MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 5, No. 5, pp. 430-434, 1992

Research Notes

Sequential Expression of Two Late Nodulin Genes in the Infected Cells of Alfalfa Root Nodules

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Received 6 January 1992. Revised 5 May 1992. Accepted 18 May 1992.

The Nms-22 and leghemoglobin (Lb) genes are expressed exclusively in the infected cells of alfalfa root nodules. Expression of these two late nodulin genes originated at distinct cellular boundaries within the symbiotic region of the nodule. The Nms-22 gene was expressed in all infected cells, including those just adjacent to the meristematic region. Lb gene expression was induced in older infected cells and was most prominent in the mature region of the nodule. Despite this temporal separation of gene expression, both the Nms-22 and Lb genes were expressed in nodules elicited by *bacA* mutants in which bacteroid development has been blocked just after release from the infection thread.

Additional keywords: *in situ* hybridization, *Medicago sativa*, symbiosis.

Bacteria of the genus *Rhizobium* interact specifically with a host legume to form highly specialized root nodules within which the rhizobia fix nitrogen. A most compelling aspect of the *Rhizobium*-legume symbiosis is the "communication" between the bacteria and plant. Bacterial and plant mutants that block nodulation at different stages have been isolated, indicating that nodule formation is a multistep process that requires information from the legume host and the bacterial symbiont. The availability of genetic markers for different stages of either plant or bacterial development has made it possible to address questions about the coordinated expression of symbiotic genes.

Many *Rhizobium* genes have been identified as essential for nodulation and nitrogen fixation. In *Rhizobium meliloti* Dangeard, mutations in the *nod* region result in the inability 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 exopolysaccharides elicit the growth of an empty nodule devoid of bacteria (Inf^-) (Leigh *et al.* 1985; Finan *et al.* 1985). Bacteria defective for heme production also elicit Fix^- nodules; some strains show the formation of infection threads that fail to release the bacteria. (Dickstein *et al.* 1991). *Rhizobium* mutants causing blocks at various stages of nodule development are a powerful tool and could help identify specific gene products involved in the signaling mechanisms between bacteria and plant.

A number of nodule-specific plant genes, characterized as either early or late, have also been identified by screening cDNA libraries. In alfalfa, early nodulin genes have been identified by their expression in nodules blocked at an early stage of development, such as the empty nodules induced by *R. meliloti exo* mutants (Finan *et al.* 1985; Dickstein *et al.* 1988). Late nodulin genes are expressed during later stages of nodule development when bacterial infection of the plant cells, via an infection thread, has occurred. The best studied late nodulin is leghemoglobin (Lb), in which gene expression is initiated from days 10–14 post-infection and located in the symbiotic region of the nodule (Dunn *et al.* 1988; Nap and Bisseling 1990; Allen *et al.* 1991). The temporal expression of several late nodulin genes has been described relative to the expression of the leghemoglobin genes. In soybean, nodulin-16 (related to nodulin-24) is expressed at the same time as Lb, but its activity peaks earlier (Nirunsuksiri and Sengupta-Gopalan 1990). In alfalfa, other late nodulin genes appear to be expressed concomitantly with Lb, suggesting a mechanism of coordinated control (Dunn *et al.* 1988; Kiss *et al.* 1990).

We report here the sequential expression of Nms-22, a late nodulin of unknown function (Dunn *et al.* 1988), and Lb in the infected cells of alfalfa root nodules. We have also examined the expression of these two genes in nodules in which bacteroid development has been aborted. Our results suggest that although the regulation of Nms-22 and Lb gene expression is quite different, induction of both genes appears to be independent of bacteroid maturation.

