MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 2, pp. 139-146, 1991

Cells Expressing ENOD2 Show Differential Spatial Organization During the Development of Alfalfa Root Nodules

Ted Allen, Sandhya Raja, and Kathleen Dunn

Department of Biology, Boston College, Chestnut Hill, MA 02167 U.S.A.
Received 17 July 1990. Accepted 23 October 1990.

We have used *in situ* hybridization to examine the spatial organization of cells expressing the early nodulin gene (ENOD2) during the development of alfalfa root nodules. ENOD2 gene expression was found in the nodule parenchyma, uninfected cells surrounding the symbiotic region of both effective and ineffective nodules. However, in empty nodules, ENOD2 gene expression was found in a mass of parenchyma cells at the base of the nodule. Similar results were also observed in 11-day-old nodules that contained infected cells but that had not yet begun to express leghemoglobin. Although early events of nodulation result in the induction of ENOD2 expression in cells at the nodule base, the pattern of cells expressing ENOD2 during nodule growth appears to be correlated with the development of other peripheral tissues.

Additional keywords: Medicago sativa, nodulins.

Infection of legumes by *Rhizobium* bacteria results in the formation of root nodules, unique plant structures within which differentiated bacteria reside and nitrogen fixation takes place. Nodule growth is a developmentally controlled process, requiring new gene expression from both the plant and the bacteria (reviewed by Ausubel *et al.* 1987; Long 1989).

Nodule development in alfalfa is initiated by the specific attachment of *Rhizobium meliloti* to root hairs, resulting in root hair curling and deformation. This is followed by enclosure of the attached bacteria and the formation of a hollow infection thread through which the rhizobia travel to the inner cortical cells of the root. Eventually the rhizobia are released within the plant cells, are surrounded by a peribacteroid membrane of plant origin, and differentiate into nitrogen-fixing bacteroids. Concomitant with bacterial attachment is the stimulation of root cortical cell division and the formation of a nodule meristem (for review, see Nap and Bisseling 1990).

The indeterminate nodules of alfalfa or pea are organized into several layers of progressively aging cells. The apical meristem provides a continual source of cells that are added to the cortical and central tissues. Next to the meristem is the invasion zone, an area of enlarging cells susceptible to invasion by *Rhizobium*. The early symbiotic region lies adjacent to the invasion zone where there is further differentiation of the bacteria within the infected cells. This area is followed by the late symbiotic zone where nitrogen fixation and ammonia assimilation take place. The most proximal zone lying adjacent to the root is the senescent zone where both plant cells and bacteroids degenerate. A series of vascular bundles branching from the root are found in the periphery of the nodule. A nodule endodermis surrounds the internal core of the nodule and a vascular endodermis surrounds each of the vascular bundles. Sandwiched between the nodule endodermis and the zone of infection are several layers of small uninfected cells, recently termed the nodule parenchyma (van de Weil *et al.* 1990).

Both bacterial and plant genes involved in nodule formation have been identified. In *R. meliloti*, mutations in the *nod* region result in failure to initiate a meristem (Nod$^-$), whereas mutations in the *nif* or *fix* regions produce a nodule that is structurally normal but incapable of fixing nitrogen (Fix$^-$) (for review see Long 1989). Fix$^-$ nodules are also elicited by *R. meliloti* carrying mutations in the *ntrA* (Ronson *et al.* 1987) and *dctA* (Finan *et al.* 1988) genes. *R. meliloti exo* mutants that are deficient in the production of exopolysaccharide elicit the growth of an empty nodule devoid of bacteria (Inf$^-$) (Leigh *et al.* 1985; Finan *et al.* 1985).

Nodule-specific plant genes are expressed throughout the development of the nodule and are referred to as early or late nodulins, depending on the temporal course of their expression. Early nodulins in pea and soybean have been identified that are involved in either nodule morphogenesis (Franssen *et al.* 1987, 1988) or the infection process (Scheres *et al.* 1990a). In alfalfa, early nodulins have been identified based on their expression in nodules that are arrested at an early stage in development, such as the empty nodules elicited by *Rhizobium exo* mutants (Dickstein *et al.* 1988). Alfalfa late nodulins, such as leghemoglobin (Dunn *et al.* 1988; Kiss *et al.* 1987) or glutamine synthetase (Dunn *et al.* 1988) are detected after nodule formation and their expression is associated with bacterial invasion and bacteroid development. A number of late nodulins of unknown function have been identified in determinate and indeterminate nodules (Fuller *et al.* 1983; Govers *et al.* 1985; Gloudemans *et al.* 1987; Kiss *et al.* 1990; Dunn *et al.* 1988), some of which are located in the peribacteroid membrane (Fortin *et al.* 1985; Fortin *et al.* 1987; Jacobs *et al.* 1987; Sandal *et al.* 1987).

Corresponding author: Kathleen Dunn, Dept. of Biology, Boston College, Chestnut Hill, MA 02167 U.S.A.

The spatial organization of cells expressing nodulin genes has been determined by using labeled cDNA clones and specific antibodies to probe the location of nodulin transcripts and corresponding proteins (van den Bosch and Newcomb 1986, 1988; Scheres *et al.* 1990a, 1990b). Leghemoglobin protein has been found in the infected cells of the symbiotic zone of pea nodules (Robertson *et al.* 1984; Nap *et al.* 1989). Recently, ENOD2 transcripts from pea and soybean were located in the nodule parenchyma (van de Wiel *et al.* 1990).

In this study, we examined the spatial organization of nodule cells expressing the early nodulin gene, ENOD2, to better understand the functional organization of alfalfa root nodules. We have determined the relationship of this organization to the development of nodules elicited by mutant *R. meliloti*. We show that early events of nodulation result in the induction of ENOD2 expression in cells at the nodule base and that the pattern of cells expressing ENOD2 in mature nodules is correlated with the development of other peripheral tissues.

