The Plant Cell, Vol. 6, 215–225, February 1994 © 1994 American Society of Plant Physiologists

Morphogenetic Rescue of *Rhizobium meliloti* Nodulation Mutants by *trans*-Zeatin Secretion

James B. Cooper [a,b,1] and Sharon R. Long [a]

[a] Department of Biological Sciences, Stanford University, Stanford, California 94305
[b] Division of Molecular, Cellular, and Developmental Biology, Department of Biological Sciences, University of California, Santa Barbara, California 93106

The development of nitrogen-fixing nodules is induced on the roots of legume host plants by *Rhizobium* bacteria. We employed a novel strategy to probe the underlying mechanism of nodule morphogenesis in alfalfa roots using pTZS, a broad host range plasmid carrying a constitutive *trans*-zeatin secretion (*tzs*) gene from *Agrobacterium tumefaciens* T37. This plasmid suppressed the Nod⁻ phenotype of *Rhizobium* nodulation mutants such that mutants harboring pTZS stimulated the formation of nodulelike structures. Alfalfa roots formed more or fewer of these nodules according to both the nitrogen content of the environment and the position along the root at which the pTZS⁺ bacteria were applied, which parallels the physiological and developmental regulation of true *Rhizobium* nodule formation. This plasmid also conferred on *Escherichia coli* cells the ability to induce root cortical cell mitoses. Both the pattern of induced cell divisions and the spatially restricted expression of an alfalfa nodule-specific marker gene (*MsENOD2*) in pTZS-induced nodules support the conclusion that localized cytokinin production produces a phenocopy of nodule morphogenesis.

INTRODUCTION

Soil bacteria in the genus *Rhizobium* form an important endosymbiotic partnership with specific legume host plant roots resulting in the development of unique organs called root nodules. Differentiated bacteria reside within root nodules where they function to fix atmospheric N_2 and supply the host plant with reduced nitrogen. A variety of investigations have been aimed at establishing the detailed understanding of nodule development required for devising rational strategies to extend the host range of N_2-fixing symbioses to nonlegume crops. Much of this work has recently culminated in the discovery of an elaborate system of interspecific chemical communication by which symbiotic partners regulate each other's development and gene expression (Dénarié and Roche, 1992; Fisher and Long, 1992; Spaink, 1992), and this interspecific signaling system has already become a paradigm for plant–microbe communication (Long and Staskawicz, 1993).

From a developmental perspective, the interaction of alfalfa roots with *R. meliloti* is one of the best understood among all legume-*Rhizobium* symbioses. On the host side of this interaction, a developmental timetable for the early events in nodule development has been established using both flood inoculation and microinoculation techniques (Hirsch et al., 1982; Dudley et al., 1987; Wood and Newcomb, 1989). Within 20 hr of inoculation, cells in the inner cortex of the alfalfa root fill with cytoplasm, the nuclei take on a spherical shape and migrate to the center of the cortical cells, and within 24 hr mitoses are initiated in the innermost layer of cortical cells. These cell divisions proliferate throughout the root cortex over the next 2 to 3 days, leading to the formation of a visible swelling on the root. Concurrently, within hours of inoculation, *Rhizobium* bacteria on the root surface deform growing root hair cells and initiate specialized infection threads that penetrate into the host root and ramify throughout the dividing mass of plant cells. Bacteria use these infection threads to invade into the nodule tissue and are ultimately released into the host cytoplasm where they differentiate into N_2-fixing bacteroids surrounded by an envelope. These early morphological responses of host roots are accompanied by the induced expression of novel host genes called early nodulins, several of which encode unusual repetitive (hydroxy)proline-rich cell wall proteins (see Franssen et al., 1992). Expression of some early nodulin genes (e.g., *ENOD5* and *ENOD12*) is correlated with nodule infection events (Scheres et al., 1990a, 1990b). Other early nodulin genes (e.g., *ENOD2* and *ENOD40*) appear to be involved in nodule morphogenesis because they are expressed in developing nodules independent of *Rhizobium* invasion (Dickstein et al., 1988; Yang et al., 1993). These early host responses can be blocked both by mutations in the *R. meliloti* nodulation (*nod*) genes (Hirsch et al., 1982; Debellé et al., 1986; Dudley et al., 1987; Dickstein et al., 1988) and by mutations in *nod* genes of the host (Dudley and Long, 1989; Utrup et al., 1993).

[1] To whom correspondence should be addressed at: Department of Biological Sciences, University of California, Santa Barbara, CA 93106.

From the bacterial side of this interaction, many of the *R. meliloti* symbiosis genes have been identified and characterized (reviewed in Long, 1992). Even before *Rhizobium* contacts the root surface, specific flavonoid signals secreted by host roots are used to activate the expression of the *nod* genes. The *nod* gene products in turn act to send signals called Nod factors that elicit specific responses in the host root. Cell-free supernatants of *R. meliloti* are able to cause both root hair deformation (initiating nodule invasion) and cortical cell divisions (initiating nodule morphogenesis), depending on the nodulation genotype of the bacterial strain (Van Brussel et al., 1986; Faucher et al., 1988, 1989; Truchet et al., 1991). Furthermore, *nod* gene expression is correlated with the biosynthesis of modified lipooligosaccharide Nod factors that are both necessary and sufficient to elicit the early host responses (Lerouge et al., 1990; Truchet et al., 1991). Genes involved in the synthesis of surface exopolysaccharides (*exo* genes) are also required for the invasion of nodule tissues by *R. meliloti* but are not required for the initiation of infection or for nodule morphogenesis (Finan et al., 1985; Leigh et al., 1985; Yang et al., 1992).

A working hypothesis for nodule initiation is that Nod factors regulate the endogenous mechanisms that normally control cell division in root cortical cells. Indeed, the idea that endogenous plant hormones control nodule development has been suggested for many years (Thimann, 1936; Libbenga et al., 1973). The recent discovery of the spontaneous formation of nodules (which express *ENOD2*) in alfalfa indicates that nodule formation can occur without exogenous signal compounds, thus lending support to this general model (Truchet et al., 1989a). Two classes of plant hormones, auxins and cytokinins, are generally thought to play roles in regulating the plant cell division cycle. Recently, attention has focused again on the potential involvement of these hormones in nodule development. A.M. Hirsch and colleagues observed that auxin transport inhibitors (ATIs) induce the formation of nodulelike structures on legume roots that express some early nodulin genes, including *ENOD2* and *ENOD12* (Hirsch et al., 1989; Scheres et al., 1992). Cytokinins have also been shown to specifically induce the expression of *ENOD2* in *Sesbania* roots (Dehio and de Bruijn, 1992).

To further explore the potential role of cytokinins in nodule initiation, we constructed a broad host range plasmid, pTZS, which constitutively expresses an isopentenyl transferase gene from *Agrobacterium tumefaciens*, and used bacteria harboring pTZS as sources of continuous *trans*-zeatin secretion. This novel strategy circumvents the potential problems of rapid metabolism and redistribution of exogenously applied chemical cytokinins that have been observed in legume roots (Morris, 1981). In this paper, we provide evidence that pTZS$^+$ bacteria, when inoculated onto alfalfa roots, induce cortical cell divisions that imitate at least some of the morphological and molecular events in nodule initiation. In this sense, pTZS complements the morphogenetic functions of the *Rhizobium nod* genes. Preliminary results from this study were reported in Long and Cooper (1988).

