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Developmental Regulation of Nodule-Specific Genes in Alfalfa Root Nodules

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We have cloned alfalfa nodule-specific cDNAs that code for leghemoglobin (Lb), glutamine synthetase (GS), and three unidentified nodulins. Hybrid-select translation of nodule RNA followed by 2-D gel electrophoresis showed that the Lb-specific cDNA corresponded to at least four Lb species of 12 kDa. One of the unidentified cDNA clones (N-32/34) corresponded to at least five polypeptides of 32–34 kDa; a second unidentified cDNA clone (N-14) corresponded to an individual polypeptide of 14 kDa. The *in vitro* translation product(s) of the RNA hybrid selected by the third unidentified cDNA clone (N-22) formed a single band at 22 kDa on a one-dimensional gel. Northern and dot blot analyses of RNA isolated from wild-type nodules and from defective nodules elicited by a variety of *Rhizobium meliloti* mutants showed that 1) RNAs corresponding to the Lb, nodule-specific GS, and three unidentified nodulins were coordinately expressed during the course of nodule development, and 2) all five nodulins were expressed in Fix$^-$ nodules that contained infection threads and bacteroids but were not expressed in nodules that lacked infection threads and intracellular rhizobia.

Additional key words: Glutamine synthetase, nodulins, *Rhizobium*-legume symbiosis

In the *Rhizobium*-legume symbiosis, nitrogen fixation occurs in nodules, highly differentiated organs that typically form on the root of the host plant in response to infection by an appropriate *Rhizobium* species. In the case of the *Rhizobium meliloti*-alfalfa symbiosis, nodulation begins with rhizobial attachment to root hairs and root hair curling, followed by division of root cortical cells, leading to the formation of a nodule meristem. Concomitant with nodule meristem formation, rhizobia invade root cortical cells by means of infection threads, hollow cellulose conduits synthesized by the plant. Eventually, rhizobia are released from the infection threads and differentiate into intracellular nitrogen-fixing bacteroids, nondividing cells that are dedicated to fixing nitrogen and secreting the fixed nitrogen in the form of ammonia to the surrounding plant cytoplasm (for recent reviews see Vincent 1980 and Long 1984).

Both rhizobial and plant mutants that block nodulation at specific stages have been isolated, indicating that nodule formation is a multistep process requiring information from both the legume host and rhizobial endosymbiont (Vincent 1980; Long 1984). *R. meliloti* symbiotic mutants can be grouped into two broad phenotypic classes. Nod$^-$ mutants fail to initiate nodule meristem development, whereas Fix$^-$ mutants elicit the development of defective nodules that are incapable of reducing di-nitrogen (N_2) to ammonia. A number of studies indicate that the *nod* genes (mutations of which lead to the Nod$^-$ phenotype) are the only *Rhizobium* symbiotic-specific genes required for nodule meristem induction (Downie *et al.* 1983; Hirsch *et al.* 1984, 1985). In the case of *R. meliloti*, a plasmid carrying the *nod* gene region is sufficient to elicit an organized nodule when transferred into *Agrobacterium tumefaciens* (Hirsch *et al.* 1984, 1985). These defective nodules lack infection threads and intracellular bacteroids and are designated as having an "empty" nodule phenotype.

The Fix$^-$ phenotype can be generated by a variety of mutations, including mutations in *nif, fix,* and *exo* genes. *Nif* genes code for functions involved directly in the nitrogen fixation process. By definition, *fix* genes code for functions required for the formation of effective (nitrogen-fixing) nodules, but the precise biochemical role of the function has not yet been determined. All *R. meliloti nif* and *fix* mutants that have been studied—*nifH, nifD, nifK, nifB, nifA, fixA,* and *fixB*—elicit nodules that contain infection threads and intracellular bacteria (Hirsch *et al.* 1983; Hirsch and Smith 1987). *Exo* genes code for proteins involved in the biosynthesis of extracellular polysaccharides (exopolysaccharides). In contrast to *nif* and *fix* mutants, *exo* mutants elicit "empty" nodules that contain a nodule meristem but lack infection threads and intracellular bacteria (Leigh *et al.* 1985; Finan *et al.* 1985).

Host proteins that are expressed specifically in nodules are called nodulins; a nodulin found in all legumes is leghemoglobin (Lb), a myoglobinlike protein that transports oxygen to the bacteroids and regulates nodule oxygen tension (Whittenberg *et al.* 1974). Other nodulins are involved in ammonia assimilation; these include uricase in soybean (Bergmann *et al.* 1983) and a nodule-specific glutamine synthetase (GS) in beans (Cullimore *et al.* 1984).

In contrast to Lb and GS, most nodulins have not been correlated with a specific biochemical function; rather, they have been identified by *in vitro* translation of nodule mRNA (Legocki and Verma 1980; Govers *et al.* 1985; Gloudemans *et al.* 1987) and/or by using nodule-specific antisera (Bisseling *et al.* 1983; Lang-Unnasch and Ausubel 1985; Vance *et al.* 1985). In addition, nodule-specific cDNAs (and their corresponding genomic equivalents) have been isolated by screening nodule cDNA libraries with root and

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nodule mRNA (cDNA) probes (Fuller *et al.* 1983; Govers *et al.* 1987b). Altogether, approximately 20 nodulins and nodule-specific cDNA clones have been identified in soybean, pea, and alfalfa (for review see Govers *et al.* 1987a). Most of these nodulins are expressed coordinately during nodule development, although at least two "early" nodulins have recently been identified in soybean and pea nodules (Govers *et al.* 1986).

An unanswered question concerning the regulation of nodule-specific GS genes in particular and of other nodule-specific genes in general is whether these genes are expressed in response to bacteroid production of ammonia or whether they are expressed as part of a nodule developmental program. We have used *R. meliloti* symbiotic mutants blocked at specific stages of nodule development to investigate this question.

