motility was assayed by spot-inoculation of bacteria to the center of LB plates containing 0.3% agar. The average diameter of the colony produced by each motile strain on the semi-solid agar was the same.

^f Not determined.

The pattern of MsENOD2 expression was identical to that previously observed in wild type *R. meliloti*-induced nodules (van de Wiel *et al.* 1990).

Studies of Lb expression in nodules elicited by wild type Rm1021 served as a point of reference for analyzing the expression in the mutant-induced nodules. In 2-wk-old wild type *R. meliloti*-induced nodules, Lb transcripts were found in all zones of the nodule excluding the meristematic and invasion zones (Fig. 6A,B). However, a stronger signal was detected in the region of the nodule known as interzone II-III (Vasse *et al.* 1990). The signal strength decreased with increasing distance from the nodule apex. The disparity in signal strength was more apparent in older nitrogen-fixing nodules (3–4 wk old) (Bochenek and Hirsch 1990; de Billy *et al.* 1991).

Lb transcripts were detected in a zone comparable in distance behind the nodule meristem to interzone II-III in the ineffective, white nodules elicited by Rm5610 NS6 (Fig. 7A,B) and Rm1021N (data not shown). Cells in this region were frequently expanded in size.

DISCUSSION

In the process of using the strategy of genetic complementation of *R. meliloti* nodulation mutants by *Frankia* DNA sequences, we isolated a *Nm⁺ Nod⁺ Fix⁻* *R. meliloti* mutant that is blocked at a critical stage of nodule development—after the release of rhizobia from infection threads and before bacterial elongation. This is a relatively unstudied phenotype for alfalfa, as the majority of *R. meliloti* mutants previously described induce either “empty” nodules or *Fix⁻* nodules in which bacteria are released and elongate to form bacteroids. Rm5610 NS6 elicited nodules in which infection threads penetrated into the central tissue of the nodule, rhizobia were released from the infection threads,

and the host cells expanded in response. However, the released rhizobia did not differentiate into elongate bacteroids, and hence the nodule phenotype is *Rel⁺*, but *Bad⁻* (Vincent 1980). Nodules induced by *R. meliloti* EJ312 *fix21* are similar overall to Rm5610 NS6-induced nodules, but *fix21* bacteria differentiate more than NS6 bacteria within the host nodule cells. In addition, the morphology of EJ312 *fix21*-induced nodules was more varied, ranging from nodules that were short and broad with limited meristematic activity to typical club-shaped indeterminate nodules (Norris *et al.* 1988). Similarly, nodules elicited by *R. meliloti* *exoH* mutants display a diversity of phenotypes. A few nodules contain infection threads that penetrate the internal tissues, but in the majority of nodules, infection threads are arrested in the superficial cells of the nodule (Leigh *et al.* 1987).

The characteristics of Rm5610 NS6 that we have examined also indicate that it is different from other previously described ineffective *R. meliloti* mutants. Although the nodules induced by Rm5610 NS6 resembled those induced by the leucine auxotrophs of *R. meliloti* (Truchet *et al.* 1980), NS6 was prototrophic unlike the *Leu⁻* strain. Furthermore, *Leu⁻* rhizobia were not released from infection threads in contrast to NS6 bacteria. When grown on plates containing Calcofluor, colonies of NS6 fluoresced