RESULTS

Zeatin Secretion Induces the Cytological Events in Nodule Initiation

For our experiments on the potential role of cytokinins in nodule development, we constructed a broad host range *trans*-zeatin secretion plasmid called pTZS, the structure of which is shown in Figure 1. The *tzs* gene from pTiT37 of *A. tumefaciens* encodes an isopentenyl transferase that catalyzes the rate-limiting step in the biosynthesis of two naturally occurring cytokinins, isopentenyl-adenine and zeatin (Akiyoshi et al., 1985). We placed the *tzs* gene under the transcriptional regulation of the *Salmonella typhimurium trp* promoter in the vector pTE3, which is regulated by *trpR* in *E. coli* and functions as a strong constitutive promoter in *R. meliloti* (Egelhof and Long, 1985). Transconjugant strains of *R. meliloti* harboring pTZS behaved as functional adenine auxotrophs, presumably because of a massive flux of AMP into the cytokinin biosynthesis pathway.

As previously reported, and as shown in Table 1, spot inoculation of emerging alfalfa root hairs with wild-type *R. meliloti* induced inner cortical cell mitoses, which after 3 to 4 days led to a distinct swelling at the inoculation site termed nodule initiation (Noi) (Dudley et al., 1987). These cortical cell divisions continued, leading to the morphogenesis of root nodules, although in some cases (~5%) wild-type inoculations aborted at this Noi stage (Table 1). By comparison, spot inoculation of emerging alfalfa root hairs with Nod$^-$ bacteria harboring pTZS, either *E. coli* LE392 or *R. meliloti* GMI255 (a 250-kb deletion strain lacking the *nod* gene region in pSymA), also caused swelling of root tissue at the inoculation site within 3 to 4 days, as shown in Figure 2A and summarized in Table 1. At least superficially, this swelling resembled the Noi response elicited by wild-type *R. meliloti*. No visible changes were

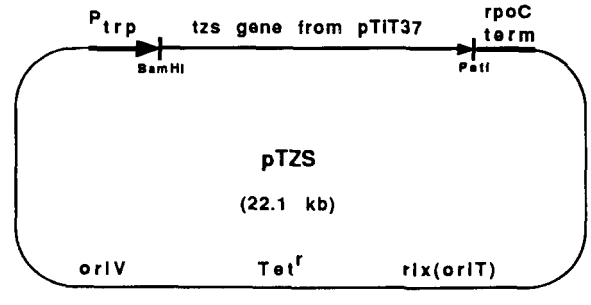

Figure 1. Structure of the Plasmid pTZS.

pTZS contains the trans-zeatin secretion gene (tzs) from A. tumefaciens pTiT37 cloned under the regulation of the trp promoter (P_{trp}) from S. typhimurium (constitutively expressed in *Rhizobium*) and the *rho*-independent transcriptional terminator sequences (term) from the *E. coli rpoC* gene. This IncP plasmid also carries a tetracycline resistance gene (Tet^r), the broad host range *oriV* replication origin, and the *oriT* (*rlx*) sequences from pRK2 allowing for conjugal mobilization.

Table 1. Morphogenetic Complementation of Noo Mutations by pTZS

Strain	Mutation	pTZS	Nodulation Phenotype[a]		
			Mac	Ndv	LegHb
1021	Wild type	–	95/101 (94%)	90/101 (89%)	90/101 (89%)
LE392	(*E. coli*, *trp*R)	–	0/32	0	0
		+	39/80 (49%)	0	0
GMI255	250-kb deletion	–	0/36	0	0
	(Δ *nod nif fix*)	+	125/169 (74%)	8/169 (5%)	0
TJ8A2	*nod*A::Tn*5*	–	0/39	0	0
		+	83/95 (87%)	67/95 (70%)	0
TJ2B2	*nod*B::Tn*5*	–	0/21	0	0
		+	34/36 (94%)	31/36 (86%)	0
7055	*exo*F::Tn*5*	–	11/12 (92%)	10/12 (83%)	0

[a] Bacteria were spot inoculated onto the emerging root hair zone of 3-day-old alfalfa seedlings. After 7 days, roots were scored for Mac (mitotic activation, defined as a visible swelling at the inoculation site); after 14 days, roots were scored for Ndv (nodule development, defined as continued cell divisions to produce a nodulelike structure larger than the root diameter) and for LegHb (leghemoglobin production, defined as pink-colored nodules). Data represent the numbers (and %) of spot inoculation sites showing each phenotype.

observed on alfalfa roots that were spot inoculated with *E. coli* LE392 or *R. meliloti* GMI255 harboring the parental vector pTE3.

To determine the extent to which swellings elicited by pTZS$^+$ bacteria imitated the early events in nodule initiation, we examined serial longitudinal sections of spot inoculated roots and also scored inoculated roots for root hair deformation and root hair curling. Inoculation with Nod$^-$ bacteria harboring pTZS had no effect on root hair morphology (deformation or hair curling) when scored in a double-blind assay (n = 8, data not shown). By contrast, as shown in Figures 2B and 2C, root swellings induced by inoculation with pTZS$^+$ bacterial strains were due to mitotic activity in the cortical cell region in all cases (n = 11), not by cell enlargement or mitoses in other cell types. As was the case with Noi events elicited by wild-type *Rhizobium* (see Figure 2C in Dudley et al., 1987), pTZS$^+$ bacteria induced periclinal mitoses in the innermost cortical cell layers (arrowhead in Figure 2B) and more randomly oriented (primarily anticlinal) mitoses in the outer cortical cell layers. Mitoses proliferated throughout the cortical region of the root to give rise to a mass of dividing cells (Figure 2C). The innermost elongated cells, derived from the initial periclinal divisions, differentiated into xylem tissue at the base of developing pTZS-induced nodules (X in Figure 2C), and the initiation of the nodule peripheral vascular system was often observed in the mass of dividing cells induced by pTZS$^+$ bacteria as cells elongating out into the periphery of the developing nodule (arrowheads in Figures 2C and 2E).

Zeatin Secretion Complements *nod* Gene Mutations for Nodule Morphogenesis and Early Nodulin Gene Expression

As described above, Nod$^-$ bacterial strains harboring a constitutive *tzs* gene induced cell divisions in the same root cells in which mitoses were induced by *Rhizobium* inoculation. The extent to which such zeatin-induced cell divisions imitated the activity of Nod factors was tested by mobilizing pTZS into a variety of specific *nod* gene mutants deficient in the production of Nod factors. In all cases, pTZS rescued the ability of *Rhizobium nod* gene mutants to form nodulelike structures on alfalfa roots (Table 1 and data not shown). In general, the mitoses induced by single *nod* gene mutants harboring pTZS developed into much larger nodules than those formed by either LE392/pTZS or GMI255/pTZS (Figure 2D). Larger nodules developed an extensive bifurcating vascular system that was easily observed in cleared whole mounts (Figure 2E). The anatomy of pTZS-induced nodules was similar to that described for empty nodules formed by Exo$^-$ mutants of *R. meliloti* (Van de Wiel et al., 1990b). As shown in Figures 2 and 3, pTZS-induced nodules consisted of an outer nodule cortex, one to several diffuse "meristem-like" regions (yellowish regions in Figures 2D and 2F) consisting of small dividing cells (asterisks in Figure 3), an enlarged central region of tightly packed parenchyma cells (see Esau, 1977; Van de Wiel et al., 1990a, 1990b) including both highly vacuolated cells and cells that stained intensely with acidic toluidine blue (Figures 3B and 3C), and a bifurcating vascular system differentiating from the root stele (Figures 2E and 3B). In some cases, as shown in Figure 2F, branched nodules were induced by pTZS$^+$ bacteria, indicating that the meristem-like regions in pTZS nodules can develop into persistent meristems, unlike the case with Exo$^-$ nodules (Yang et al., 1992).