MATERIALS AND METHODS

Plant material. Alfalfa seeds (*Medicago sativa* L. 'Iroquois') were sterilized for 2 hr in ethanol and 20 min in bleach, rinsed 10–12 times in sterile water, and then soaked for 1–2 hr in sterile water. Seeds were germinated and grown on slants of Nod medium (Meade *et al.* 1982) containing 1.2% agar in 16 × 150 mm borosilicate culture tubes. After 5 days of germination, the seedlings were inoculated with 1×10^7 bacteria per plant, placed in a growth chamber at 20° C on a 16-hr light/8-hr dark cycle. Nodules were harvested 4 wk postinoculation; then they were either immediately frozen in liquid nitrogen and stored at −70° C before use or fixed and embedded as outlined in the following section.

Bacterial strains. The mutant strains of *R. meliloti* used in this study are outlined in Table 1. The wild-type strain of *R. meliloti* (1021) is a streptomycin-resistant derivative strain of SU47 (Meade *et al.* 1982).

RNA preparation and northern blot analysis. Total RNA preparation and northern blots were prepared as described previously (Dunn *et al.* 1988), except that cDNA clones were labeled with Random Primer Kit (Boehringer Mannheim, Indianapolis, IN).

RNA antisense probes. The ENOD2 cDNA clone, pBI-A2ENOD2, has a 0.3-kilobase (kb) insert cloned into pBS+ (Stratagene, La Jolla, CA) at the *Eco*RI site (Dickstein *et al.* 1988). The leghemoglobin cDNA clone is a 0.4-kb insert subcloned into the *Pst*I site of pGEM-3Zf(-) (Promega, Madison, WI) from a previously isolated clone (Dunn *et al.* 1988). Determination of the antisense strands for *in situ* hybridization was made by probing northern blots of total nodule RNA with [^{35}S]UTP-labeled *in vitro* RNA transcripts from the T3 and T7 promoters of pBS+ and from the Sp6 and T7 promoters of pGEM-3Zf(-).

***In situ* hybridization.** The protocol for the *in situ* hybridizations was a variation of the procedure used by Martineau and Taylor (1986). Nodules were fixed with Bouin's fixative at 4° C for 2 hr, dehydrated through a graded ethanol-butanol series, and embedded in Paraplast. Sections, 7 μm thick, were attached to chrome-alum with gelatin-subbed slides and allowed to dry overnight on a 37° C slide-warming rack.

Slides carrying nodule tissue sections prepared as described above were deparaffinized in xylene and then hydrated by passing them through an ethyl alcohol hydration series (100, 95, 85, 70, 50, and 30%) and then through distilled water twice, 5 min in each solution. The slides were then incubated in 100 mM triethanolamine-HCl (pH 8.0) with 0.25% acetic anhydride, washed briefly in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), rinsed in distilled water for 1 min, and allowed to air dry. Those slides to be used for RNase controls had 200 μl of 2× SSC with 10 μg/ml DNase-free RNase added, covered with a coverslip, and incubated at 37° C for 30 min. Control slides were then rinsed in 2× SSC, twice in distilled water, and air dried.

Hybridization probes were labeled with [^{35}S]UTP (1,000–1,500 Ci/mmol, New England Nuclear, Boston) by using either Sp6, T7 (Promega), or T3 (Stratagene) polymerase. Hybridization of nodule sections was carried out in 50% deionized formamide, 0.3M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.02% bovine serum albumin, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 500 μg/ml yeast tRNA, and 10% dextran sulfate as described by Cox *et al.* 1984. To 9 volumes of hybridization buffer, 1 volume of probe (1×10^6 cpm) was added so the RNA probe concentration was 0.5 ng/μl. The diluted probe was then added to pretreated slides at approximately 20 μl of solution per square centimeter of specimen. The slides were then covered with a 22-mm coverslip and placed in a moist chamber. Hybridization was allowed to proceed at 50° C for 2 hr.

After the incubation period, the slides were washed in 2× SSC at room temperature and the coverslips removed. The slides were then treated with RNase A in 2× SSC at 37° C for 30 min and then washed in the same buffer at 50° C for 15 min. The final wash was done twice in 2× SSC at room temperature. The slides were then dehydrated through an ethanol series 30, 50, 70, 85, 90, and 100%, containing 300 mM ammonium acetate, and allowed to air dry.

The slides were dipped in Kodak NTB-2 emulsion, diluted 1:1 with water, allowed to dry in complete darkness for 1–2 hr, and then stored at 4° C in a lighttight box. Slides were developed within 3–5 days in D-19 developer for 5 min at 17° C, rinsed briefly in water, and fixed in Kodak fixer for 5 min. After fixation, the slides were rinsed in water, stained for 5 min in 0.05% toluidine blue O, destained for 1 min in water, allowed to air dry, and permanently

Table 1. *Rhizobium meliloti* strains

Strain	Genotype	Relevant phenotypes[a]	Reference
1021	*str*-21	Nod$^+$, Fix$^+$, Bad$^+$	Meade *et al.* 1982
F642	Dct 14::Tn*5*	Nod$^+$, Fix$^-$, Bad$^+$	Yarosh *et al.* 1989
5011	*ntrA*::Tn*5*	Bid$^+$, Fix$^-$, Bad$^+$	Finan *et al.* 1988
7023	*exoA*::Tn*5*	Nod$^+$, Fix$^-$, Inf$^-$	Leigh *et al.* 1985

[a]Nod, nodule formation; Fix, nitrogen fixation; Bad, bacteroid development; Inf, bacterial infection.

mounted with Permount (Sigma).

Sections were examined through an Olympus microscope and photographed with Kodak Ektachrome 160 (tungsten) film.