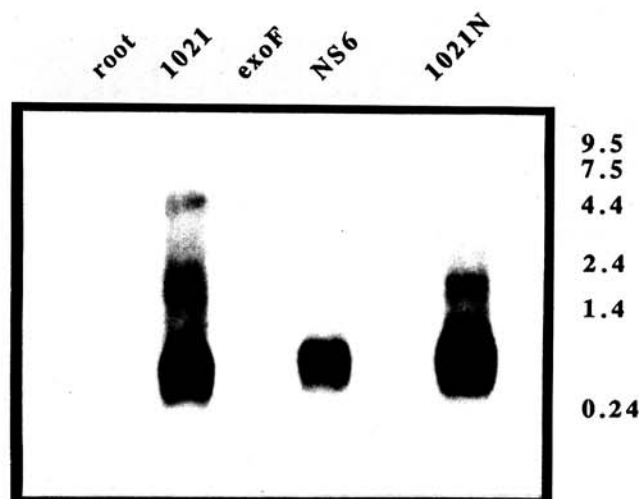


Fig. 4. Northern blot of poly(A)⁺ RNA from alfalfa roots and from nodules elicited by various *Rhizobium meliloti* strains. One microgram of poly(A)⁺ RNA from uninoculated plants (root) and nodules induced by wild type *R. meliloti* (1021), and the mutants indicated above each lane, was subjected to gel electrophoresis and blotted to a Nytran membrane. The blot was probed with insert from pMsENOD12-1. The sizes (in kilobases) of RNA molecular weight standards are shown on the right hand side of the blot.

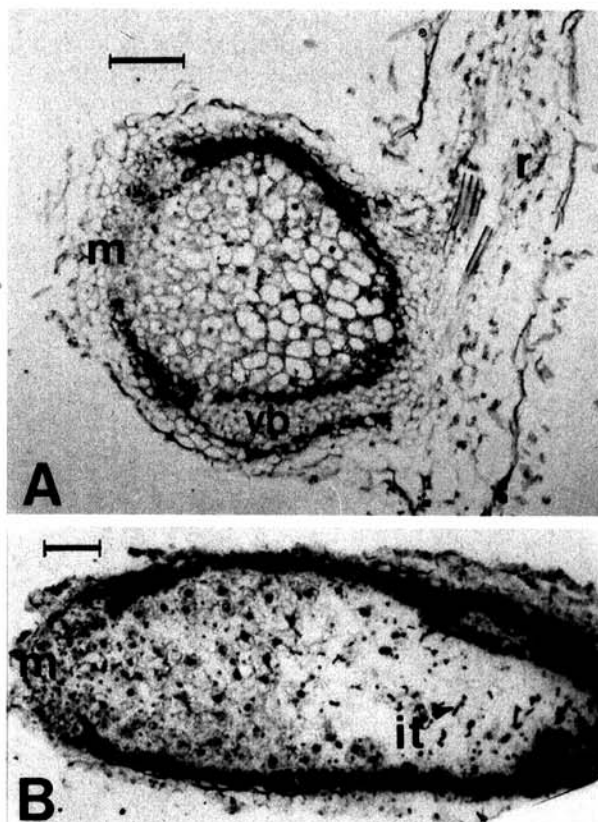


Fig. 5. Detection of MsENOD2 transcripts in alfalfa nodules by *in situ* hybridization. Bright field photographs of nodules elicited by A, Rm5610 NS6; and B, Rm1021N. Transcripts that hybridized to pA2ENOD2 insert are localized to parenchyma cells along the periphery of the nodules and also at the base of the nodules. Abbreviations: m, meristem; vb, nodule vascular bundle; r, root; it, infection thread. Bar = 100 μ m

under ultraviolet light, indicating that the strain produced acidic exopolysaccharide and was therefore unlike the exopolysaccharide (*exo*) mutants of *R. meliloti* (Leigh *et al.* 1985; Finan *et al.* 1985). The mutant did not have a rough colony morphology, nor did it autoagglutinate in LB medium like some *lps* mutants (Noel *et al.* 1987). NS6 was able to grow in the presence of deoxycholate, unlike the lipopolysaccharide mutant strain *R. meliloti* EJ312 *fix21* (Kieber *et al.* 1987). Motility of NS6 was comparable to that of the wild type strain, thereby suggesting that NS6 is unlike mutants that fail to produce β -1,2 glucan (Geremia *et al.* 1987; Dylan *et al.* 1985).

There are several possibilities that could explain the mutant phenotype of Rm5610 NS6 on plants. We have tested two of the more likely alternatives closely. The first possibility is that there was a change in the *nodA* protein, brought about either by the excision of Tn5 or by reiteration of the *Rhizobium nodA* sequence at the site where Tn5 was inserted (Berg *et al.* 1981; Auerswald *et al.* 1981). This change could have resulted in a defective NodA protein that has a deleterious effect on subsequent steps in the symbiosis. The other possibility was that the spontaneous acquisition of neomycin resistance caused the Fix⁻ phenotype. To discriminate between these two possible explanations, we attempted to complement a genetic defect in *nodA*, if present, by introducing functional common *nod* genes on pRmJ30 into a Tc^r derivative of Rm5610 NS6. We found that the resultant transconjugants were still Fix⁻. In addition, we were unable to detect by Southern hybridizations any gross changes, such as the transposition of ISRm1, an insertion sequence known to transpose preferentially into *nif* genes (Ruvkun *et al.* 1982a), in either the *nod* or *nif* operons.