In this paper we report the cloning of an alfalfa nodule-specific GS cDNA. We also report the identification of three unidentified alfalfa nodulins by cDNA cloning. The nodule-specific GS gene and the three unidentified nodulins were coordinately expressed with Lb during nodule development and were expressed in Fix^- nodules elicited by a variety of *R. meliloti* mutants. On the other hand, these nodulins were not expressed in alfalfa nodules elicited by *R. meliloti exo* mutants that lack infection threads and intracellular bacteria.

MATERIALS AND METHODS

Bacterial strains. *R. meliloti* strain 1021 is a symbiotically effective, streptomycin-resistant derivative of strain SU47 (Meade *et al.* 1982). Symbiotic mutants 1491 (*nifH*), 1308 (*nifD*), 1334 (*fixA*), and 1354 (*nifA*) contain Tn*5* insertions within the *nod-fix* region of pSym1 (Zimmerman *et al.* 1983), mutant 5011 is deficient in succinate transport (Finan *et al.* 1986) and was recently determined to be an *ntrA* mutant (T. Finan, personal communication), and mutant 7023 (*exoA*) is deficient in exopolysaccharide production (Leigh *et al.* 1985). The characteristics of these strains are summarized in Table 1.

RNA preparation. Alfalfa (*Medicago sativa* cv. Iroquois) nodules, roots, and leaves were harvested into liquid nitrogen and stored at −70° C before RNA isolation. Total RNA was prepared in one of two ways: Frozen tissue (10 g) was ground to a fine powder with liquid nitrogen in a coffee grinder, transferred to an extraction buffer (4 M guanidinium thiocynate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M mercaptoethanol), and centrifuged in a GSA rotor at 9,000 rpm for 30 min. The supernatant solution was layered over 5.7 M CsCl and centrifuged in a Beckman SW27 rotor at 25,000 rpm for 16–20 hr. The RNA pellet was resuspended in 7.5 M guanidinium-HCl, heated for 2 min at 65° C, and centrifuged at 5,000 rpm for 10 min, and the RNA was precipitated from the supernatant solution by adding 0.025 volumes of 1 M glacial acetic acid and 0.5 volumes of ethanol and storing overnight at −20° C. $PolyA^+$ RNA was isolated by oligo-dT cellulose chromatography according to Aviv and Leder (1972).

Alternatively, frozen nodules were ground in 2.5 volumes of extraction buffer (0.2 M sodium acetate, pH 6.5, 1% sodium dodecyl sulfate [SDS], 10 mM EDTA) using a Polytron homogenizer, extracted twice with phenol:chloroform, and extracted once with chloroform. The aqueous phase was precipitated with two volumes of ethanol at −20° C, and the RNA was purified on CsCl as described above.

Mutant nodules (100–500 μl) were ground in liquid nitrogen using a mortar and pestle and extracted with an equal volume of SDS extraction buffer (described above) and two volumes of phenol:chloroform, followed by a second extraction with phenol:chloroform and a final extraction with chloroform. The aqueous phase was precipitated at 4° C overnight by the addition of one-third volume of 8 M LiCl, followed by ethanol precipitation. The integrity and concentration of the RNA was determined by observing the ribosomal RNA bands on neutral agarose gels.

cDNA cloning. $PolyA^+$ nodule RNA was primed with oligo-dT, and a cDNA copy was synthesized using AMV reverse transcriptase (Life Sciences) and *Escherichia coli* DNA polymerase I (large fragment, Bethesda Research Laboratories) as previously described (Maniatis *et al.* 1982). The cDNA was treated with S1 nuclease (BRL), poly-C tailed with terminal transferase (BRL), and inserted into the *Pst*I site of a G-tailed pBR322 vector from New England Nuclear. Colonies were screened for growth on tetracycline and for absence of growth on ampicillin.

A GS-specific probe was generated by first digesting a *Phaseolus* GS cDNA clone (pcvPvNGS-01) (Cullimore *et al.* 1984) with *Pst*I and then gel-purifying the cDNA insert. To further eliminate pBR322 contamination, the purified *Pst*I fragment was digested with *Hae*III, and the resulting 364 and 378 bp fragments were eluted from a 5% polyacrylamide gel.

Colony blot hybridization. Bacterial colonies were individually grown in microtiter dishes and transferred to nitrocellulose as previously described (Maniatis *et al.* 1982). Colony blots were prehybridized in 0.1 mg/ml sheared calf thymus DNA, 10% dextran sulfate, 1 M NaCl, and 1× P buffer (0.2% bovine serum albumin; 0.2% polyvinyl-pyrrolidone, M_r 40,000; 0.2% Ficoll, M_r 400,000; 50 mM Tris-HCl, pH 7.5; 0.1% sodium pyrophosphate; 1% SDS) at 65° C. Probe (specific activity = 10^8cmp/μg) was added to the filters to give a final concentration of 10^6cpm/ml and hybridized overnight at 65° C.

DNA sequencing. Appropriate restriction fragments from selected cDNA clones were subcloned into M13mp10 and M13mp18 (Messing 1983) and sequenced by the method of Sanger *et al.* (1977).