The similarities between the responses of alfalfa roots to pTZS$^+$ bacteria and to *Rhizobium* were further examined using in situ hybridization experiments to test for the pTZS-induced expression of alfalfa *MsENOD2*. The expression of *ENOD2* has been observed both in nodules formed by wild-type *Rhizobium* and in invasion-deficient nodules formed by Exo$^-$ and nodule development (Ndv$^-$) *Rhizobium* mutants (Dickstein et al., 1988; Hirsch et al., 1989). In normal alfalfa nodules, *ENOD2* expression is restricted to the cells of the

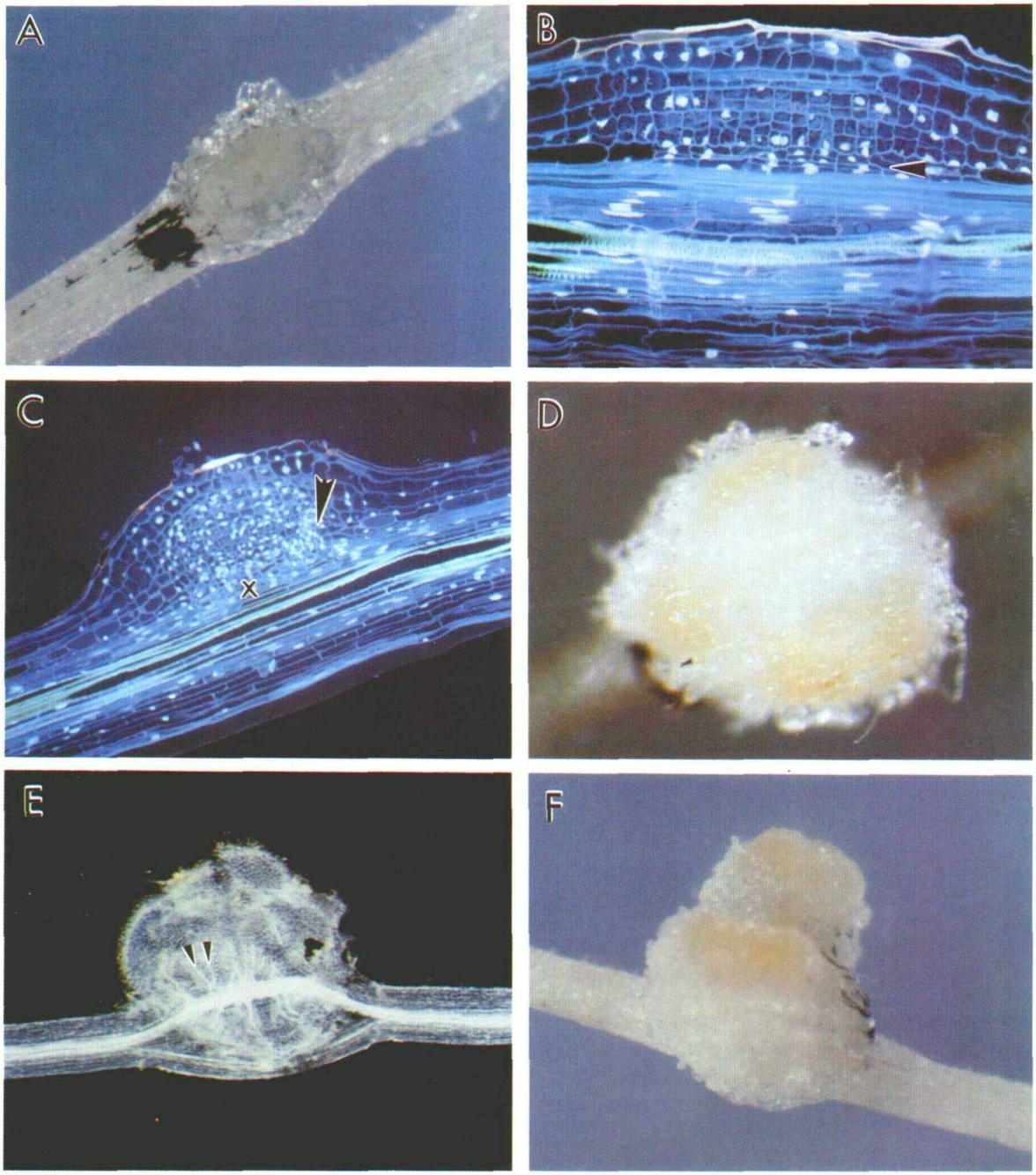

Figure 2. Structure of pTZS-Induced Alfalfa Nodules.

(A) Swelling observed at microinoculation site on an alfalfa root 5 days after inoculation with GMI255/pTZS.
(B) and **(C)** Fluorescence photomicrographs of longitudinal sections of pTZS-induced swellings elicited 36 hr **(B)** and 7 days **(C)** after inoculation with GMI255/pTZS. Sections were stained with 4',6-diamidino-2-phenylindole and acridine orange. The X indicates the xylem tissues at the base of the developing pTZS-induced nodule, and the arrowheads indicate peripheral elongated cells in the early stages of vascular differentiation.
(D) pTZS-induced alfalfa nodule formed 14 days after inoculation with TJ2B2/pTZS, a *nodB*::Tn*5* mutant harboring the zeatin secretion plasmid. Note the yellowish color of the multiple "meristem-like" regions.
(E) Dark-field view of a cleared pTZS-induced nodule 14 days after inoculation with TJ2B2/pTZS. The nodule was cleared with lactic acid, whole mounted, and squashed under a coverslip. Arrowheads indicate the bifurcating vascular system. The harsh clearing and squashing conditions usually led to some fragmentation of the nodules.
(F) Branching pTZS-nodule formed 21 days after inoculation with GMI255/pTZS. Note the yellowish color of the two meristematic regions.