To test the second possibility, we selected for a spontaneous Nm^r mutant of *R. meliloti*, and isolated Rm1021N, which is identical phenotypically to Rm5610 NS6 in all

the traits examined (Table 1). Thus, the mutation resulting in resistance to neomycin was pleiotropic with an effect on nodule development. This effect has been observed previously with other *Rhizobium* strains (Schwinghamer 1964; Yakovleva 1981).

We do not know the nature of the Nm^r defect in *R. meliloti*. Neomycin resistance can result either from impaired transport of neomycin across membranes or from an inhibition of 30S ribosome function. The Nm^r phenotype appeared to involve more than a single gene mutation, because it was not transducible by phage M12, which is able to transfer up to 190 kb of DNA (Finan *et al.* 1984). Another explanation is that the neomycin-sensitive phenotype is dominant and therefore a longer expression time than was used in our transduction experiments is required for the resistance phenotype to be apparent. However, no transductants appeared even after growth on nonselective medium for 24 hr. The low frequency of spontaneous neomycin resistance that occurred does not allow us to discriminate between these two possibilities. However, we favor the interpretation that more than a single gene is mutated.

Using the strategy of genetic complementation of *R. meliloti* nodulation mutants, we had reported previously that the transconjugant Rm5610 NS6, which contains a cosmid clone with *Frankia* HFPCc13 DNA, appeared to complement the *nodA*::Tn5 mutation of Rm5610 (Reddy *et al.* 1988). Tn5 mutants of *R. meliloti* are reported to be remarkably stable (Meade *et al.* 1982) and have been used previously to isolate complementing clones by the same strategy (Long *et al.* 1982; Marvel *et al.* 1985). Thus, we did not anticipate that Tn5 would be excised from *nodA*. Furthermore, our initial attempts to complement a naive strain of Rm5610 were promising, and the *Frankia* cosmid clone showed hybridization to a *nodABC* probe under conditions that allow 35–40% mismatch. However, our