Northern blots. Two to four micrograms of total RNA (from nodules, roots, or leaves) were denatured with formamide and formaldehyde and electrophoresed through a 6% formaldehyde, 1.5% agarose gel (Maniatis *et al.* 1982) and transferred to a Gene Screen filter as described by the vendor (New England Nuclear). The filters were prehybridized in 1× P buffer (described above), containing 50% (v/v) formamide, 10% dextran sulfate, and 0.1 mg/ml sheared salmon sperm DNA, for 6 hr at 42° C. Probe was added to the prehybridization solution to give a final

Table 1. *Rhizobium meliloti* strains

Strain	Genotype	Relevant phenotypes[a]	Reference
1021	*str-21*	Nod^+ Fix^+ Bad^+	Meade *et al.* 1982
1354	*nifA*::Tn*5*	Nod^+ Fix^- Bad^+	Zimmerman *et al.* 1983
1491	*nifH*::Tn*5*	Nod^+ Fix^- Bad^+	Zimmerman *et al.* 1983
1308	*nifD*::Tn*5*	Nod^+ Fix^- Bad^+	Zimmerman *et al.* 1983
1334	*fixA*::Tn*5*	Nod^+ Fix^- Bad^+	Zimmerman *et al.* 1983
5011	*ntrA*::Tn*5*	Nod^+ Fix^-	T. Finan, personal communication
7023	*exoA*::Tn*5*	Nod^+ Fix^- Inf^-	Leigh *et al.* 1985

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

concentration of less than 10 ng/ml (10^5–10^6 cpm/ml), and the filters were hybridized overnight. Filters were washed twice in 2× standard saline citrate (SSC) (1× SSC = 0.015 M sodium citrate, 0.15 M sodium chloride) at room temperature for 5 min, followed by two 30-min washes in 2×SSC, 1% SDS, and then by two 30-min washes in 0.1% SSC at room temperature.

For tissue expression of GS, polyA$^+$ RNA isolated from alfalfa nodules, roots, and leaves, and from alfalfa tissue culture cells resistant to the glutamine analogue L-phosphinothricin (PPT) (Donn *et al.* 1984) was denatured with glyoxal in 8%, electrophoresed through a 1.5% agarose gel (2 μg per lane), and transferred to nitrocellulose as described by Maniatis *et al.* (1982). The filters were prehybridized for 8 hr in 3× SSC, 10× Denhardt's solution, 50% (v/v) formamide, and 0.1 mg/ml sheared salmon sperm DNA. Probe was added to the prehybridization solution to give a final concentration of 10^6 cpm/ml, and the filters were hybridized overnight. One filter was hybridized with synthetic oligonucleotide specific for the 3′-untranslated region of the nodule GS cDNA clone (5′-TTACACTA-CACCAGGTTTAGGTTTC-3′) (synthesized in the lab of J. Smith, Massachusetts General Hospital), and the other was hybridized with a 419 bp GS coding sequence fragment from λGSA19 (Tischer *et al.* 1986). The oligonucleotide probe was labeled at the 5′ end with T4 polynucleotide kinase and γ-^{32}P-dATP to a specific activity of 6×10^8 cpm/μg (Maniatis *et al.* 1982). An *Eco*RI-*Bam*HI fragment (1,014 bp) of λGSA19 containing the coding region of GS was subcloned into pSP64 (to yield pSP64-A) and linearized with *Eco*RI. RNA transcripts of 419 bp, extending from the *Bam*HI site to the internal *Eco*RI site of pSP64-A, were synthesized *in vitro* using phage SP6 RNA polymerase and labeled with α-^{32}P-GTP according to Melton *et al.* (1984). Prehybridization and hybridization were at 42° C for the filter probed with the *in vitro*-synthesized RNA and at 25° C for the filter probed with the oligonucleotide. The filter hybridized with the oligonucleotide was washed three times, 15 min each, in 3×SSC at 25° C, followed by a 30-min wash in 1× SSC and 0.1% SDS. The RNA-probed filter was washed three times, 15 min each, in 3× SSC at 65° C, followed by two 30-min washes in 0.1× SSC and 0.1% SDS.

Cell-free translation and gel electrophoresis. One to two micrograms of total RNA was translated *in vitro* in the presence of ^{35}S-methionine using a rabbit reticulocyte lysate translation system (New England Nuclear). Incorporation of radiolabel was determined according to the manufacturer's instructions. Translation reactions were treated with RNase A (2 mg/ml) for 15 min at 37° C, acetone precipitated, and resuspended in 25–50 μl of lysis buffer (O'Farrell 1974). Two-dimensional gels were run according to Chory *et al.* (1987), except that the isoelectric focusing dimension was run for 12 hr at 400 V, followed by 1 hr at 800 V (O'Farrell 1974) One dimensional gels were 12% SDS-PAGE (Laemmli 1970).

Hybrid-released translation. Hybrids were selected as previously described by Miller *et al.* (1983). The hybrid-selected RNA was translated, and the products were examined by gel electrophoresis as described above.

RNA dot blots. For dot blot analysis, 2–4 μg of RNA in 6% formaldehyde, 1 M NaCl, 30 mM sodium phosphate, pH 7.0, was heated to 55° C, immobilized on nitrocellulose with a Schleicher and Schuell microdot blotter, and washed three times with 20× SSC. Filters were prehybridized at least 1 hr in 100 μg/ml sheared calf thymus DNA, 10% dextran sulfate, 1 M NaCl, and 1× P buffer (described above) at 65° C. cDNA probe as described above was added to the prehybridization solution; the filter was hybridized overnight and washed as described above.

Determination of GS activity. GS activity was determined by the synthetase reaction as monitored by the formation of γ-glutamyl-hydroxamate (Rhodes *et al.* 1975). The assay mixture contained 5 mM adenosine 5′-triphosphate, 75 mM glutamate, 10 mM hydroxylamine, 100 mM magnesium sulfate in 50 mM imidazole buffer, pH 7.2. The assay was initiated by the addition of enzyme and incubated at 30° C for 30 min. The reaction was stopped by the addition of an equal volume of 4% trichloroacetic acid, 3.2% ferric chloride in 0.5 N HCl. After centrifugation, the absorbance of the supernatant was measured at 500 nm.