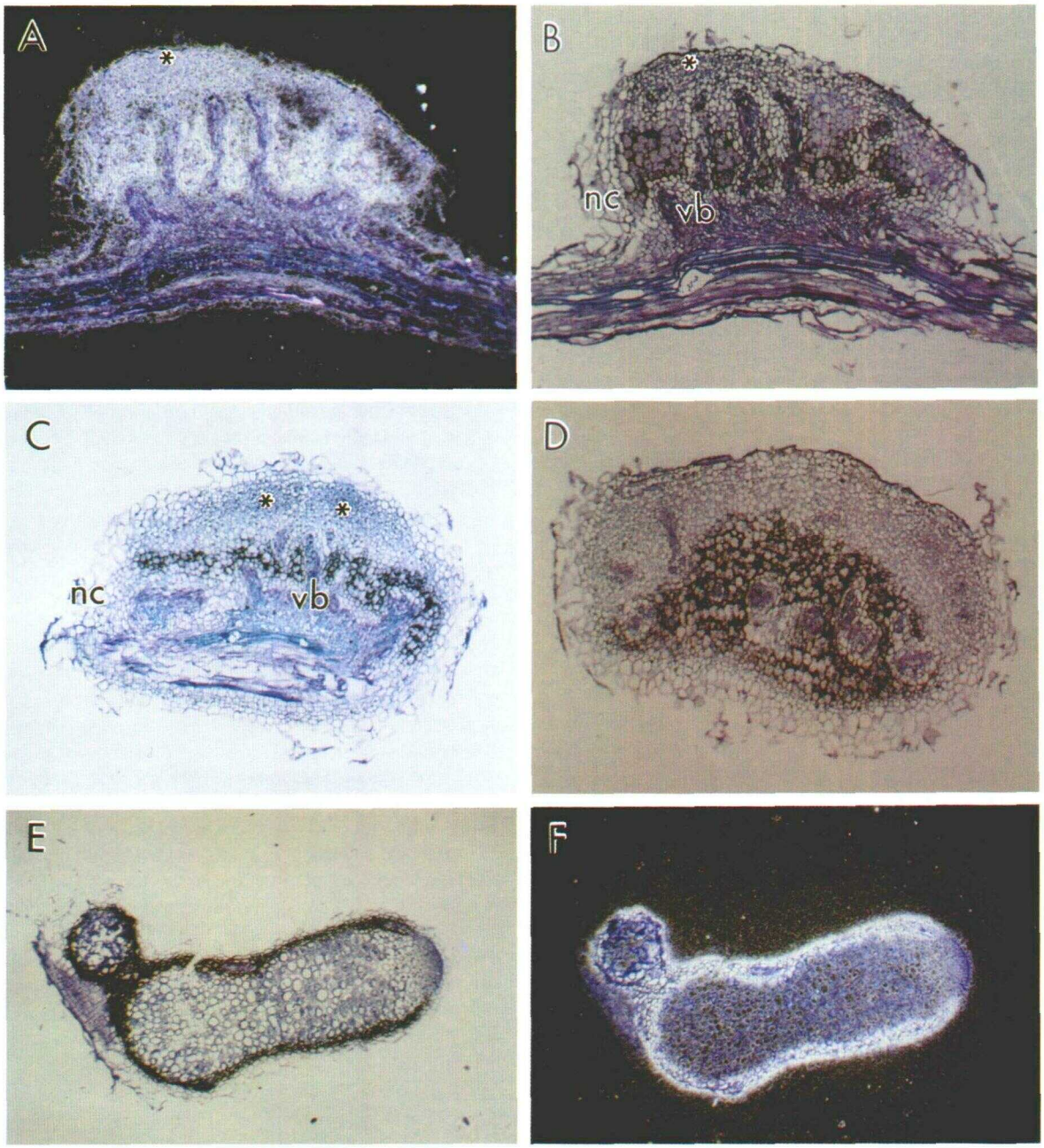

Figure 3. Expression of *ENOD2* in pTZS-Induced and Wild-Type Alfalfa Nodules.

In situ hybridizations of alfalfa nodule sections with a ^{35}S-labeled antisense *MsENOD2* probe. Sections were counterstained with acidic toluidine blue. No hybridization was observed using sense strand *ENOD2* probes.

(A) and **(B)** Dark-field and bright-field photomicrographs, respectively, showing expression of *ENOD2* in a longitudinal section of a 21-day-old nodule formed by SL44/pTZS. Asterisks indicate "meristem-like" regions consisting of small dividing cells; vb, vascular bundles; nc, nodule cortex.

(C) Bright-field photomicrograph showing expression of *ENOD2* in an oblique section of a 21-day-old nodule formed by SL44/pTZS.

(D) Bright-field photomicrograph showing expression of *ENOD2* in a transverse section of a 21-day-old nodule formed by SL44/pTZS. Expression of *ENOD2* in pTZS-induced nodules is restricted to the parenchyma cells surrounding the differentiating nodule vascular system.

(E) and **(F)** Bright-field and dark-field photomicrographs, respectively, showing *ENOD2* expression in a mature nodule formed by wild-type *R. meliloti*, indicating that *ENOD2* expression is also restricted to the nodule parenchyma tissue at the base of the nodule and surrounding the peripheral vascular tissue. No hybridization was observed in the nodule meristem, the nodule cortex, or the central symbiotic zone.

peripheral nodule parenchyma that separate the nodule vascular system from the root and nodule cortical cells on the outside and from the nodule symbiotic zone on the inside (Van de Wiel et al., 1990a). In pTZS-induced nodules, as shown in Figures 3A to 3D, high levels of *ENOD2* expression were detected primarily in the vacuolated parenchyma cells surrounding the differentiating vascular bundles at the base of the nodule. This observed pattern of *ENOD2* expression was virtually identical to that observed previously in empty nodules produced by *Rhizobium exo* mutants (Van de Wiel et al., 1990b; Allen et al., 1991). The parenchyma tissue in Exo$^-$ nodules fails to "organize" around a central symbiotic zone (Allen et al., 1991), and *ENOD2* expression is restricted to the parenchyma cells at the base of the nodule (Van de Wiel et al., 1990b). No *ENOD2* expression was detected in the nodule cortex or in the vascular bundles (Figures 3A to 3D), and low levels of expression were detected in the small meristem-like cells in the distal region of pTZS-induced nodules (asterisks in Figures 3A to 3C). As was also observed with Exo$^-$ nodules (see Figure 2F in Van de Wiel et al., 1990b), a layer of nonexpressing cells separated the vascular bundles from the parenchyma cells expressing *ENOD2* (Figures 3C and 3D). The pattern of *ENOD2* expression in pTZS-induced nodules differed spatially, but not developmentally, from that observed in wild-type nodules formed by *R. meliloti* in which the development of nodule parenchyma is primarily peripheral (Figures 3E and 3F; Van de Wiel et al., 1990a, 1990b; Allen et al., 1991).

Response to Zeatin Secretion Is Regulated by Host Plant Factors

To further investigate the similarity between nodule initiation and pTZS-induced cell divisions in alfalfa roots, we examined the extent to which the nodulation regulatory systems in host roots also regulate the response of root cortical cells to bacteria harboring pTZS. It has long been known that nodule formation in legumes is blocked by the presence of reduced nitrogen in the soil (Thornton, 1936; Munns, 1968). The data in Table 2 confirm that the presence of 10 mM nitrate inhibited nodule initiation and indicate that 10 mM nitrate also inhibited the response of alfalfa roots to pTZS$^+$ bacteria. In this respect, the response of alfalfa roots to pTZS$^+$ bacteria is regulated in the same manner as the formation of nodules in the absence of *Rhizobium* (Truchet et al., 1989a). We also observed that nodule development (i.e., continued cell divisions) was more sensitive to nitrate than nodule initiation (data not shown).

In alfalfa, the response of root cells to *Rhizobium* inoculation (which leads to nodule initiation) is dependent on the stage of development (Bhuvaneswari et al., 1981). Specifically, the region of emerging root hairs is known to be most competent to respond to inoculation, whereas the mature regions of the root are much less competent (Table 2). This same pattern of developmental regulation was observed in response to inoculations with Nod$^-$ bacterial strains harboring pTZS, indicating that the sensitivity of alfalfa root cells to *Rhizobium* Nod factors correlates with the sensitivity of root cortical cells to zeatin (Table 2).