Nms-22 gene expression occurs before the expression of the Lb genes. The temporal expression of the nodulin genes, Nms-22 and Lb, was compared on northern blots of total RNA from 4-wk-old root nodules (*Medicago sativa* L. 'Iroquois') probed with ^{32}P-labeled cDNA clones; previously described procedures were used (Dunn *et al.* 1988). As shown in Figure 1, Nms-22 gene expression was detected approximately 11 days post-infection, whereas Lb gene expression was not observed until 15 days post-infection. We

obtained similar results with *in situ* hybridization of nodules harvested various times post-infection by using antisense ^{35}S-labeled RNA from the Nms-22 and Lb cDNA clones (data not shown). However, Lb expression was observed at 13 days post-infection, a timepoint not included in the northern analysis.

Nms-22 and Lb genes are expressed sequentially at distinct cellular boundaries in infected cells. We used *in situ* hybridization to compare the cellular localizations of Nms-22 and Lb gene expression. ^{35}S-RNA was transcribed in the sense and antisense direction from the Nms-22 and Lb cDNA clones (Dunn *et al.* 1988) as previously described (Allen *et al.* 1991). Nodules were fixed and sectioned according to the procedure of van de Wiel *et al.* (1990) and hybridized under conditions described by Allen *et al.* (1991). When serial longitudinal sections of 4-wk-old nodules were hybridized with the Nms-22 and Lb probes, the antisense probes hybridized to the symbiotic region of the nodule (Fig. 2A,B), whereas there was no significant signal from the sense probes (data not shown). Nms-22 gene expression began in the cell layers just adjacent to the invasion zone and continued throughout the symbiotic and senescing regions (Figure 2A–D). A magnification of the infected region from Figure 2C verified that Nms-22 gene expression is restricted to cells infected with bacteria (Fig. 3).

In contrast to the results described for Nms-22, Lb gene expression began at a distinct boundary of "older" infected cells, several cell layers proximal to the expression of the Nms-22 gene (Fig. 2G,H). The induction of the Nms-22 and Lb genes at two distinct cell layers parallel to the meristem was consistent with northern blot analysis that showed expression of the Nms-22 gene before the Lb gene. Lb gene expression was most prominent in the mature region of the nodule and severely reduced in the senescing region (Fig. 2E,F). The localization of Nms-22 and Lb gene expression to the infected cells is consistent with previous studies showing that these genes are not expressed in nodules in which intracellular infection has not occurred. Neither gene is expressed in empty nodules elicited by *Rhizobium exo* mutants (Dunn *et al.* 1988) or in nodules induced by *hemA* mutants in which bacteria are not released from the infection threads (Dickstein *et al.* 1991).

The data presented here indicate that the Nms-22 and Lb genes are differentially regulated within the infected

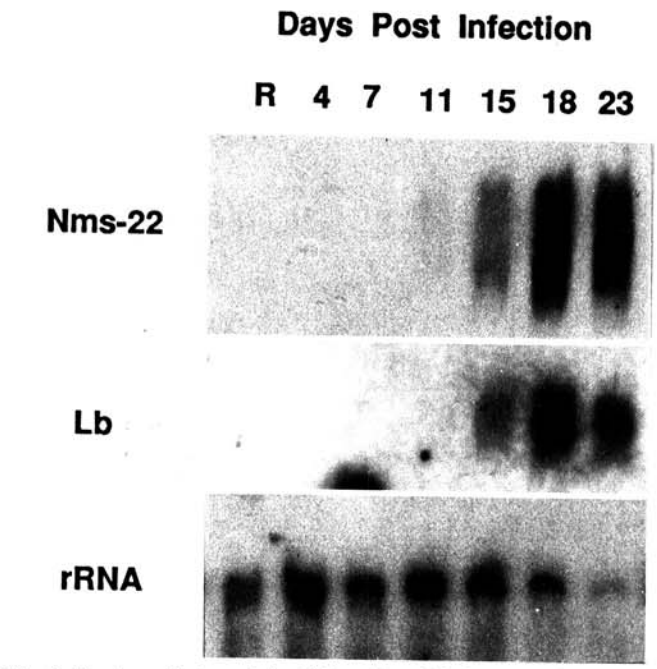

Fig. 1. Northern blot analysis of Nms-22 and leghemoglobin (Lb) gene expression at various times post-infection. Approximately 2 μg of total RNA isolated from infected roots was blotted onto a nylon membrane and probed with ^{32}P-labeled cDNA clones. R indicates total RNA isolated from uninfected root. Ribosomal RNA (rRNA) shows the relative amounts of RNA in each lane.