RESULTS

Localization of ENOD2 transcripts in wild-type nodules. We have used *in situ* hybridization to determine the cell

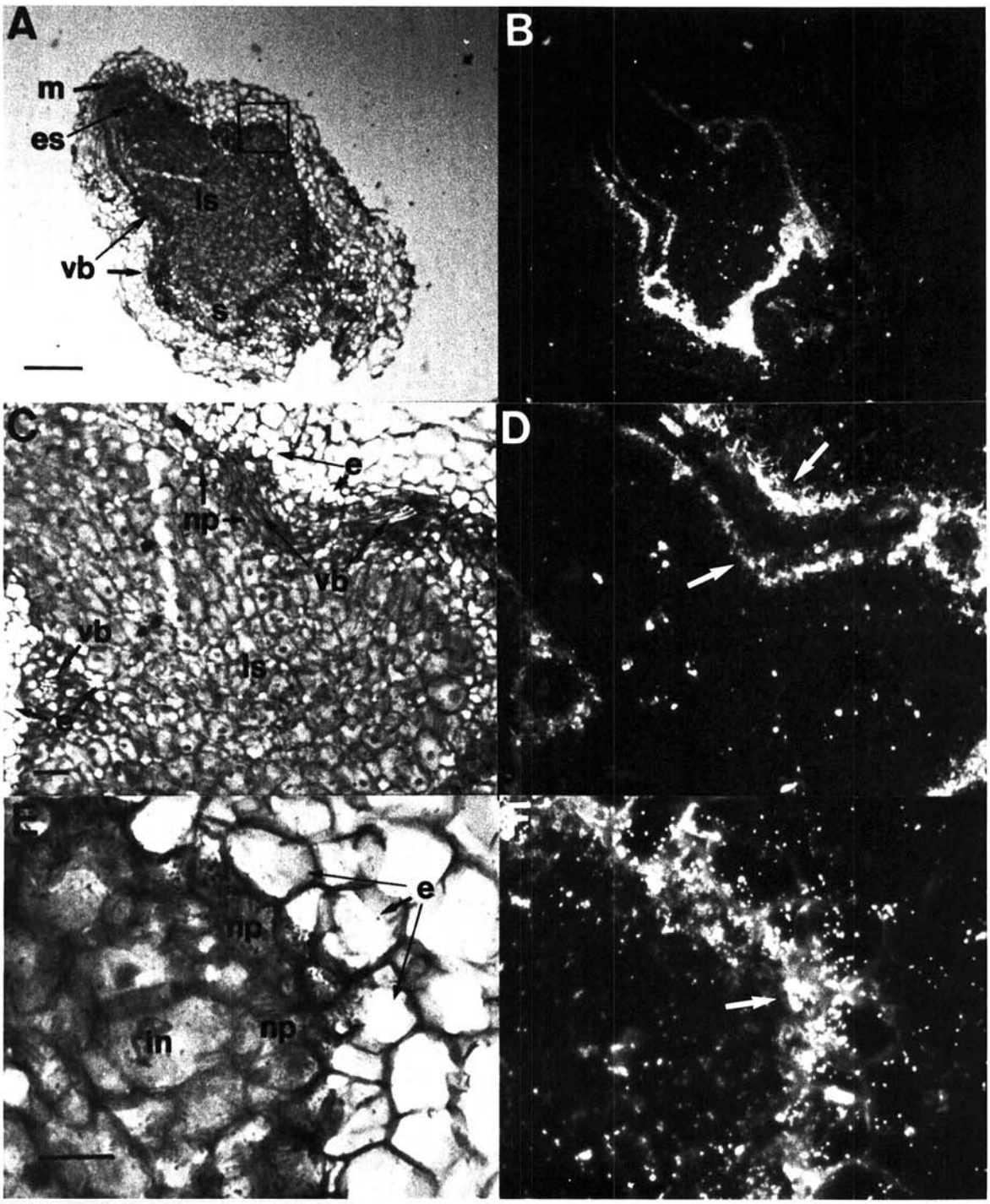

Fig. 1. Localization of ENOD2 transcripts by *in situ* hybridization in alfalfa. **A,** Bright field micrograph of a longitudinal section through a 28-day-old wild-type root nodule from alfalfa showing an apical meristem (m); early symbiotic (es); late symbiotic (ls); and senescent (s) zones. Peripheral vascular tissue is designated as (vb). Bar = 200 μm. **B,** Dark field micrograph of the same section as in **A** showing autoradiographic signal, appearing as white grains, localized in cells surrounding the zone of infection and the vascular tissue. **C,** Detail of the late symbiotic zone. The central tissue is almost completely surrounded by several layers of uninfected nodule parenchyma (np), which are internal to the endodermis (e). Parenchyma cells and endodermis also completely surround the vascular bundles (vb). Bar = 100 μm. **D,** Dark field micrograph of the same section as in **C;** arrows point to autoradiographic signal around the vascular bundle. **E,** Detail of late symbiotic zone outlined in **A** by black box showing central core of infected cells (in), layer of nodule parenchyma (np), and the layer of endodermis (e). Bar = 50 μm. **F,** Dark field micrograph of the same section as in **E,** arrow points to autoradiographic signal localized within the uninfected nodule parenchyma and not in the endodermis.

specific pattern of ENOD2 expression. Hybridization of ENOD2 antisense RNA to longitudinal sections of 4-wk-old alfalfa nodules showed that ENOD2 gene expression was confined to the nodule periphery (Fig. 1A, B). ENOD2 expression was not seen in the infected or uninfected cells of the symbiotic region of the nodule but was also internal to the endodermis and around the peripheral vascular bundles. (Fig. 1C, D). At a higher magnification, the cells transcribing ENOD2 were identified as the nodule parenchyma, uninfected cells that lie adjacent to the endodermis and that surround the central zone (Fig. 1E, F).

In contrast to ENOD2 gene expression, leghemoglobin gene expression is found in the central tissue (Fig. 2A, B). Higher magnification revealed that leghemoglobin expression is confined to the infected cells (Fig. 2C, D). No signal was detected with either probe in root tissue (data not shown).

Localization of ENOD2 transcripts in nodules infected with *Rhizobium* defective for nitrogen fixation. We examined nodules elicited by two Fix$^-$ strains of *R. meliloti* to determine the requirement for nitrogen fixation on the organization of cells expressing the ENOD2 gene. *R. meliloti* strain F642 is defective in dicarboxylic acid transport (Yarosh *et al.* 1989), and nodules elicited by this mutant are ineffective. Although the morphology of these nodules is similar to the wild-type nodules, there is a smaller zone of symbiosis and an enlarged zone of senescence (Fig. 3; S. Raja and K. Dunn, unpublished results). Strain 5011 carries a Tn*5* insertion within the *ntrA* gene (Finan *et al.* 1988). Nodules elicited by these mutants show a reduced level of nodulin gene expression (Dunn *et al.* 1988) and, although infection threads are present, the zone of symbiosis is less defined with few infected cells (S. Raja and K. Dunn, unpublished results). The *Rhizobium* strains and their characteristics are summarized in Table 1.