DISCUSSION

Phenotype analyses have demonstrated that the *nod* genes of *R. meliloti* are strictly required for both root hair deformation and cortical cell divisions in alfalfa (Hirsch et al., 1982; Debellé et al., 1986; Dudley et al., 1987). These genes are involved in the production of signal molecules that act as host-specific nodulation signals (Lerouge et al., 1990). The structures of the Nod factors are novel lipooligosaccharides (Lerouge et al., 1990), and thus are structurally unlike any of the well-characterized classes of phytohormones, including zeatin or other cytokinins. The primary Nod factor produced by *R. meliloti* is NodRmIV (Ac,S) consisting of a tetrameric β-1,4-linked-*N*-acetyl glucosamine backbone, modified on the reducing end with sulfate and on the nonreducing end with an *N*-acyl fatty acid substitution (Lerouge et al., 1990; Roche et al., 1991). In alfalfa roots, Nod factors cause a rapid membrane

Table 2. Host Root Regulation of pTZS-Induced Mitotic Activation

Inoculation/Growth Conditions	Mitotic Activation (No. Inoculations[a]) Strain: 1021	LE392/pTZS	GMI255/pTZS
Emerging RH[b] zone, 0 mM NO_3	88/107 (88%)	34/66 (52%)	59/77 (77%)
Emerging RH zone, 10 mM NO_3	10/35 (28%)	0/17 (0%)	5/23 (22%)
Mature RH zone, 0 mM NO_3	18/60 (30%)	8/42 (19%)	ND[c]

[a] Number of spot inoculation sites showing a visible swelling 7 days after inoculation.
[b] RH, root hair.
[c] ND, not determined.

depolarization in root hair cells (Ehrhardt et al., 1992), root hair branching and distortion (Lerouge et al., 1990), and nodule organogenesis (Truchet et al., 1991). With the purification and biochemical description of the *Rhizobium* nodulation signals, a primary goal in nodulation research is now focused on understanding the mechanism by which Nod factors, such as NodRmIV (Ac,S), elicit appropriate responses in specific host plant root cells.

In this study, we found that a plasmid constitutively expressing the *tzs* gene could replace the *Rhizobium nod* genes for one function: namely, the stimulation of mitoses in root inner cortical cells and the consequent organogenesis of root nodules. Several features of the response of alfalfa roots to pTZS$^+$ bacteria indicate a relationship to true nodulation: first, the initial zeatin-induced cell divisions occurred in the inner root cortical cells, the same cells in which cell divisions are induced by *Rhizobium*; second, pTZS-induced nodules express an early nodulin gene (*MsENOD2*) in a pattern that mimics *ENOD2* expression in infection-deficient nodules formed by Exo$^-$ mutants of *R. meliloti*; third, as is the case with *Rhizobium* nodulation, the cell divisions and consequent nodulelike structures were statistically more likely to form when pTZS$^+$ bacteria were inoculated onto the emerging root hair zone and were less likely to form elsewhere; and fourth, the pTZS-induced mitoses were partially suppressed by environmental nitrate.

Our results extended previous observations that exogenous application of cytokinins, but not other plant hormones, rapidly induced *ENOD2* expression in Sesbania roots (Dehio and de Bruijn, 1992) and that *ENOD2* was expressed in legume hairy root tumors that have an increased sensitivity to endogenous cytokinins (Govers et al., 1990). The pattern of cell divisions and vascularization induced by bacteria harboring pTZS is distinctive for nodule initiation and differs significantly from the patterns of cell division that lead to lateral root formation (Libbenga and Harkes, 1973; Hirsch et al., 1982; Dudley et al., 1987; Truchet et al., 1989b). The spatial pattern of *ENOD2* expression in pTZS-induced nodules is virtually identical to that described previously in root nodules formed by Exo$^-$ *Rhizobium* mutants that secrete Nod factors but lack an exopolysaccharide required for nodule invasion (Van de Wiel et al., 1990b; Allen et al., 1991). A simple interpretation of these results is that the inner cortical cells of alfalfa roots serve as target cells for mitotic activation by cytokinins and that the anatomical effects of zeatin secretion on root cells mimic the morphogenetic activity of Nod factors. The bifurcating vascular system of root nodules may develop as a secondary response to continued mitoses in the cortical cell region of alfalfa roots.

Unlike wild-type *Rhizobium*, Nod$^-$/pTZS$^+$ bacterial strains had no significant effect on root hair morphology (branching, distortion, or curling). It is unlikely that this negative result relates to the adenine auxotrophy we observed in pTZS$^+$ *Rhizobium* strains (which we interpret as an indication that *tzs* expression depletes cytoplasmic pools of adenine and/or intermediates in the purine biosynthetic pathway). Nodule formation by *Rhizobium* purine auxotrophs is reportedly blocked early in development (Kerppola and Kahn, 1988; Noel et al., 1988), and we have observed that an *R. meliloti* adenine auxotroph (Rm2103) induced the formation of large ineffective nodules on alfalfa (J.B. Cooper, unpublished results). Although bean nodules elicited by purine auxotrophs lack infection threads, this effect was not specific for adenine, and purine auxotrophy had no effect on root hair curling (Noel et al., 1988).

Rhizobium species are known to synthesize and secrete cytokinins (Phillips and Torrey, 1972; Taller and Sturtevant, 1991), but none of the genetic data has indicated that cytokinin production is required for nodule development (i.e., no Nod$^-$ mutants are known to affect cytokinin production). Furthermore, the structural identification of NodRmIV (Ac,S) provides strong evidence against the early hypothesis that nodule development is initiated by cytokinins produced by *Rhizobium* (Phillips and Torrey, 1972; Libbenga et al., 1973; Schmidt et al., 1988). The fact that Nod$^-$/pTZS$^+$ *Rhizobium* strains imitate one effect (nodule initiation) of the modified lipooligosaccharide signal molecules, such as NodRmIV (Ac,S), may indicate instead that Nod factors and cytokinins affect or participate in the same fundamental response system in the plant. Thus, nodule initiation may be regulated by hormonal mechanisms common among the angiosperms. In this context, it is interesting to note the report that kinetin application induces the formation of "pseudonodules" on tobacco roots (Arora et al., 1959).

Application of auxin transport inhibitors (2,3,5-triiodobenzoic acid [TIBA] or *N*-[1-naphyl]phthalamic acid) to legume roots also induced the formation of nodulelike structures that express early nodulin genes, including *ENOD2* (Hirsch et al., 1989; Scheres et al., 1992). In Sesbania roots, TIBA induced very low levels of *ENOD2* expression, whereas cytokinins induced high levels of *ENOD2* expression (Dehio and de Bruijn, 1992). By inhibiting transport of endogenous auxins from the shoot to the root, ATIs might act to increase the cytokinin-to-auxin ratio in root cortical cells, just as inoculation with pTZS$^+$ bacteria would increase this ratio by a converse mechanism. Two significant differences between the response of alfalfa roots to zeatin and to ATIs must be accounted for, however. First, the response of roots to bacteria harboring pTZS is regulated by environmental nitrogen, as is the case with both normal nodules and spontaneous nodules; however, the response to ATIs is reportedly insensitive to reduced nitrogen (Hirsch et al., 1989). Second, both the anatomy and the pattern of *ENOD2* gene expression in pTZS-induced nodules mimic that observed in empty nodules formed by infection-deficient *Rhizobium* mutants, whereas the anatomy and *ENOD2* gene expression pattern observed in ATI-induced nodules mimic that in fully infected nodules formed by wild-type (Nod$^+$ Exo$^+$) *Rhizobium* (Van de Wiel et al., 1990b).