RESULTS

Identification of nodulin cDNA clones. PolyA$^+$ RNA isolated from nitrogen-fixing alfalfa root nodules was used to construct a cDNA library in pBR322 (see Materials and Methods for details). From this library, 3,000 clones, stored in microtiter dishes, were screened by the colony hybridization procedure for homology to a *Phaseolus vulgaris* (bean) GS cDNA (Cullimore *et al.* 1984) and a *Pisum sativum* (pea) Lb cDNA (Bisseling *et al.* 1983). Clones that did not hybridize to these probes were further screened with ^{32}P-labeled cDNA probes made from polyA$^+$ root or nodule RNA. Among the 3,000 clones tested, one hybridized to the GS probe and approximately 150 hybridized to the Lb probe. Approximately 400 clones that hybridized to the nodule cDNA probe but not to the Lb probe or the root cDNA probe were rescreened with nodule, root, and Lb cDNA probes. Two clones were determined to be ribosomal cDNA clones (as judged by their banding patterns on northern gels). Twenty-five clones that did not hybridize to the Lb probe and exhibited the strongest differential signals to root and nodule probes were selected for further study.

The 25 clones that hybridized to nodule but not to root cDNA were further tested by the Southern and northern hybridization procedures. The 25 cDNA clones were used as hybridization probes against total alfalfa DNA digested with *Eco*RI and against total RNA isolated from alfalfa roots or nodules (data not shown). Three of these cDNA clones that were determined to be nodule-specific and appeared to correspond to single-copy alfalfa genes or small gene families (clones 2B, 6D, and 9A with insert sizes of ~260, ~440, and ~415 bp, respectively) were chosen for further study.

To confirm that the alfalfa cDNA clone (pAnGS1), which hybridized to the *Phaseolus* GS cDNA, coded for an alfalfa GS gene, the DNA sequence of pAnGS1 was determined and compared with the previously determined DNA sequence of an alfalfa GS cDNA from alfalfa tissue culture cells (Tischer *et al.* 1986). For the DNA sequence analysis, pAnGS1 was digested with either *Hind*III or *Bam*HI, recloned into either M13mp10 or M13mp18, respectively, and sequenced using the dideoxy sequencing method. As illustrated in Figure 1, pAnGS1 contains 255 bp of presumptive GS coding sequence that is 81% homologous to the GS cDNA isolated from tissue culture cells. The nodule GS cDNA also contains 139 base pairs of the 3′-untranslated region that shows only 39% homology to the corresponding region of the tissue culture GS sequence. These results show that pAnGS1 corresponds to an alfalfa GS gene distinct from the GS gene previously identified in alfalfa tissue

culture cells.

A subset of the 150 Lb clones was analyzed by restriction enzyme digestion and gel electrophoresis. A comparison of the restriction sites and fragment size suggested that at least three distinct Lb genes are represented in this collection of clones (data not shown). Previous work on the Lb polypeptides showed five distinct isomers (Jing *et al.* 1982), and hybrid selection of the Lb mRNA followed by *in vitro* translation and 2-D gel analysis revealed four distinct polypeptides (Fig. 2E).

Tissue-specific expression of nodulins. Figure 3 shows that the mRNAs corresponding to Lb and to the three newly identified nodulins (2B, 6D, and 9A) were present in wild-type nodules but could not be detected in roots and leaves. The mRNAs corresponding to Lb, 2B, and 9A are approximately 410, 540, and 710 bases, respectively (as determined from the RNA size markers, Fig. 3), and are thus capable of coding for proteins ranging in size from 15 to 30 kDa. The mRNA corresponding to 6D consistently appeared as a heterogeneous population between 450 and 600 nucleotides. An ethidium bromide stain of this gel revealed equal amounts of RNA in the root and nodule lanes as determined by the intensity of the ribosomal RNA bands (data not shown).

To determine whether the GS cDNA (pAnGS1) was specifically expressed as part of the nodulation process, an oligonucleotide homologous to 25 bases in the 3′-untranslated region of the pAnGS1 cDNA clone was synthesized (Fig. 1). This 25-base region was chosen to minimize homology with the 3′-untranslated region of the tissue culture GS cDNA clone. The synthetic oligonucleotide was radiolabeled and used to probe northern blots containing RNA isolated from several different organs (see Materials and Methods).

Figure 4 shows that when 2 μg of polyA$^+$ RNA from leaf, root, nodule, or tissue culture cells was probed with a nearly full-length GS cDNA probe from alfalfa tissue culture cells, hybridizing GS mRNA was detected in all tissues, although a considerably higher level of GS mRNA was found in nodules and tissue culture cells than in leaves. This line of alfalfa tissue culture cells contains a GS gene that has been amplified (Tischer *et al.* 1986). In contrast to the coding region GS probe, the 3′-specific synthetic oligonucleotide from pAnGS1 hybridized only to nodule RNA and did not detect RNA from the other plant tissues (Fig. 4). This demonstrates that expression of the GS gene corresponding to pAnGS1 is nodule-specific and presumably contributes

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1    AAGCTTAGAT TGAGGCATAA GGAGCACATT GCTGCTTATG GAGAAGGAAA TGAGAGACGT   60
814  AAGCTTGGGA AGAAGCACAA GGAGCACATT GCTGCTTATG GAGAAGGCAA CGAGCGTAGA  873

61   CTCACTGGAA AACATGAAAC AGCAGACATC AACACCTTTT CTTGGGGTGT GGCAAACCGT  120
874  TTGACAGGGC GACATGAGAC AGCTGACATT AACACCTTCT TATGGGGTGT TGCAAACCGT  933

121  GGTGCCTCAG TTAGGGTTGG AAGAGACACA GAAAAAGATG GCAAAGGTTA CTTTGAGGAC  180
934  GGTGCGTCGA TTAGAGTTGG AAGGGACACA GAGAAAGCAG GGAAAGGTTA TTTCGAGGAT  993

181  AGAAGGCCTT CTTCTAATAT GGATCCCTAT GTGGTCACTT CCATGATTGC TGAAACTACC  240
994  AGGAGGCCAT CATCTAACAT GGATCCATAT GTTGTTACTT CCATGATTGC AGACACCACC 1053

241  CTTCTATGGA AACCATGAAG TCACAACCAC TTGTCTTTGG ATTTGGACCA CATCACATTA  300
1054 ATTCTCTGGA AACCATAAGC CACCACACAC ACATGCATTG AAGTATTTGA AAGTCATTGT 1113