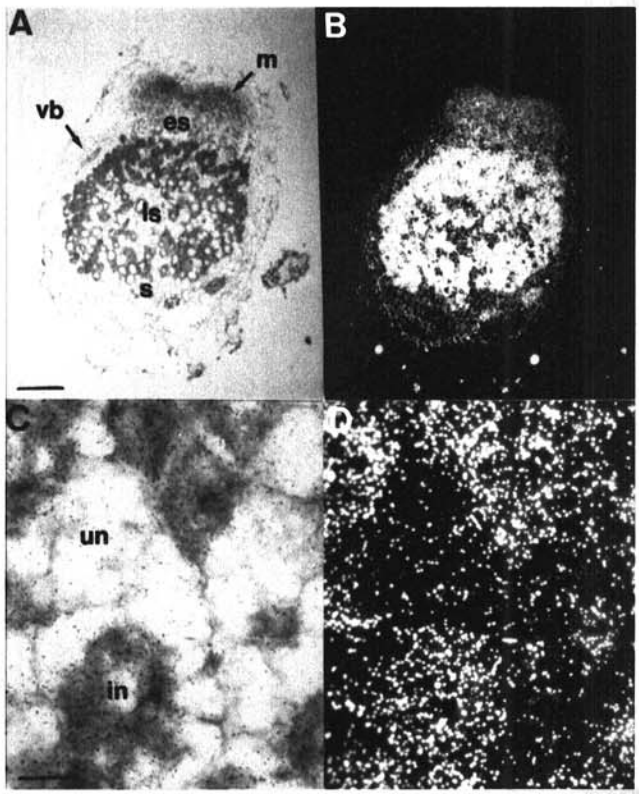

Fig. 2. Localization of leghemoglobin transcripts by *in situ* hybridization in alfalfa. **A,** Longitudinal section through a 28-day-old wild-type root nodule with the following zones of growth: the apical meristem (m), early symbiotic (es), late symbiotic (ls), and senescent region (s). The peripheral vascular tissue is designated as (vb). Bar = 200 μm. **B,** Dark field micrograph of the same section as in **A** showing the autoradiographic signal, appearing as white grains, localized within the late symbiotic region of the nodule. **C,** Higher magnification of a section of the late symbiotic zone from **A** showing those cells infected (in) with bacteria and those uninfected (un). **D,** Dark field micrograph of the same section as **C** showing signal localized only in those cells infected with bacteria. Bar = 50 μm.

Longitudinal sections of nodules induced by mutant bacteria were examined for ENOD2 gene expression (Fig. 3). As in wild-type nodules, ENOD2 gene transcription in nodules induced by *R. meliloti* strain F642 (*dctA*) was found localized in the nodule parenchyma (Fig. 3A, B). The nodule parenchyma in the F642-induced nodules lies adjacent to a well-defined nodule endodermis. The cells expressing the ENOD2 gene surrounded the symbiotic zone, but were considerably reduced around the senescing region, which is greatly enlarged in these mutants. ENOD2 gene transcription was similarly organized in nodules elicited by strain 5011 (*ntrA*) where the zone of senescence is also quite large (Fig. 3C, D). However, the symbiotic zone in these nodules is quite reduced and the nodule parenchyma surrounding this zone contains more cells, has an irregular border, and appears less organized.

Failure of cells expressing the ENOD2 gene in empty nodules to organize around a central core. The cellular organization of ENOD2 gene expression was examined in nodules elicited by *R. meliloti exo* mutants deficient in exopolysaccharide to determine the influence of intracellular bacteria infection. The nodules elicited by *exo* mutants are very small and characterized by a lack of intracellular bacteria and infection threads (Finan *et al.* 1985). Nodules elicited by *exoB* mutants were hybridized with labeled ENOD2 antisense transcripts and the ENOD2 gene expression was found localized in cells at the base of the nodule, surrounded by endodermis (Fig. 3E, F). The vascular bundles were found near the base of the nodule or transversing the nodule periphery, and the endodermis had developed such that it completely surrounded a central core consisting of a variable number of uninfected cells (Fig. 3E). Similar results were also observed with *exoA* nodules except that the nodule tissue appeared more fragile and rarely survived the hybridization procedure intact. In no instance did we observe either peripheral expression of the ENOD2 gene or peripheral organization of the vascular bundles. On the contrary, vasculature of the empty nodules was quite disorganized, was located more toward the proximal end of the nodule, and often transversed the nodule periphery. These observations were also confirmed by light micrographs of thin sections (data not shown).

Location of cells expressing the ENOD2 gene during early development. We examined nodules at various stages of their development to determine the temporal coordination of ENOD2 gene expression. Northern blot analysis of RNA extracted from roots at various times post-inoculation showed that ENOD2 gene expression was first detected at 11 days and continued throughout nodule

development (Fig. 4). At this stage, nodules were just visible with very few infected cells and there was no indication of leghemoglobin mRNA (Fig. 4).