Several distinct models for nodule initiation are consistent with our results. One model is that NodRmIV (Ac,S) directly causes an increase in the cytokinin-to-auxin ratio of root cortical cells or in the sensitivity of cortical cells to these endogenous growth regulators. A second model is that

NodRmIV (Ac,S) (and other Nod factors) act on alfalfa root cells by regulating the same cell cycle control mechanisms that are normally regulated by endogenous cytokinins. In this sense, the cytokinin and Nod factor signal transduction pathways may "intersect" (i.e., share a common step), and the influence of nitrogen and developmental position must be exerted downstream of this common step. Potential target mechanisms include protein kinases, phosphatases, and cyclins homologous to yeast and mammalian cell cycle–related genes (see Jacobs, 1992), endogenous calcium flux regulators (Saunders and Hepler, 1982, 1983), dehydroconiferyl glucosides (Binns et al., 1987), and novel cell cycle regulatory compounds such as trigonelline (Evans et al., 1987). Yet a third model is that NodRmIV (Ac,S) induces nodule morphogenesis by a mechanism involving a novel unknown pathway and that cytokinins indirectly affect one of the steps of this novel pathway.

It is not known whether lipooligosaccharides represent a completely novel class of plant regulators or whether they are structurally similar to a class of endogenous hormones that has not been previously characterized (Fisher and Long, 1992). The recent report that *Rhizobium* Nod factors were able to rescue a carrot embryogenesis mutant (De Jong et al., 1993) is sure to stimulate further interest in the mechanism of action of these bacterial signal molecules. Regardless of the actual mechanism of action, it is likely that the identification of plant receptor(s) for NodRmIV (Ac,S) and for other *nod* gene–associated factors, and elucidation of the mechanism(s) by which NodRmIV (Ac,S) controls mitosis in alfalfa root cortical cells will contribute greatly to our understanding of the regulation of plant cell division by endogenous cytokinins. Conversely, elucidation of the signal transduction pathway by which cytokinins regulate the plant cell–division cycle should help to illuminate the symbiotic control of nodule morphogenesis.

METHODS

DNA Manipulations and Strain Constructions

Bacterial strains and plasmids used in this study are listed in Table 3. The broad host range expression plasmid pTZS carrying a constitutive *trans*-zeatin secretion (*tzs*) gene was constructed using standard recombinant DNA methods (Maniatis et al., 1989). Specifically, the cohesive ends of the 1.4-kb BamHI-HindIII fragment from pDA112-1 containing the *tzs* gene (Akiyoshi et al., 1985) were filled in using the Klenow fragment of DNA polymerase, and this fragment was subcloned into the HincII site of pUC18 to create an intermediate plasmid with appropriate cloning sites. The BamHI-PstI fragment of this intermediate plasmid was then cloned into the expression vector pTE3 (Egelhof and Long, 1985). pTZS contains ~270 bp of *tzs* sequences upstream of the 729-bp open reading frame encoding the isopentenyl transferase (Akiyoshi et al., 1985). *Rhizobium meliloti* was grown on TY plates with streptomycin (500 mg/L) (Meade et al., 1982) or on AB minimal plates (Chilton et al., 1974). Broad host range plasmids were mobilized from *Escherichia coli* into *R. meliloti* using triparental matings with the Tra^+ Mob^+ helper plasmid pRK2013 (Ditta et al., 1980). Transconjugants overexpressing the *tzs* gene behaved as functional adenine auxotrophs (i.e., grew slowly on minimal medium and normally on minimal medium supplemented with 0.2 mg/mL adenine).

Plant Inoculations

Alfalfa roots were inoculated using the spot inoculation technique (Dudley et al., 1987). Seeds of *Medicago sativa* cv AS13 (Ferry Morse Seed Co., Mountain View, CA) were surface sterilized (35 min in 70% EtOH followed by 35 min in 5.25% hypochlorite), rinsed thoroughly with sterile H_2O, and imbibed overnight. Imbibed seeds were germinated for 22 to 24 hr on inverted 0.8% agar plates and planted on square Petri plates containing Nod3, a buffered nodulation medium (2 mM

Table 3. Bacterial Strains and Plasmids Used in This Study

Strain/Plasmid	Relevant Phenotype[a]	Source
E. coli		
LE392	*trp*R55	Murray et al. (1977)
R. meliloti		
1021	Wild type; Sm^r	Meade et al. (1982)
GMI255	250-kb deletion of pSymA; Sm^r Nm^r	Truchet et al. (1985)
SL44	8.7-kb deletion of *nodD1ABC*; Sm^r Nm^r	Fisher et al. (1988)
TJ8A2	*nod*A::Tn*5*; Sm^r Nm^r	Jacobs et al. (1985)
TJ2B2	*nod*B::Tn*5*; Sm^r Nm^r	Jacobs et al. (1985)
7055	*exo*F::Tn*5*; Sm^r Nm^r	Leigh et al. (1985)
2013	Adenine auxotroph derivative of 1021	Meade et al. (1982)
Plasmids		
pRK2013	tra^+ mob^+	Ditta et al. (1980)
pDA112-1	*tzs* gene from pTiT37 of *A. tumefaciens*	Akiyoshi et al. (1985)
pTE3	Broad host range expression vector	Egelhof and Long (1985)
pA2ENOD2	Alfalfa cDNA clone for ENOD2	Dickstein et al. (1988)
pTZS	Broad host range *tzs* expression plasmid	This study

[a] Sm^r, streptomycin resistant; Nm^r, neomycin resistant.

$CaSO_4$, 1 mM $MgSO_4$, 0.5 mM K_2HPO_4, 1 mM 2-[*N*-morpholino]ethane sulfonic acid, pH 6.5, and trace elements of Murashige–Skoog [1962] medium lacking KI). Three days later, roots were microinoculated on the emerging root hair zone with small droplets of bacteria ($\sim 10^{10}$ cells per mL) suspended in 10 mM $MgSO_4$, as described previously by Dudley et al. (1987).

Microscopy

For microscopy, pTZS-induced nodules were fixed overnight (4% formaldehyde in PBS), dehydrated through an EtOH series, and embedded in JB4 resin (Polysciences, Warrington, PA). Sections (4 μm) were cut with a glass knife microtome and stained with 4′,6-diamidino-2-phenylindole and acridine orange, as described previously by Dudley et al. (1987). For visualizing the vascularization patterns in nodules, 8 to 10 nodules were cleared overnight in 85% lactic acid at 65°C, and nodule whole mounts were squashed under a coverslip (Dudley et al., 1987). Photomicrographs were taken with an epifluorescence microscope (Alphaphot; Nikon, Garden City, NY) or with a dissecting microscope (model M5S; Wild, Heerbrugg, Switzerland).

In Situ Hybridizations

In situ hybridization experiments were performed essentially as described by Meyerowitz (1987) and Smith et al. (1987). Tissues were fixed in 20 mM KPi, 200 mM KCl containing 1% glutaraldehyde at 0°C for 4 hr, dehydrated through an EtOH series, and embedded in Paraplast. Sections (10-μm thick) cut with a rotary microtome were dried onto slides at 45°C overnight, deparaffinized with toluene, rehydrated through an EtOH series, treated with 0.2 M HCl for 20 min at room temperature, 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 8) for 30 min at 70°C, proteinase K (1 mg/mL in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.6) for 30 min at 37°C, and 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at room temperature and then dehydrated through an EtOH series and air dried. Slides were incubated overnight at 45°C with ^{35}S-labeled RNA probes in 50% formamide, 100 mg/mL dextran sulfate, 0.6 M NaCl, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 × Denhardt's solution (0.02% Ficoll, 0.02% PVP, 0.02% BSA), 37.5 mM DTT, 1 mg/mL poly(A) RNA, and 0.8 mg/mL yeast tRNA.