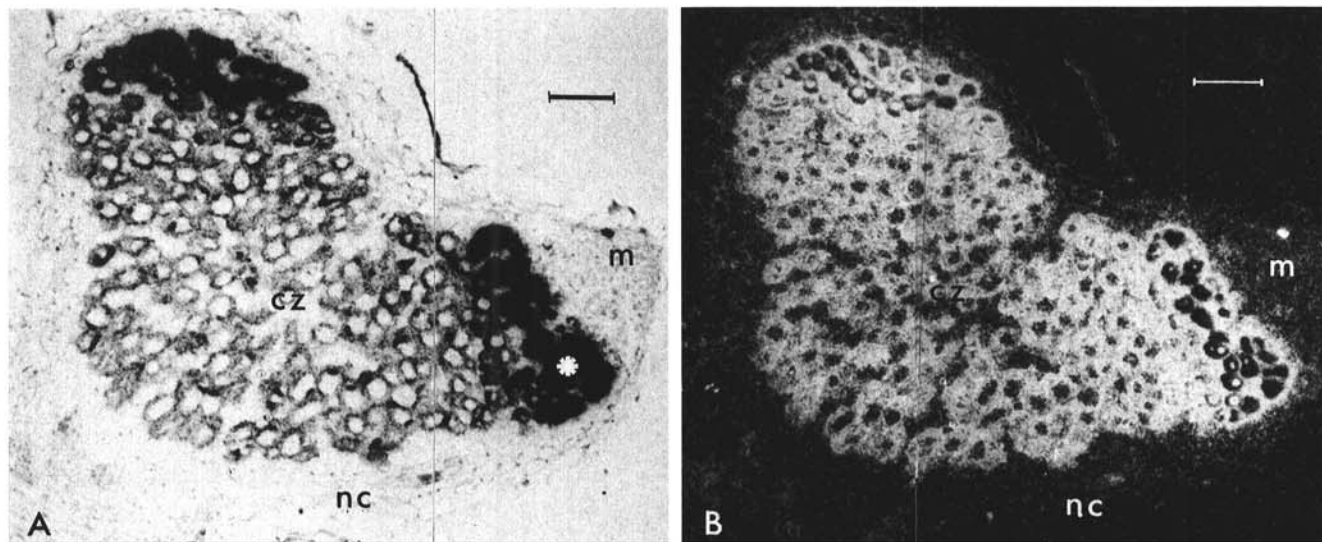


Fig. 6. Detection of leghemoglobin (Lb) transcripts in alfalfa nodules by *in situ* hybridization. **A**, bright field photograph of a 2-wk-old bilobed nodule elicited by wild type *Rhizobium meliloti* 1021. Transcripts that hybridized to Lb are evident in all zones of the nodule excluding the meristematic zone. A stronger signal was detected in interzone II-III of the nodule (*). The signal strength decreases with increasing distance from the nodule apex. **B**, dark field photograph of (A) in which the Lb transcripts are visualized by the brightly reflecting silver grains. Abbreviations: nc, nodule cortex; cz, central zone; m, meristem. Bar = 100 μ m.

subsequent experiments as described in this paper showed that Tn5 had indeed excised from *nodA*, and that the transconjugant spontaneously acquired Nm^r (50 μ g/ml), which appears to occur infrequently in *R. meliloti* (this paper). We also found no linkage between the complementing activity and the cosmid clones present in the prototrophic transconjugants. Based on the genetic data, we conclude that the strategy of complementing *R. meliloti* mutants cannot be used to uncover *Frankia* HFPCc13 nodulation genes. Therefore, other means of studying *Frankia* genes are required.

Although we did not know the exact nature of the genetic defect in the Nm^r *R. meliloti* strains, we were interested in examining the pattern of nodulin gene expression in these mutant-induced nodules, because they were arrested at a later stage of development than that previously described for *exo* or *ndv* mutants. We found that MsENOD2 and MsENOD12-1, as well as Lb were expressed in these developmentally arrested nodules. Although MsENOD2 is expressed in a diversity of "empty" nodules (van de Wiel *et al.* 1990; Dickstein *et al.* 1988), MsENOD12-1, a (hydroxy)proline-rich protein presumed to be in the walls of cells of the invasion zone, is not expressed in bacteria-free nodules (M. Löbner and A. M. Hirsch, unpublished).

Because MsENOD12-1 transcripts were detected in nodules containing *R. meliloti* 5610 NS6 and 1021N, we suggest that the induction of genes coding for MsENOD12-1 may be correlated with infection thread penetration into internal tissues of the nodule. Moreover, the presence of MsENOD12-1 mRNAs in ineffective nodules further demonstrates that this nodulin is involved in nodule morphogenesis rather than nodule function.

Similarly, Lb is also expressed before the onset of nitrogen fixation. Lb has been detected in ineffective alfalfa nodules that are elicited by various symbiotically defective mutants. Nodules elicited by the lipopolysaccharide mutant Rm5121 (EJ312 *fix21*) (Norris *et al.* 1988), and those elicited by *nifA*, *nifD*, *nifH*, *fixA*, and *ntrA* mutants of *R. meliloti* (Dunn *et al.* 1988) were found to contain Lb transcripts, whereas Hoving *et al.* (1990) reported that Rm1045-elicited nodules sometimes contained small amounts of immunologically detectable leghemoglobin late in development. Lb transcripts were also evident in ineffective soybean nodules (Morrison and Verma 1987). In this report, we have described the detection of Lb transcripts in wild type *R. meliloti*-induced alfalfa nodules, and their localization to the distal part of zone III (interzone II-III; Vasse *et al.* 1990; de Billy *et al.* 1991), in those cells that had expanded in response to infection thread penetration. Lb transcripts were also present in the more mature regions of the nodule, but at a lower intensity. Surprisingly, ineffective nodules elicited by Rm5610 NS6 and Rm1021N exhibited the same spatial localization of Lb transcripts as wild type *R. meliloti*-induced nodules. However, Lb transcripts dropped off sharply in the proximal cells of the mutant-induced nodules, presumably because the released bacteria did not differentiate into bacteroids and nitrogen fixation did not occur.