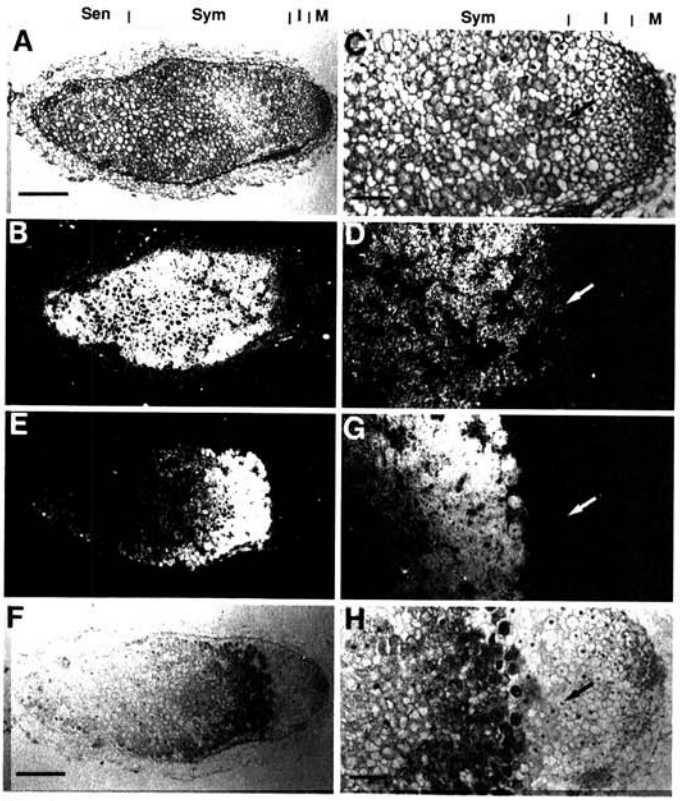

Fig. 2. Localization of Nms-22 and leghemoglobin (Lb) transcripts by *in situ* hybridization in alfalfa root nodules. **A,** Bright field micrograph of a longitudinal section through a 28-day-old wild-type root nodule from alfalfa hybridized with Nms-22 ^{35}S-labeled RNA antisense probe shows an apical meristem (M); infection (I); symbiotic zone (Sym); and senescent zone (Sen). Bar represents 350 μm. **B,** Dark field micrograph of the same section as in A shows autoradiographic signal, which appears as white grains localized in infected cells beginning in cell layers just adjacent to the invasion zone and continuing throughout the symbiotic region. **C,** Detail of the early symbiotic region. Arrow indicates interface of infection and symbiotic zones. Bar represents 100 μm. **D,** Dark field micrograph of the same section as in C shows autoradiographic signal beginning in the first layer of infected cells. **E,** Dark field micrograph of the same section as in F shows the Lb autoradiographic signal localized predominantly in the symbiotic region of the nodule. **F,** Bright field micrograph of a serial section of the same nodule as in A hybridized with Lb ^{35}S-labeled RNA antisense probe. Bar represents 350 μm. **G,** Dark field micrograph of the same section as in E shows autoradiographic signal localized at a distinct boundary of infected cells, several layers from the beginning of infection. **H,** Detail of the early symbiotic region. Arrow indicates interface of infection and symbiotic zones. Bar represents 100 μm.

cells. Because the Nms-22 gene is expressed initially in cells just after infection with the bacteria, its induction may be independent of further development of either the bacteria or the plant. Comparatively, the onset of Lb gene expression is delayed, suggesting a requirement for further maturation of bacteria or plant components in the newly infected cells.