Probes were produced by in vitro transcription of the PvuII fragment from pA2ENOD2 (Dickstein et al., 1988) with T7 or T3 polymerase using an in vitro transcription kit (Stratagene), hydrolyzed to 50 to 100 bases in length using 0.2 M $NaCO_3$ buffer, pH 10.2, at 60°C, and denatured before use at 95°C for 2 min. Following hybridization, slides were washed with 4 × SSPE, 10 mM DTT for more than 60 min, treated with RNase A (20 μg/mL in 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA) for 60 min at 37°C, and washed with this same buffer lacking RNase (30 min, 37°C), 3 × SSPE (30 min, 50°C), twice with 2 × SSPE (30 min, room temperature), and once with 0.75M SSPE (>60 min, room temperature); slides were then hydrated through an EtOH series containing 0.3 M $(NH_4)_2OAc$ and air dried. Slides were coated with NTB2 nuclear emulsion (Kodak), exposed at 4°C for an appropriate time, and developed using Kodak D19. Sections were counterstained after autoradiography with toluidine blue O. Bright- and dark-field photomicrographs were taken with a light microscope (Axioskop; Zeiss, Thornwood, NY).

ACKNOWLEDGMENTS

This work was supported by a generous gift from the Du Pont Company and by National Science Foundation Presidential Young Investigator awards to S.R.L. and J.B.C. We are indebted to Donna Akiyoshi for the gift of pDA112-1 and to Rebecca Dickstein for the gift of pA2ENOD2. We also thank Mark Dudley for help with JB4 sectioning, advice on the double staining technique, and for providing the cover micrograph; Debi Fisher for assistance with paraffin sectioning; Page Ericson for advice concerning autoradiography; Alexandra Bloom for typing; and Ruth Finkelstein for critically reading the manuscript.

Received August 23, 1993; accepted December 9, 1993.

REFERENCES

Akiyoshi, D.E., Regier, D.A., Jen, G., and Gordon, M.P. (1985). Cloning and sequencing of the *tzs* gene from *Agrobacterium tumefaciens* strain T37. Nucl. Acids Res. **13,** 2773–2788.

Allen, T., Raja, S., and Dunn, K. (1991). Cells expressing ENOD2 show differential spatial organization during the development of alfalfa root nodules. Mol. Plant-Microbe Interact. **2,** 139–146.

Arora, N., Skoog, F., and Allen, O.N. (1959). Kinetin-induced pseudonodules on tobacco roots. Am. J. Bot. **46,** 610–613.

Bhuvaneswari, T.V., Bhagwat, A.A., and Bauer, W.D. (1981). Transient susceptibility of root cells in four common legumes to nodulation by *Rhizobia.* Plant Physiol. **68,** 1144–1149.

Binns, A.N., Chen, R.H., Wood, H.N., and Lynn, D.G. (1987). Cell division promoting activity of naturally occurring dehydroconiferyl glucosides: Do cell wall components control cell division? Proc. Natl. Acad. Sci. USA **84,** 615–619.

Chilton, M.-D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P., and Nester, E.W. (1974). *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc. Natl. Acad. Sci. USA **9,** 3672–3676.

Debellé, F., Rosenberg, C., Vasse, J., Maillet, F., Martinez, E., Dénarié, J., and Truchet, G. (1986). Assignment of symbiotic developmental phenotypes to common and specific nodulation genetic loci of *Rhizobium meliloti.* J. Bacteriol. **168,** 1075–1086.

Dehio, C., and de Bruijn, F.J. (1992). The early nodulin gene SrENOD2 from *Sesbania rostrata* is inducible by cytokinin. Plant J. **2,** 117–128.

De Jong, A.J., Heidstra, R., Spaink, H.J., Hartog, M.V., Meijer, E.A., Hendriks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., Van Kammen, A., and De Vries, S.C. (1993). *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell **5,** 615–620.

Dénarié, J., and Roche, P. (1992). *Rhizobium* nodulation signals. In Molecular Signals in Plant-Microbe Communications. D.P.S. Verma, ed (Boca Raton, FL: CRC Press), pp. 295–324.

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

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980). Broad host range DNA cloning system for gram-negative bacteria:

Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77,** 7347–7351.

Dudley, M.E., and Long, S.R. (1989). A non-nodulating alfalfa mutant displays neither root hair curling nor early cell division in response to *Rhizobium meliloti*. Plant Cell **1,** 65–72.

Dudley, M.E., Jacobs, T.W., and Long, S.R. (1987). Microscopic studies of cell divisions induced in alfalfa roots in response to *Rhizobium meliloti*. Planta **171,** 289–301.

Egelhof, T.T., and Long, S.R. (1985). *Rhizobium meliloti* nodulation genes: Identification of *nodDABC* gene products, purification of NodA protein, and expression of *nodA* in *Rhizobium meliloti*. J. Bacteriol. **164,** 591–599.

Ehrhardt, D.W., Atkinson, E.M., and Long, S.R. (1992). Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science **256,** 998–1000.

Esau, K. (1977). Anatomy of Seed Plants. (New York: John Wiley and Sons), pp. 61–70.

Evans, L.S., Tramontano, W.A., and Gill, R. (1987). A natural substance that regulates the cell cycle in complex plant tissues. Phytochemistry **26,** 2891–2893.

Faucher, C., Maillet, F., Vasse, J., Rosenberg, C., Van Brussel, A.A.N., Truchet, G., and Dénarié. J. (1988). *Rhizobium meliloti* host range *nodH* gene determines production of an alfalfa-specific extracellular signal. J. Bacteriol. **170,** 5489–5499.

Faucher, C., Camut, S., Dénarié, J., and Truchet, G. (1989). The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *R. leguminosarum* bv. *viciae* and determine changes in the production of plant-specific extracellular signals. Mol. Plant-Microbe Interact. **2,** 291–300.

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

Fisher, R.F., and Long, S.R. (1992). *Rhizobium*-plant signal exchange. Nature **357,** 655–660.

Fisher, R.F., Egelhof, T.T., Mulligan, J.T., and Long, S.R. (1988). Specific binding of proteins from *Rhizobium meliloti* cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. Genes Dev. **2,** 282–293.

Franssen, H.J., Nap, J.-P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., 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., Nap, J.-P., and Bisseling, T. (1992). Nodulins in root nodule development. In Biological Nitrogen Fixation, G. Stacey, R.H. Burris, and H.J. Evans, eds (New York: Chapman and Hall), pp. 598–624.

Govers, F., Moerman, M., Downie, J.A., Hooykaas, P.J.J., Franssen, H.J., Louwerse, J., Van Kammen, A., and Bisseling, T. (1990). Function and regulation of the early nodulin gene ENOD2. In Genetic Engineering of Crop Plants, G.W. Lycett and D.W. Grierson, eds (London: Butterworths), pp. 259–269.

Hirsch, A.M., Long, S.R., Bang, M., Haskins, N., and Ausubel, F.M. (1982). Structural studies of alfalfa roots infected with nodulation mutants of *Rhizobium meliloti*. J. Bacteriol. **151,** 411–419.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA **86,** 1244–1248.

Jacobs, T. (1992). Control of the cell cycle. Dev. Biol. **153,** 1–15.

Jacobs, T.W., Egelhof, T.T., and Long, S.R. (1985). Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. J. Bacteriol. **162,** 469–476.