Longitudinal sections of 11-day-old nodules revealed that the ENOD2 transcripts were concentrated in the parenchyma cells just above the nodule base, adjacent to a partially formed nodular endodermis (Fig. 5A, B). This was similar to what was observed in empty nodules except that the endodermis did not completely surround the ENOD2 expressing cells. In the 11-day-old nodules, the

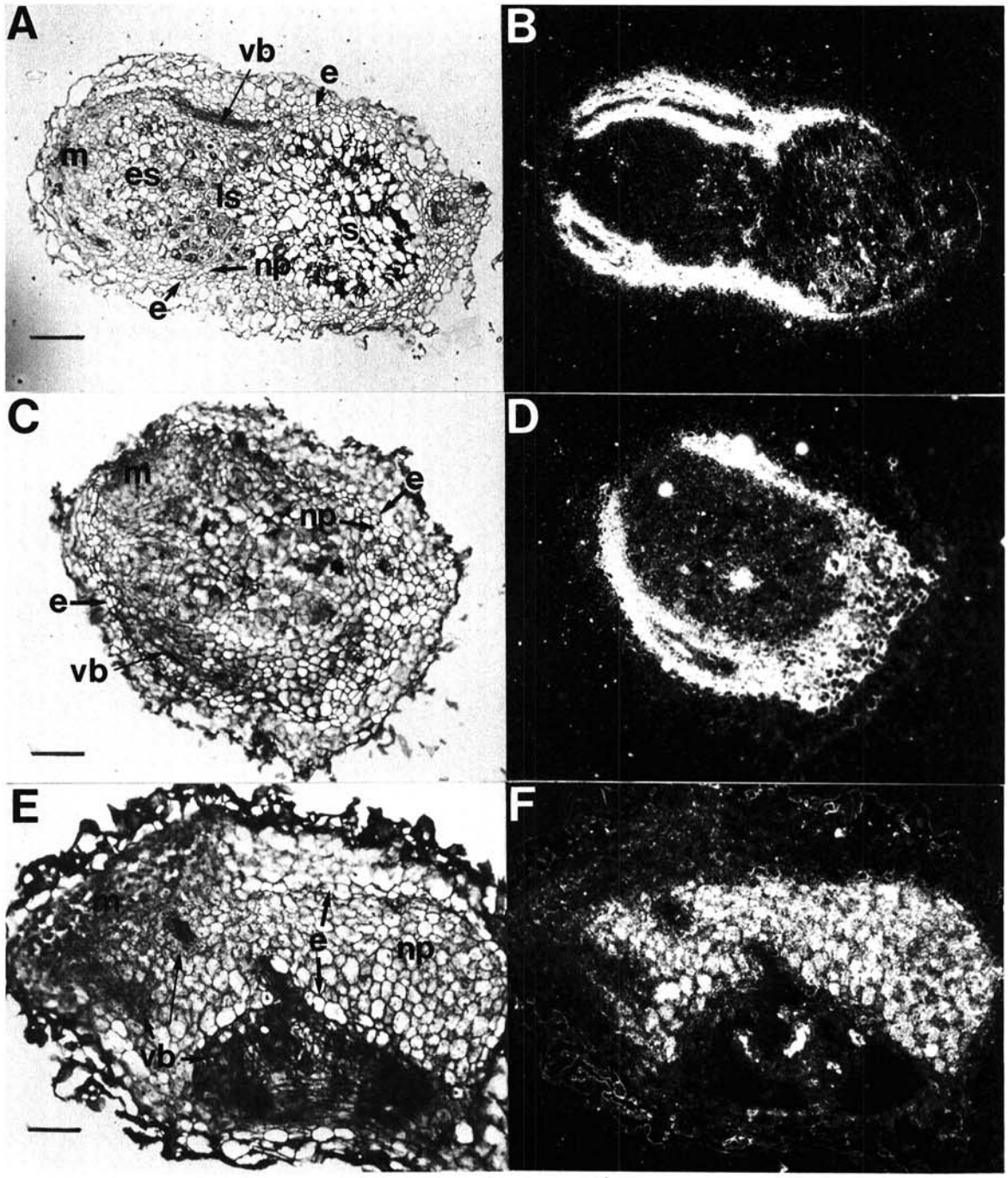

Fig. 3. Localization of ENOD2 transcripts by *in situ* hybridization in nodules induced by mutant *Rhizobium meliloti*. **A,** Bright field micrograph of a longitudinal section through a 28-day-old root nodule induced by F642 showing an apical meristem (m); early symbiotic (es); late symbiotic (ls); and senescent (s) zones. Toward the periphery of the nodule are the vascular bundles (vb) and the endodermis (e). Nodule parenchyma is designated as (np). Bar = 200 μm. **B,** Dark field micrograph of the same section as in **A** showing autoradiographic signal, appearing as white grains, localized in cells surrounding the zone of infection and the vascular tissue. **C,** Localization of ENOD2 transcripts in a nodule induced by strain 5011. Abbreviations as in A. Bar = 75 μm. **D,** Dark field micrograph of the same section as in **C** showing autoradiographic signal. **E,** Localization of ENOD2 transcripts in a nodule induced by 7094. The nodule parenchyma (np) is completely surrounded by the nodule endodermis (e); other abbreviations as in **A**. Bar = 75 μm. **F,** Dark field micrograph of the same section as in **E** showing autoradiographic signal in the mass of uninfected parenchyma cells at the base of the nodule surrounded by endodermis.

zone of symbiosis is just beginning to emerge and expression of the leghemoglobin gene was not detected (data not shown).

In 15-day-old nodules, ENOD2 gene expression was located in the parenchyma cells adjacent to a small symbiotic zone but was absent in the cells of the meristem (Fig. 5C, D). By this time, leghemoglobin gene expression was evident within the cells of the infected zone (as shown for wild-type nodules in Fig. 2B) and a well-formed endodermis was found surrounding the nodule. Although not observed in this section, serial sections of this same nodule show the beginnning of peripheral vasculature.

Finally, at 18 days, peripheral vascular bundles were observed and the cells expressing ENOD2 remained closely associated with the endodermis and were found from an area of highest concentration at the base of the nodule to the edge of the meristem (Fig. 5E, F). Again, serial sections of this same nodule showed further peripheral development of the vascular bundles (data not shown). These results further demonstrate the close association of the cells expressing the ENOD2 gene with the endodermis and suggest that the spatial organization of these cells in mature nodules is correlated with peripheral organization of the vascular tissue.

DISCUSSION

We have used *in situ* hybridization to determine the cellular location of early nodulin gene transcription (ENOD2) within developing nodules of *M. sativa*. Our results indicate that ENOD2 gene transcription is initiated in cells at the base of the nodule and is ultimately found in the nodule parenchyma, uninfected cells that surround the zone of infection. A similar pattern of ENOD2 gene expression was also observed in the indeterminate nodules from pea (van de Wiel *et al.* 1990).