Bacterial infection but not bacteroid development is required for induction of Nms-22 and Lb gene expression. The differential expression of the Nms-22 and Lb genes raises questions about the influence of bacteroid development on the differentiation of the infected plant cell. In a previous study, neither nitrogenase nor its regulatory protein NifA was required for the induction of the Lb or Nms-22 genes (Dunn *et al.* 1988). Because nodules elicited by the *nifA* and *nifH* mutants show substantial bacteroid development (Hirsch *et al.* 1983), we examined the expression of these genes in nodules in which bacteroid development has been aborted. Rm8386 carries a mutation in *bacA* and produces nodules with intracellular bacteria that fail to elongate or develop into mature bacteriods (Long *et al.* 1988; J. Glazebrook and G. Walker, unpublished). An electron micrograph of bacteroids from *bacA*-induced and wild-type nodules illustrates the *bacA* phenotype (Fig. 4).

Nodules elicited by *bacA* mutants were used to determine if there is a relationship between the progression of bacteroid development and the expression of the Nms-22 and Lb genes. A northern blot of RNA from nodules elicited by the *bacA* mutant was probed with ^{32}P-labeled inserts from the Nms-22 and Lb cDNA clones. A weak but distinct signal was observed (data not shown). For visualizing nodulin gene induction in individual nodules, we used *in situ* hybridization to examine Nms-22 and Lb gene expression in longitudinal sections of nodules elicited by *bacA* mutants. Interestingly, cells showing a significant level of Nms-22

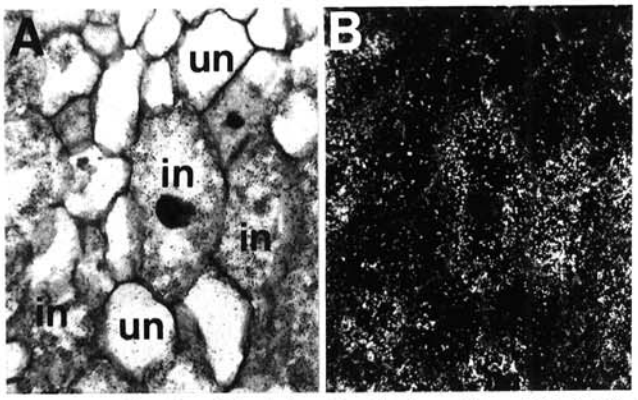

Fig. 3. A magnification of the infected region from Figure 2C. **A,** Bright field micrograph of a section of the late symbiotic region from Figure 2C shows infected (in) or uninfected (un) cells. **B,** Dark field micrograph of the same section as A shows Nms-22 signal only in those cells infected with bacteria. Magnification ×400.