In summary, although the technique of using genetic complementation of *Rhizobium* mutants as a means of isolating *Frankia* nodulation genes was not successful, we have fortuitously isolated a mutant that induced an interesting alfalfa nodule phenotype. *R. meliloti* mutants, like Rm5610 NS6 and 1021N, that arrest nodule development at different stages may prove to be very useful tools in elucidating the signaling process that occurs between the two symbionts when forming an effective nitrogen-fixing association. Furthermore, from the experiments described here, it appears that the early nodulin MsENOD12-1 and the late nodulin Lb are both expressed as a result of cues that are independent of the extent of effectiveness of the symbiotic association. The identity of these cues is as yet unknown.

ACKNOWLEDGMENTS

We acknowledge the generosity of Agway, Inc., Syracuse, NY, for seeds of *Medicago sativa* 'Iroquois.' The *Rhizobium meliloti* Tn5-containing strains used in these experiments were generously provided by Ethan R. Signer and Shashi B. Sharma, Massachusetts Institute of Technology and by Jean Dénarié, INRA CNRS, France. The alfalfa A2ENOD2 clone was a gift from Rebecca Dickstein, Drexel University, and the alfalfa Lb clone was given to us by Kathleen Dunn, Boston College. We would like to thank Juliana Wong for recloning the Lb cDNA clone in the pBS vector. We are especially grateful to Marian Löbner for the alfalfa MsENOD12-1 clone that was used in these experiments, and also to John G. Torrey, Ethan R. Signer, and members of our laboratory for their help and encouragement during this project and for their comments on the manuscript.

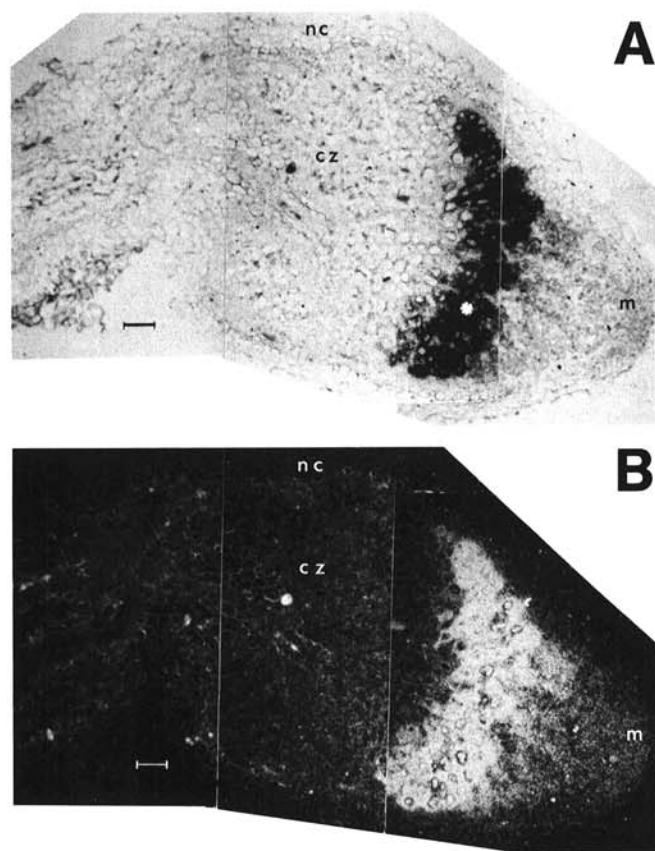


Fig. 7. Detection of leghemoglobin (Lb) transcripts in ineffective alfalfa nodules by *in situ* hybridization. **A**, bright field photograph of a 4-wk-old nodule elicited by Rm5610 NS6. A strong signal detecting transcripts that hybridized to Lb is seen in an area comparable to interzone II-III of a wild type-induced nodule (*). **B**, dark field photograph of (A). Abbreviations: nc, nodule cortex; cz, central zone; m, meristem. Bar = 100 μ m.

This material is based on work supported by the U.S. Department of Agriculture under grant 86-FSTY-9-0142 to J. G. Torrey and A. M. Hirsch, and 88-37262-3979 to A. M. Hirsch.

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