301  TGTGTTCAAT AAATAGTCTA GTTTATATCT ATCTATTATC ATTATGCAAA GTGTTGCTTC  360
1114 TGATTCCGCA TTAGAATTTG GTCATTGTTT TTTCTAGGAT TTGGATTTGT GTTATTGTTA 1173

361  ATTAGTAGTA ATTGTCCAAG TGGTCTTTTA A  391
1174 TGGTTCACAC TTTGTTTGTT TGAATTTGAG G 1204
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Fig. 1. DNA sequence of the nodule-specific glutamine synthetase (GS) cDNA clone (top) compared to a GS cDNA clone from the amplified alfalfa tissue culture (Tischer *et al.* 1986). The translational stop codons are boxed and a bar identifies the sequence used in the synthesis of a nodule-specific oligonucleotide.

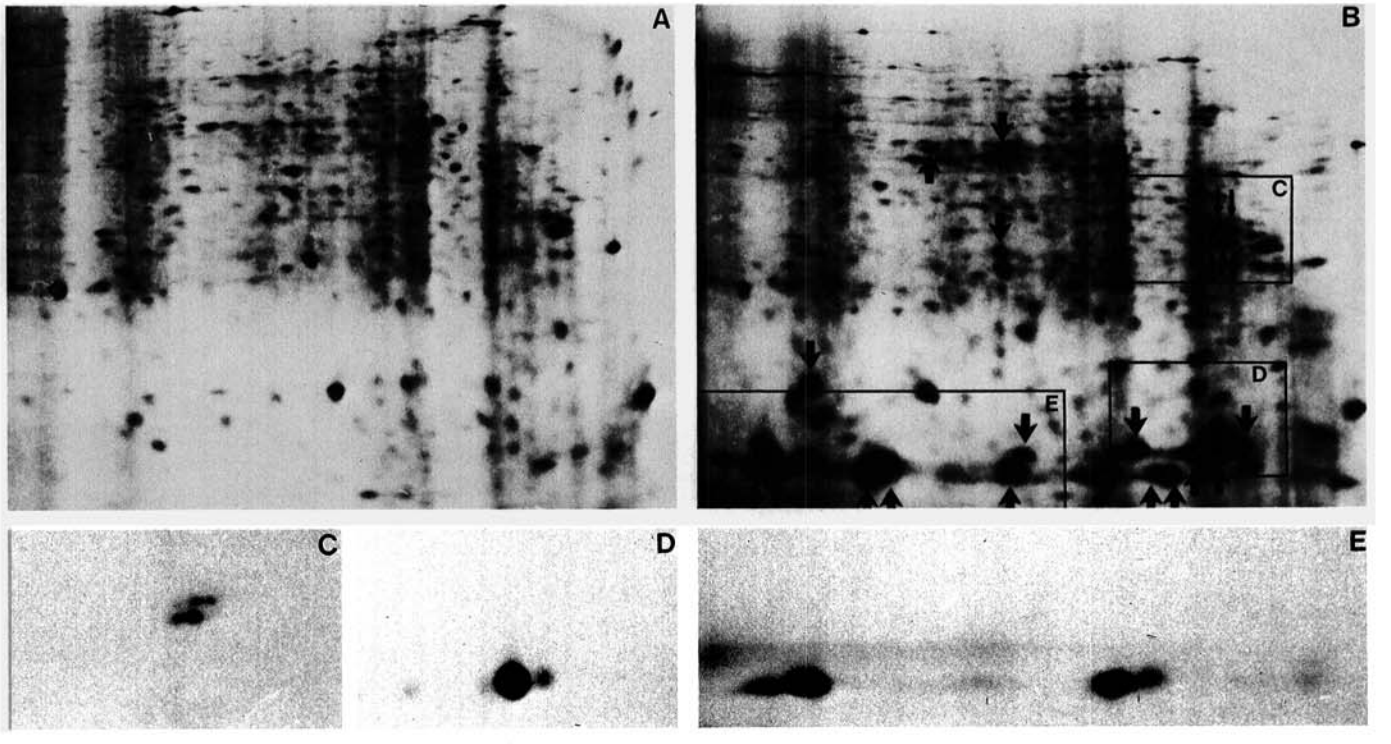

Fig. 2. Identification of nodule-specific *in vitro* translation products. RNA from root (**A**) and nodule (**B**) was translated *in vitro*, and the translation products were analyzed by 2-D gel electrophoresis. Arrows in (B) denote nodule-specific translation products. Nodule-specific cDNA clones were used to hybrid select nodule RNA. The cDNA clones used were 9A (**C**), 2B (**D**), and leghemoglobin (**E**). Boxes in panel B indicate the area of the gel shown in the corresponding C, D, and E panels.

to the increase in enzyme activity found in nodule tissue (Table 2).

Two-dimensional gel analysis of *in vitro*-translated nodule mRNA. To correlate the three unknown nodule-specific cDNA clones (2B, 6D, and 9A) with specific nodule proteins (nodulins), we used the cDNA clones to hybrid-select nodule mRNA, translated the RNA *in vitro*, and then examined the translation products by 2-D gel electrophoresis. Figures 2A and B show the *in vitro* translation products of uninfected root and nodule RNA, respectively. Approximately 20 nodule-specific polypeptides (indicated with arrows in Fig. 2B) ranging in size from 12 to 80 kDa were identified in both the acidic and basic regions of the isoelectric focusing gel. When 30 μg of Lb, 2B, or 9A cDNA was immobilized on nitrocellulose filters and incubated with total nodule RNA and then the hybridizing RNA was eluted from the filters and translated *in vitro*, the translation products shown in Figures 2C, D, and E were obtained. Figure 2E shows that Lb cDNA hybrid-selected RNA that codes for at least four distinct polypeptides with molecular masses of about 12 kDa. Clone 2B selected a single mRNA coding for 14 kDa polypeptide (Fig. 2D) and clone 9A selected mRNAs coding for five polypeptides ranging in size from 32 to 34 kDa (Fig. 2C). The nodulins corresponding to the Lb and to the 2B and 9A cDNAs are boxed in Figure 2B.