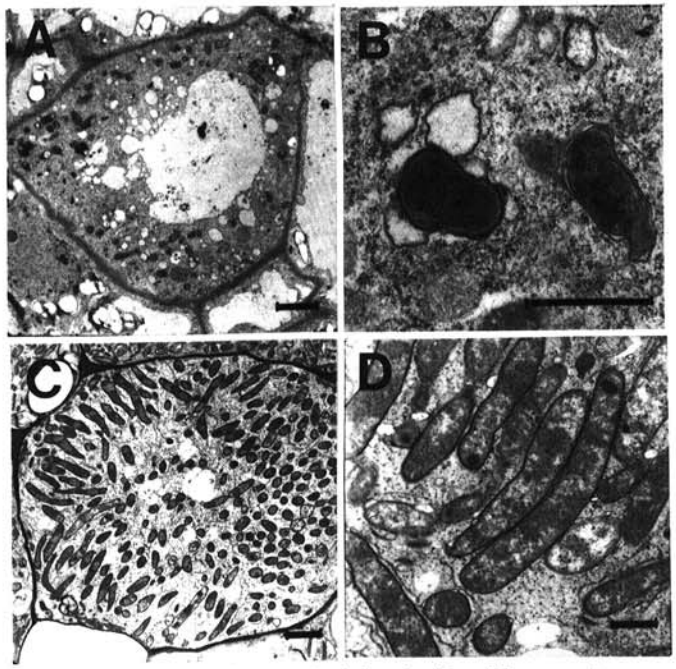

Fig. 4. Ultrastructure of host cells infected with wild-type and mutant *Rhizobium meliloti*. **A,** Transmission electron micrograph (TEM) of an infected nodule cell elicited by *R. meliloti* strain 8386 (*bacA*). Bar represents 10 μm. **B,** Enlargement of A shows aborted bacteroid development. Bar represents 1 μm. **C,** TEM of a symbiotic zone nodule cell elicited by wild-type *R. meliloti* strain 1021. Bar represents 10 μm. **D,** Enlargement of C shows normal bacteroid development. Bar represents 1 μm.

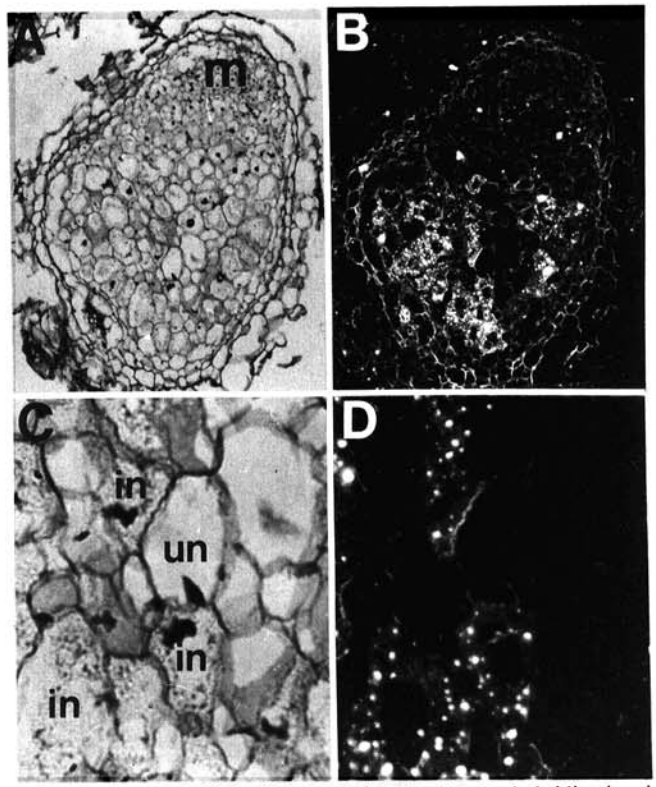

Fig. 5. Localization of Nms-22 transcripts by *in situ* hybridization in nodules induced by *Rhizobium meliloti* strain 8386 (*bacA*). **A,** Bright field micrograph of a longitudinal section through a 28-day-old root nodule showing apical meristem (m). Magnification ×100. **B,** Dark field micrograph of a serial section of the same nodule as shown in A hybridized with Nms-22 ^{35}S-labeled RNA antisense probe. **C,** Higher magnification of a section of the late symbiotic zone from A shows infected (in) and uninfected (un) cells. Magnification ×400. **D,** Dark field micrograph of the same section as C shows Nms-22 signal in all cells infected with bacteria.

or Lb gene expression were observed when nodules were examined by the more sensitive technique of *in situ* hybridization. Nms-22 gene expression appeared in all of the infected cells of the *bacA*-induced nodules (Fig. 5). Lb gene expression was also found exclusively in infected cells, but some infected cells showed no expression of these genes and were consistent with what is seen in normal nodules (Fig. 6). We have not determined the morphology of bacteriods in those infected cells that do not express the Lb gene, but it is likely that they are in senescence or have lysed. Because most of these nodules are quite small, it was not possible to observe distinct cellular boundaries for the onset of Nms-22 and Lb gene expression.