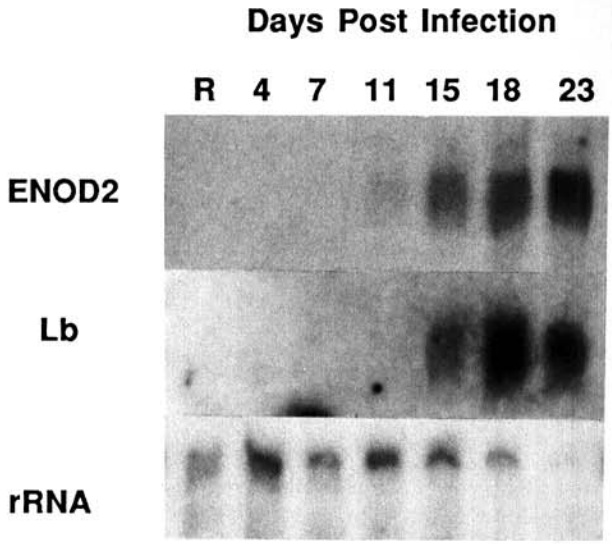

Fig. 4. Northern blot analysis of ENOD2 and leghemoglobin (Lb) gene expression at various times postinfection. Approximately 2 μg of total RNA isolated from infected roots was blotted onto a nylon membrane and probed with ^{32}P-labeled cDNA clones. R indicates total RNA isolated from uninfected root. Ribosomal RNA (rRNA) show the relative amounts of RNA in each lane.

During development, ENOD2 gene expression is found in different locations within the nodule. In 11-day-old nodules, ENOD2 gene expression is limited to a group of cells at the base of the nodule, suggesting that induction of transcription first occurs in cells most proximal to the root. Although these young nodules have visible infection threads and a few infected cells, it is not until the infection process has progressed further that the ENOD2 transcripts are found in the cells surrounding the zone of symbiosis. This indicates that further nodule development influences the localization of ENOD2 and a close association is observed with the development of the vascular tissue and the endodermis. Similarly, the Fix$^-$ nodules that have developed peripheral vasculature also showed a mature pattern of ENOD2 gene expression, demonstrating that nitrogen fixation does not influence the normal distribution of ENOD2 expression.

We examined empty nodules elicited by *exoB* and *exoA* mutants to determine if there was role for the infection process in the ultimate pattern of ENOD2 gene expression. In the smaller empty nodules, as in very young nodules where the zone of infection is extremely small, the expression of ENOD2 is limited to cells at the base of the nodule. Again, the endodermis is found in close association with the cells expressing the ENOD2 gene.

The change in location of ENOD2 expression from cells at the base of the nodule to cells surrounding the zone of symbiosis is most likely the result of continued cell division and growth of the nodule. In general, nodules elicited by the *exoB* or *exoA* mutants are often arrested in this growth process at approximately the 11-day stage, or before the normal onset of leghemoglobin gene expression. It would appear that the lack of either invading bacteria or intracellular infection is influential in the termination of this growth. In most cases, the original "trigger" for nodule initiation by *exoB* or *exoA* mutants is not sufficient to sustain the growth of a normal nodule and consequently the vasculature is disorganized. ENOD2 gene expression is confined to parenchyma cells at the nodule base but is still in close association with the endodermis.

It would be interesting to examine the organization of the nodule parenchyma in nodules elicited by other *Rhizobium* mutants, especially those where invasion has occurred but where intracellular infection has been aborted. This might distinguish aspects of the infection process that are responsible for stimulating nodule development beyond the 11-day-old stage.

An important developmental question is from which cells are new ENOD2 expressing cells derived. At the time a nodule can first be visualized, the cells expressing ENOD2 are found grouped together at the nodule base. Shortly thereafter, cells expressing ENOD2 are found surrounding the zone of infection. Because clonal proliferation, a major developmental mechanism in animals, does not normally occur during plant development, the cells expressing ENOD2 at the base of the nodule probably do not give rise to other ENOD2 expressing cells. More likely, the undifferentiated cells continually supplied from the meristem are induced to express the ENOD2 gene.

Several positioning factors may be involved in differ-

entiation of the nodule parenchyma and induction of ENOD2 expression. The close association of cells expressing ENOD2 with the endodermis could be an important factor in the regulation of ENOD2 expression. Certainly, the cells already expressing ENOD2 could play a role in signaling adjacent cells to do the same. Because the cells within the central symbiotic region are themselves differentiated, it is possible that they are unable to respond