Clone 6D hybrid selected RNA that coded for a polypeptide that was too basic to be resolved on the 2-D gel system used for the experiments shown in Figure 2. As shown in Figure 5, clone 6D hybrid selected RNA coding for a 22-kDa protein.

In accordance with nomenclature recently proposed (van Kammen 1984), the nodulins corresponding to cDNA clones 2B, 6D, and 9A were designated N-14, N-22, and N-32/34, respectively. Important features of these cDNA clones and their corresponding nodulins are summarized in Table 3.

Time course of nodulin expression. To determine when the mRNAs corresponding to Lb, N-14 (clone 2B), N-22 (clone 6D), and N-32/34 (clone 9A) were expressed during

Table 2. Levels of glutamine synthetase (GS) activities in Fix$^-$ nodules[a]

Strain	Genotype	ΔA_{500}/20 min[b]	Protein concentration (mg/ml)	Specific activity (nmol/min/mg protein)	Wild-type activity (%)
1021	Wild-type	0.33	1.4	28	100
1354	*nifA*$^-$	0.15	1.3	14	50
1334	*fixA*$^-$	0.22	2.0	13	46
1491	*nifH*$^-$	0.24	1.2	12	42
5011	*ntrA*$^-$	0.07	1.0	8	28
7023	*exo*$^-$	0.04	0.7	7	25
Uninoculated root		0.07	1.75	5	17

[a] GS activity determined by the synthetase reaction as monitored by the formation of λ-glutamyl-hydroxamate.
[b] The A_{500}/20 min was determined from the curve generated by the activity at 1-, 10-, and 20-min time points.
[c] 100 μl of nodule or root protein was used in each reaction.

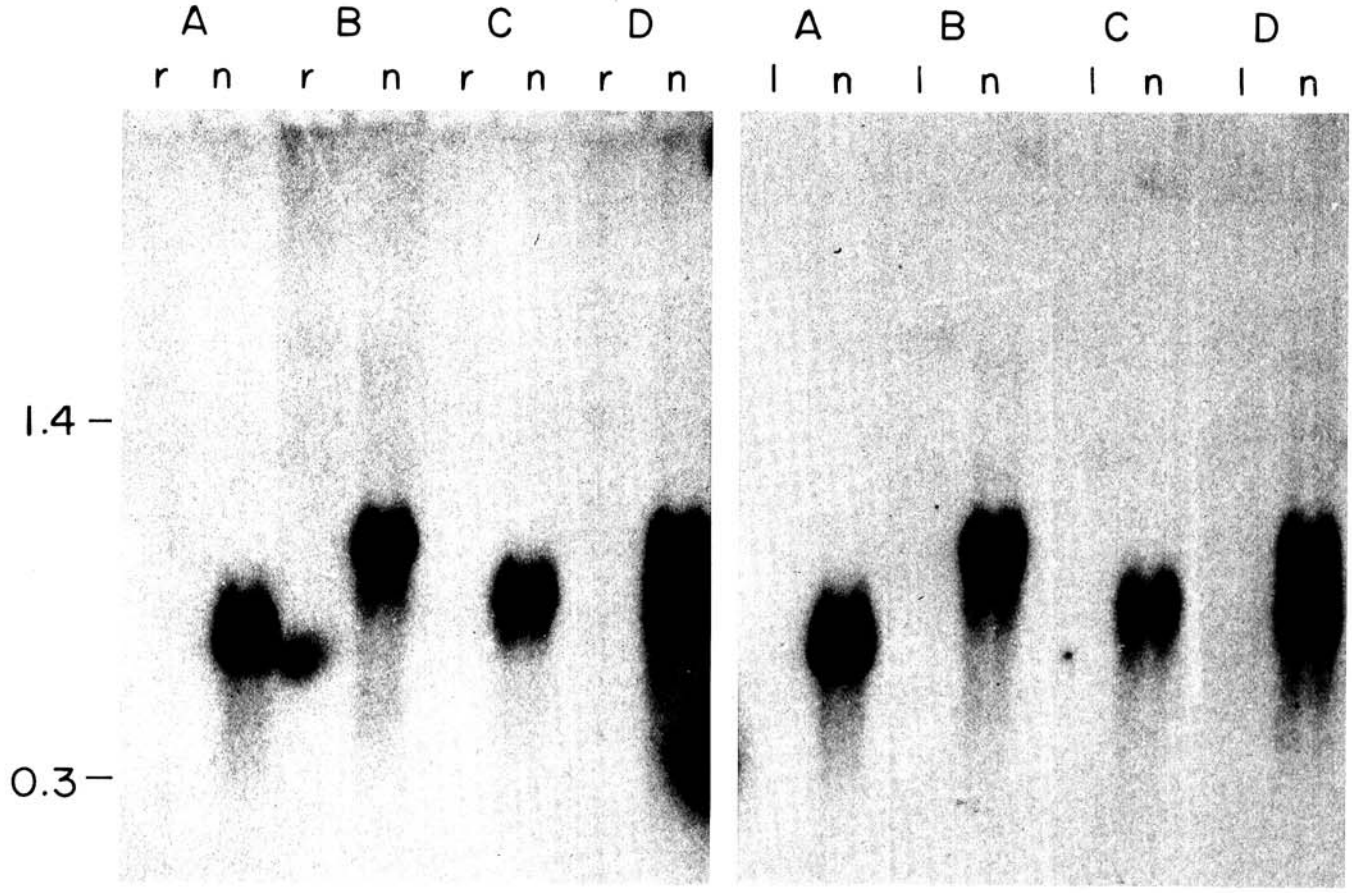

Fig. 3. Nodule-specific expression of nodulin genes. Northern blot analysis of 2–4 μg of root (r), nodule (n), and leaf (l) RNA probed with nick-translated cDNA clones of leghemoglobin (A), 9A (B), 2B (C), and 6D (D). Markers are 1.4 kb and 0.3 kb from the lambda RNA ladder of Bethesda Research Laboratory. Approximately equal amounts of RNA were present in each lane as determined by the rRNA bands visualized with ethidium bromide staining (data not shown).