These results show that both the Nms-22 and Lb genes are induced in nodules in which the bacteria fail to elongate or progress through normal stages of development. Such results were expected for the Nms-22 gene, which is expressed in newly infected cells, but not for the Lb gene, which is delayed in these cells. A previous report has localized the induction of Lb gene expression to a layer of amyloplast-rich cells containing elongated mature bacteroids (de Billy *et al.* 1991). The expression of the Lb gene in nodules elicited by *bacA* mutants shows that induction of this gene can be uncoupled from the bacteroid species with which it is normally associated. A similar observation for Lb gene expression was recently reported by Reddy *et al.* (1992) in nodules elicited by a spontaneous Fix$^-$ mutant of *R. meliloti* in which bacteroids also fail to elongate after release from the infection thread.

The data presented here are consistent with previous models for nodulin gene induction that involve a nondiffusible signal or signals found in cells after infection of the invading bacteria (Truchet *et al.* 1980). The differential expression of the Nms-22 and Lb genes may be the result of an initial signal that induces a program of development. Interestingly, we have not been able to identify nodules in which intracellular infection has occurred in the absence of either Nms-22 or Lb gene expression. A series of signals produced during the developmental process may be involved. Although both the Nms-22 and Lb genes are expressed in the absence of bacteroid maturation, this does not preclude the influence of metabolic or biochemical factors on the expression of these genes. Because the level of nodulin gene expression is reduced in all Fix$^-$ nodules, it is possible that maximum expression requires either complete bacteroid development or other environmental signals.

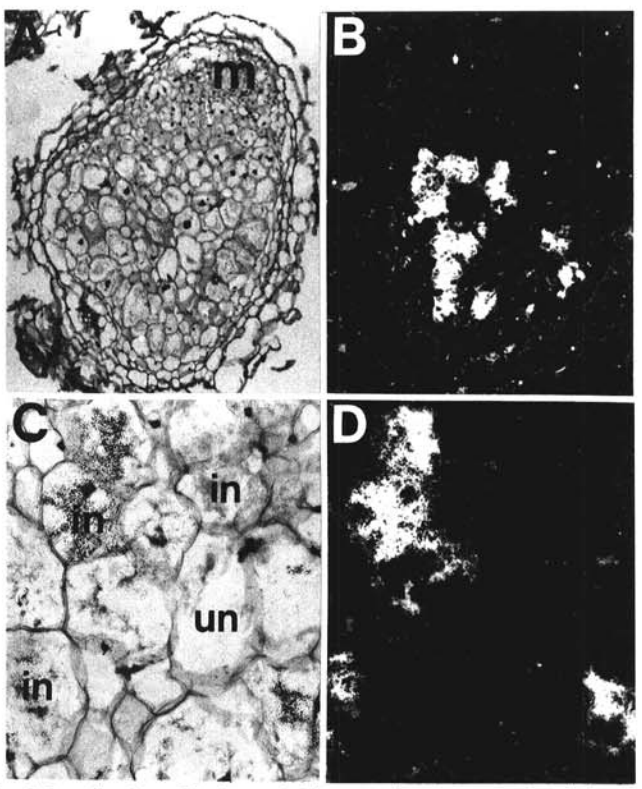

Fig. 6. Localization of leghemoglobin transcripts by *in situ* hybridization in nodules induced by *Rhizobium meliloti* strain 8386 (*bacA*). **A,** Bright field micrograph of a longitudinal section through a 28-day-old root nodule showing apical meristem (m). Magnification ×100. **B,** Dark field micrograph of a serial section of the same nodule as shown in A hybridized with leghemoglobin ^{35}S-labeled RNA antisense probe. **C,** Higher magnification of a section of the late symbiotic zone from A shows infected (in) and uninfected (un) cells. Magnification ×400. **D,** Dark field micrograph of the same section as C shows leghemoglobin signal in only some cells infected with bacteria.

ACKNOWLEDGMENTS

We thank Jacqueline Heard for a careful reading of the manuscript and Graham Walker and Jane Glazebrook for helpful discussions. This work was supported by United States Public Health Service grant GM37840 and a Research Incentive Grant from Boston College.

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