Kerppola, T.K., and Kahn, M.L. (1988). Symbiotic phenotypes of auxotrophic mutants of *Rhizobium meliloti* 104A14. J. Gen. Microbiol. **134,** 913–919.

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.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344,** 781–784.

Libbenga, K.R., and Harkes, P.A.A. (1973). Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. Planta **114,** 17–28.

Libbenga, K.R., Van Iren, F., Bogers, R.J., and Schraag-Lamers, M.F. (1973). The role of hormones and gradients in the initiation of cortex proliferation and nodule formation in *Pisum sativum* L. Planta **114,** 29–39.

Long, S.R. (1992). Genetic analysis of *Rhizobium* nodulation. In Biological Nitrogen Fixation, G. Stacey, R.H. Burris, and H.J. Evans, eds (New York: Chapman and Hall), pp. 560–597.

Long, S.R., and Cooper, J.B. (1988). Overview of symbiosis. In Molecular Genetics of Plant-Microbe Interactions, R. Palacios and D.P.S. Verma, eds (St. Paul, MN: American Phytopathological Society Press), pp. 163–178.

Long, S.R., and Staskawicz, B.J. (1993). Prokaryotic plant parasites. Cell **73,** 921–935.

Maniatis, T., Sambrook, J., and Fritsch, E.F. (1989). Molecular Cloning. A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Meade, H., 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*. J. Bacteriol. **149,** 114–122.

Meyerowitz, E.M. (1987). In situ hybridization to RNA in plant tissue. Plant Mol. Biol. Rep. **5,** 242–250.

Morris, D.A. (1981). Distribution and metabolism of root-applied cytokinins in *Pisum sativum*. Physiol. Plant. **52,** 251–256.

Munns, D.N. (1968). Nodulation of *Medicago sativa* in solution culture. III. Effects of nitrate on root hairs and infection. Plant Soil **24,** 33–47.

Murashige, T., and Skoog, F. (1962). A revised medium for the rapid growth and bioassay of tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Murray, N.E., Brammar, W.J., and Murray, K. (1977). Lambdoid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. **150,** 53–61.

Noel, K.D., Diebold, R.J., Cava, J.R., and Brink, B.A. (1988). Rhizobial purine and pyrimidine auxotrophs: Nutrient supplementation, genetic analysis, and the symbiotic requirement for de novo purine biosynthesis. Arch. Microbiol. **149,** 499–506.

Phillips, D.A., and Torrey, J.G. (1972). Studies on cytokinin production by *Rhizobium*. Plant Physiol **49,** 11–15.

Roche, P., Lerouge, P., Ponthus, C., and Promé, J.C. (1991). Structural determination of bacterial nodulation factors involved in the *Rhizobium meliloti*-alfalfa symbiosis. J. Biol. Chem. **266,** 10933–10940.

Saunders, M.J., and Hepler, P.K. (1982). Calcium ionophore A23187 stimulates cytokinin-like mitosis in *Funaria*. Science **217,** 943–945.

Saunders, M.J., and Hepler, P.K. (1983). Calcium antagonists and calmodulin inhibitors block cytokinin-induced bud formation in *Funaria*. Dev. Biol. **99,** 41–49.

Scheres, B., Van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Zwartkruis, F., Wolters, A.M., Gloudemans, T., Van Kammen, A., and Bisseling, T. (1990a). The ENOD12 gene product is involved in the infection process during 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.

Scheres, B., McKhann, H.I., Zalensky, A., Löbler, M., Bisseling, T., and Hirsch, A.M. (1992). The PsENOD12 gene is expressed at two different sites in Afghanistan pea pseudonodules induced by auxin transport inhibitors. Plant Physiol. **100,** 1649–1655.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. (1988). *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stimulate mitosis of plant cells. Proc. Natl. Acad. Sci. USA **85,** 8578–8582.

Smith, A.G., Hinchee, M., and Horsch, R. (1987). Cell and tissue specific expression localized by in situ RNA hybridization in floral tissues. Plant Mol. Biol. Rep. **5,** 237–241.

Spaink, H.P. (1992). Rhizobial lipo-oligosaccharides: Answers and questions. Plant Mol. Biol. **20,** 977–986.

Taller, B.J., and Sturtevant, D.B. (1991). Cytokinin production by rhizobia. In Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 1. H. Hennecke and D.P.S. Verma, eds (Dordrecht: Kluwer), pp. 215–221.

Thimann, K.V. (1936). On the physiology of the formation of nodules on legume roots. Proc. Natl. Acad. Sci. USA **22,** 511–515.

Thornton, H.G. (1936). Action of sodium nitrate on infection of lucerne root hairs by nodule bacteria. Proc. Roy. Soc. (Lond.) Ser. B **119,** 474–492.

Truchet, G., Debellé, F., Vasse, J., Terzaghi, B., Garnerone, A.-M., Rosenberg, C., Batut, J., Maillet, F., and Dénarié, J. (1985). Identification of a *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. J. Bacteriol. **164,** 1200–1210.

Truchet, G., Barker, D.G., Camut, S., De Billy, F., Vasse, J., and Huguet, T. (1989a). Alfalfa nodulation in the absence of *Rhizobium*. Mol. Gen. Genet. **219,** 65–68.

Truchet, G., Camut, S., De Billy, F., Odorico, R., and Vasse, J. (1989b). The *Rhizobium*-legume symbiosis. Two methods to discriminate between nodules and other root-derived structures. Protoplasma **149,** 82–88.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., De Billy, F., Promé, J.-C., and Dénarié, J. (1991). Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature **351,** 670–673.

Utrup, L.J., Cary, A.J., and Norris, J.H. (1993). Five nodulation mutants of white sweetclover (*Melilotus alba* Desr.) exhibit distinct phenotypes blocked at root hair curling, infection thread development, and nodule organogenesis. Plant Physiol. **103,** 925–932.

Van Brussel, A.N.N., Zaat, S.A.J., Canter Cremers, H.C.J., Wijffelman, C.A., Pees, E., Tak, T., and Lugtenberg, B.J.J. (1986). Role of plant root exudate and sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of TSR factor which causes thick and short roots on common vetch. J. Bacteriol. **165,** 517–522.

Van de Wiel, C., Scheres, B., Franssen, H.J., Van Lierop, M.-J., Van Lammeren, A., Van Kammen, A., and Bisseling, T. (1990a). The early nodulin ENOD2 is located in the nodule specific parenchyma (inner cortex) of pea and soybean nodules. EMBO J. **9,** 1–7.

Van de Wiel, C., Norris, J.H., Bochenek, B., Dickstein, R., Bisseling, T., and Hirsch, A.M. (1990b). Nodulin gene expression and ENOD2 localization in effective, nitrogen-fixing and ineffective, bacteria-free nodules of alfalfa. Plant Cell **2,** 1009–1017.

Wood, S.M., and Newcomb, W. (1989). Nodule morphogenesis: The early infection of alfalfa (*Medicago sativa*) root hairs by *Rhizobium meliloti*. Can. J. Bot. **67,** 3108–3122.

Yang, C., Signer, E.R., and Hirsch, A.M. (1992). Nodules initiated by *Rhizobium meliloti* exopolysaccharide mutants lack a discrete, persistent nodule meristem. Plant Physiol. **98,** 143–151.

Yang, W.C., Katinakis, P., Hendriks, P., and Smolders, A. (1993). Characterization of GmENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development. Plant J. **3,** 573–585.