nodule development, alfalfa seedlings were inoculated with wild-type *R. meliloti*. RNA was isolated from total root and nodule tissue at various times and dot-blotted to nitrocellulose filters. The dot blots were hybridized with ^{32}P-labeled cDNAs (Fig. 6). All four nodulin mRNAs were first detectable above background by at least 10 days postinoculation and reached a maximum level of expression about 22 days postinoculation. In several experiments, the point at which the nodulin mRNAs were first detectable corresponded to the time at which nodule development was first apparent and when nitrogen fixation activity (acetylene reduction) could first be detected (data not shown). Similar experiments using pAnGS1 as a hybridization probe showed that the expression of mRNA corresponding to GS followed the same developmental pattern of expression as the other nodulin mRNAs (data not shown). Although nodule-specific GS is clearly induced in root nodules (Fig. 4), we do not know to what extent other GS genes may contribute to the increase in the GS message.

Expression of nodulins in defective nodules. To determine whether the presence of specific alfalfa nodulin

Table 3. Properties of nodulin cDNA clones

cDNA clone[a]	Insert size (bp)	mRNA size (bases)	Polypeptide M_r (kDa)	Expression in		
				Root	Nodule	Empty nodule
Lb	385	410	12	−	+	−
9A	415	710	32–34	−	+	−
6D	440	450/600	22	−	+	−
2B	260	540	14	−	+	−
GS	390	1500	39	−	+	−

[a] Lb, leghemoglobin; GS, glutamine synthetase; empty nodule refers to nodules devoid of bacteria.

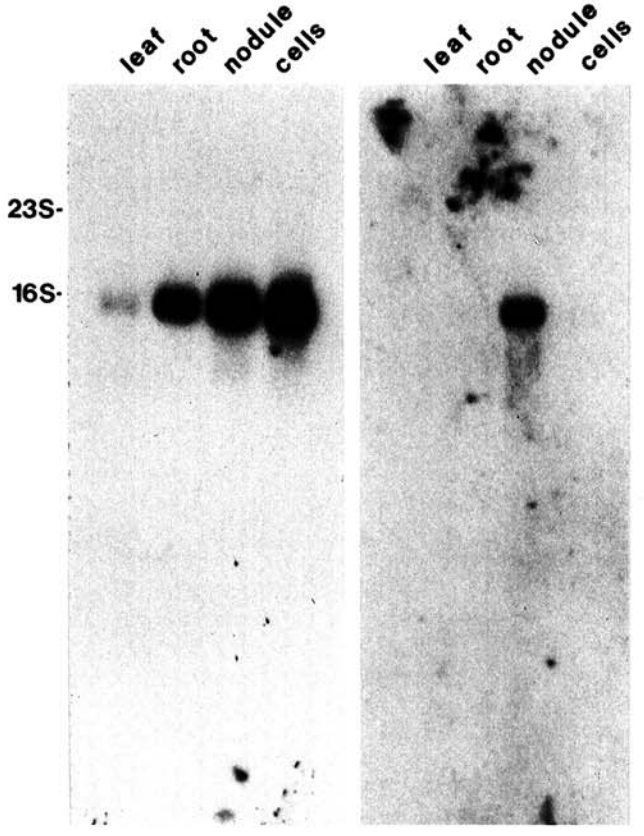

Fig. 4. Nodule-specific expression of a GS gene. Duplicate northern blots of polyA$^+$ RNA from alfalfa tissue culture cells in which GS has been amplified and root nodules, root, and leaf hybridized with a RNA probe containing coding sequences of GS (left) or with a ^{32}P-labeled oligonucleotide from the 3′-untranslated region of the nodule GS cDNA clone (right).

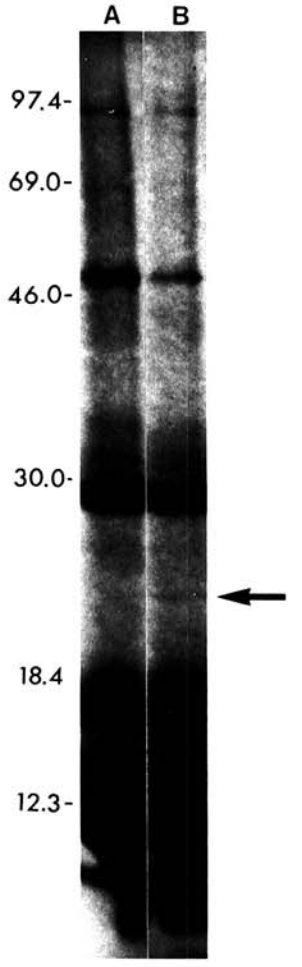

Fig. 5. SDS polyacrylamide gel of the N-22 polypeptide translated from RNA hybrid selected by the 6D cDNA clone (B). Lane A shows translation reaction in the absence of RNA. Molecular weight markers are in kDa.