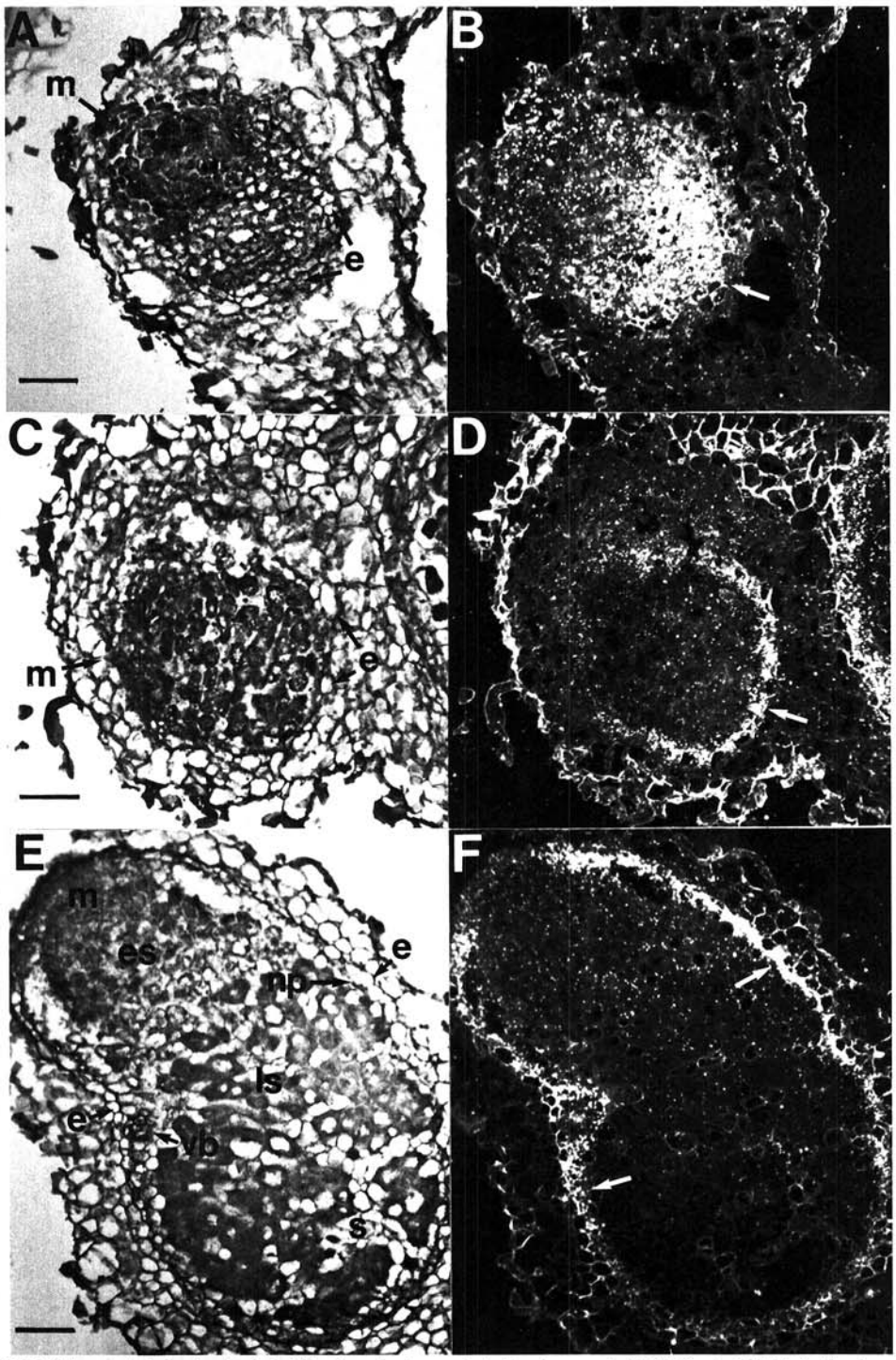

Fig. 5. Localization of ENOD2 transcripts by *in situ* hybridization during nodule development of alfalfa. **A,** Bright field micrograph of a longitudinal section through an 11-day-old wild-type root nodule showing an apical meristem (m) and endodermis (e). Bar = 100 μm. **B,** Dark field micrograph of the same section as in **A,** arrow shows autoradiographic signal appearing as white grains, at the base of the nodule in a mass of uninfected nodule parenchyma. **C,** Bright field micrograph of a longitudinal section through a 15-day-old wild-type root nodule. Abbreviations as in **A.** Bar = 100 μm. **D,** Dark field micrograph of the same section as in **C,** arrow shows autoradiographic signal in the nodule parenchyma surrounding a small zone of infected cells. **E,** Bright field micrograph of a longitudinal section through a 23-day-old wild-type root nodule showing an apical meristem (m), early symbiotic (es), late symbiotic (ls), and senescent (s) zones. Toward the periphery of the nodule are the vascular bundles (vb), the nodule parenchyma (np), and the endodermis (e). Bar = 100 μm. **F,** Dark field micrograph of the same section as in **E,** arrows show autoradiographic signal in the nodule parenchyma internal to the nodule endodermis surrounding the central zone of infected cells.

to ENOD2 induction signals. As a result, induction of ENOD2 expression is restricted to cells between the central core and the endodermis, the nodule parenchyma.

The nodule parenchyma has been identified as the major site of resistance to the diffusion of oxygen to the nodule interior (Tjepkeme and Yocum 1974; Witty *et al.* 1987). Thus, this tissue serves a major functional role in ensuring the optimal oxygen conditions for both respiration and nitrogen fixation. The structure and mechanism of this oxygen diffusion barrier remain unknown. As previously suggested, the induction of a nodule-specific gene during the morphogenesis of this tissue is consistent with a structural or functional role for ENOD2 in this process (van de Wiel *et al.* 1990). This is further supported by our findings and those in pea that the characteristic ring of ENOD2 gene expression is seen either prior to or concomitant with leghemoglobin gene expression, suggesting a correlation with functional aspects of the nodule.

ACKNOWLEDGMENTS

We would like to thank Rhonda Feinbaum for a careful reading of the manuscript, Fred Ausubel for helpful discussion, and Daniene Decker for technical assistance. This work was supported by United States Public Health Service grant GM37840.

LITERATURE CITED

Ausubel, F. M., Honma, M. A., Dickstein, R., Szeto, W. W., Nixon, B. T., and Ronson, C. W. 1987. Plant-bacterial signaling in the *Rhizobium*-legume symbosis. Pages 531-539 in: Plant Molecular Biology. D. von Wettstein and N. H. Chua, eds. Plenum Publishing, New York.

Cox, K. H., DeLeon D. V., Angerer, L. M., and Angerer, R. C. 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Dev. Biol. 101:485-502.

Dickstein, R., Bisseling, T., Reinhold, V. N., and Ausubel, F. M. 1988. Expression of nodule specific genes in alfalfa root nodules blocked at an early stage of development. Genes Dev. 2 677-687.

Dunn, K., Dickstein, R., Feinbaum, R., Burnett, B., Peterman, T. K., Thoidis, G., Goodman, H. M., and Ausubel, F. M. 1988. Developmental regulation of nodule-specific genes in alfalfa root nodules. Mol. Plant-Microbe Interact. 1:66-74.

Finan, T. M., Hirsch, A. M., Leigh, J. A., Johansen, E., Kuldau, G. A., Deegan, A., Walker, G. C., and Signer, E. R. 1985. Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell 40:869-877.

Finan, T. M., Oresnik, I., and Bottacin, A. 1988. Mutants of *Rhizobium meliloti* defective in succinate metabolism. J. Bacteriol. 170:3396-3403.

Fortin, M. G., Zelechowska, M., and Verma, D. P. S. 1985. Specific targeting of membrane nodulins to the bacteroid-enclosing compartment in soybean nodules. EMBO J. 4:3041-3046.

Fortin, M. G., Morrison, N. A., and Verma, D. P. S. 1987. Nodulin-26, a peribacteroid membrane nodulin is expressed independently of the development of the peribacteroid compartment. Nucleic Acids Res. 15:813-824.

Franssen, H. J., Nap, J.-P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., van Kammen, A., and Bisseling, T. 1987. Characterization of cDNA for nodulin-75 of soybean: A gene product involved in early stages of root nodule development. Proc. Natl. Acad. Sci. USA 84:4495-4499.

Franssen, H. J., Scheres, B., van de Wiel, C., and Bisseling, T. 1988. Characterization of soybean (hydroxy)proline-rich early nodulins. Pages 321-326 in: Molecular Genetics of Plant-Microbe Interactions. R. Palacios and D. P. Verma, eds. American Phytopathological Society, St. Paul, MN.

Fuller, F., Kunster, P. W., Nguyen, T., and Verma, D. P. S. 1983. Soybean nodulin genes: Analysis of cDNA clones reveals several major tissue-specific sequences in nitrogen-fixing root nodules. Proc. Natl. Acad. Sci. USA 80:2594-2598.

Gloudemans, T., DeVries, S. C., Bussink, H. J., Malik, N. S. A., Franssen, H. J., Louwerse, J., and Bisseling, T. 1987. Nodulin gene expression during soybean (*Glycine max*) nodule development. Plant Mol. Biol. 8:395-403.

Govers, F., Gloudemans, T., Moerman, M., van Kammen, A., and Bisseling, T. 1985. Expression of plant genes during the development of pea root nodules. EMBO J. 4:861-867.

Jacobs, R. A., Zhang, M., Fortin, M. G., and Verma, D. P. S. 1987. Several nodulins of soybean share structural domains but differ in their subcellular locations. Nucleic Acids Res. 15:1271-1280.

Kiss, F. B., Vegh, Z., and Vincze, E. 1987. Nucleotide sequence of a cDNA clone encoding leghemoglobin III (LbIII) from *Medicago sativa*. Nucleic Acids Res. 15:3620.

Kiss, G. B., Vincze, E., Vegh, Z., Toth, G., and Soos, J. 1990. Identification and cDNA cloning of a new nodule-specific gene, Nms-25 (nodulin-25) of *Medicago sativa*. Plant Mol. Biol. 14:467-475.

Leigh, J. A., Signer, E. R., and Walker, G. C. 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA 82:6231-6235.

Long, S. R. 1989. *Rhizobium*-legume nodulation: Life together in the underground. Cell 56:203-214.

Martineau, B., and Taylor, W.C. 1986. Cell-specific photosynthetic expression in maize determined using cell separation techniques and hybridization *in situ*. Plant Physiol. 82:613-618.

Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. 149:114-122.

Nap, J.-P., and Bisseling, T. 1990. Nodulin function and nodulin gene regulation in root nodule development. Pages 181-229 in: Molecular Biology of Symbiotic Nitrogen Fixation. P. M. Gresshoff, ed. CRC Press, Boca Raton, FL.

Nap, J.-P., van de Wiel, C., Spaink, H. P., Moerman, M., van den Heuvel, M., Djordjevic, M. A., van Lammeren, A. A. M., van Kammen, A., and Bisseling, T. 1989. The relationship between nodulin gene expression and *Rhizobium nod* genes in *Vicia sativa* root nodule development. Mol. Plant-Microbe Interact. 2:53-63.

Robertson, J. G., Wells, B., Bisseling, T., Farnden, K. J. F., and Johnston, A. W. B. 1984. Immuno-gold localization of leghemoglobin in cytoplasm in nitrogen-fixing root nodules of pea. Nature 31:254-256.

Ronson, C. W., Nixon, B. T., Albright, L. M., and Ausubel, F. M. 1987. *Rhizobium meliloti* ntrA (rpoN) gene is required for diverse metabolic functions. J. Bacteriol. 169:2424-2431.

Sandal, N. N., Bojsen, K., and Marcker, K. A. 1987. A small family of nodule specific genes from soybean. Nucleic Acids Res. 15:1507-1519.

Scheres, B., van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., van Eck, H., Zwartkruis, F., Wolters, A., Gloudemans, T., van Kammen, A., and Bisseling, T. 1990a. The ENOD12 gene product is involved in the infection process during the pea-*Rhizobium* interaction. Cell 60:281-294.

Scheres, B., van Engelen, F., van der Knaap, E., van de Wiel, C., van Kammen, A., and Bisseling, T. 1990b. Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell 2:687-700.

Tjepkeme, J. D., and Yocum, C. S. 1974. Measurement of oxygen partial pressure with soybean nodules by oxygen microelectrodes. Planta 115:351-360.

van den Bosch, K. A., and Newcomb, E. H. 1986. Immunogold localization of nodule-specific uricase in developing soybean root nodules. Planta 167:425-436.

van den Bosch, K. A., and Newcomb, E. H. 1988. The occurrence of leghemoglobin protein in the uninfected interstitial cells of soybean root nodules. Planta 175:442-451.

van de Wiel, C., Scheres, B., Franssen, H., van Lierop, M., van Lammeren, A., and Bisseling, T. 1990. The early nodulin transcript ENOD 2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. EMBO J. 9:1-7.

Witty, J. F., Skot, L., and Revsbech, N. P. 1987. Direct evidence for changes in the resistance of legume root nodules in O_2 diffusion. J. Exp. Bot. 38:1129-1140.

Yarosh, O. K., Charles, T. C., and Finan, T. M. 1989. Analysis of C-4 dicarboxylate transport genes in *Rhizobium meliloti*. Mol. Microbiol. 3:813-823.