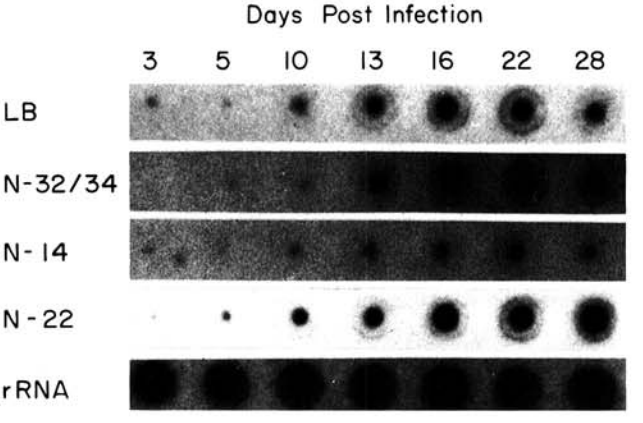

Fig. 6. Time course of nodulin gene expression. 2–4 μg of total RNA isolated from infected roots was dot-blotted onto nitrocellulose and probed with nick-translated cDNA clones.

mRNAs depends on the activity of specific *R. meliloti* symbiotic genes, we determined whether nodulin mRNAs were present in defective nodules elicited by various *R. meliloti* mutants (Table 1) blocked at various stages of nodule development. Strain 7023 (*exoA*::Tn*5*) elicits nodules that contain neither infection threads nor intracellular bacteria (Finan *et al.* 1985). Strain 1334 (*nifA*::Tn*5*) elicits numerous small nodules in which bacteroids fail to mature (Hirsch and Smith 1987). Strains 1491 (*nifH*::Tn*5*) and 1334 (*fixA*::Tn*5*) elicit nodules in which bacteroids appear to mature properly but senesce early (Hirsch *et al.* 1983). Strain 5011 is phenotypically deficient in succinate transport and was originally thought to be a dicarboxylic acid transport mutant. Recently, however, 5011 has been identified as an *ntrA* mutant (T. Finan, personal communication; Ronson *et al.* 1987). Although the ultrastructure of nodules elicited by 5011 has not yet been examined, both plant and bacterial ribosomal RNA bands are observed in ethidium-stained agarose gels. This suggests that these nodules contain intracellular bacteria, because bacterial rRNA is noticeably absent from both uninfected roots and the "empty" nodules formed with the *R. meliloti exo* mutants (K. Dunn, unpublished observation).

RNA isolated from 4-wk-old nodules elicited by wild-type and by the five mutant strains was electrophoresed on 1.5% agarose formaldehyde gel, blotted onto Gene Screen membranes, and then successively hybridized with ^{32}P-labeled cDNA corresponding to Lb and nodulins N-14 (2B), N-22 (6D), and N-32/34 (9A). Figure 7 shows that mRNAs corresponding to Lb, N-32/34, and N-14 were present in defective nodules containing intracellular bacteria (nodules elicited by wild-type and by *nifA, nifH, fixA,* and *ntrA* mutants) but were not present in nodules that lacked intracellular bacteria (nodules elicited by the *exoA* mutant). The mRNA corresponding to N-22 gave similar results, except its level was dramatically reduced in nodules formed with the *ntrA* mutant. To verify that the lane in Figure 7 corresponding to RNA isolated from nodules elicited by the *exoA* mutant contained RNA, the northern blot was also hybridized with a ribosomal cDNA probe from soybean.

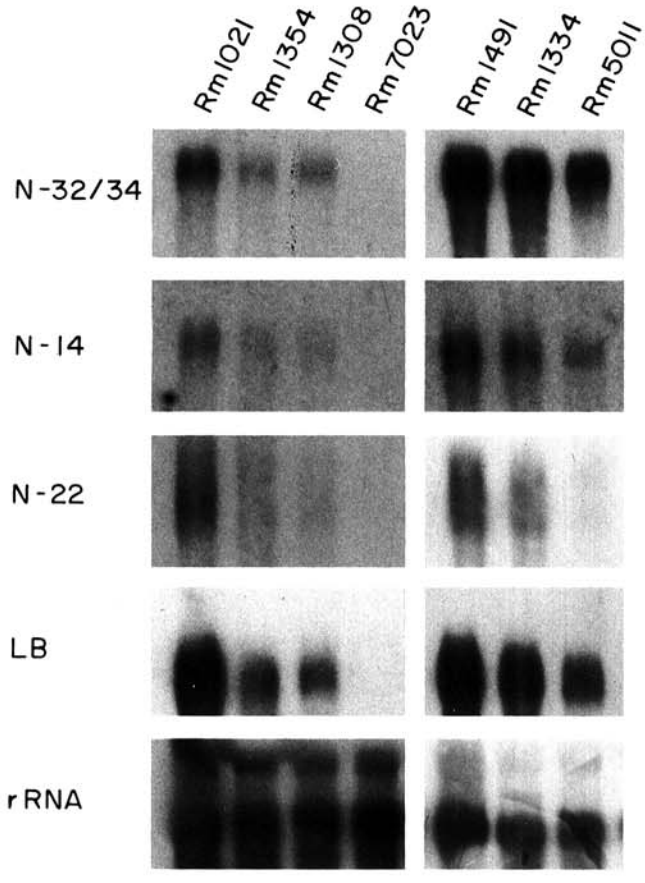

Fig. 7. Expression of nodulin genes in nodules elicited by various *Rhizobium meliloti* Fix$^-$ mutants. Two northern blots containing total RNA from nodules elicited by *R. meliloti* strains 1021 (WT), 1354 (*nifA*), 1308 (*nifD*), and 7023 (*exo*) on one blot and 1491 (*nifH*), 1334 (*fixA*), and 5011 (*ntrA*) on a second blot were sequentially probed with cDNA clones corresponding to N-32/34 (clone 9a), N-22 (clone 6D), N-14 (clone 2B), and Lb. The blots were also probed with a cDNA clone corresponding to rRna from soybean.

The northern blot shown in Figure 7 (as well as duplicate filters) were also probed with the oligonucleotide probe corresponding to the 3′-untranslated region of the nodule-specific GS cDNA clone. Unfortunately, background hybridization to ribosomal RNA bands obscured any GS specific signal. On the other hand, when the GS oligonucleotide was used to probe dot blots of total RNA isolated from the set of *R. meliloti* symbiotic mutant nodules, hybridization significantly above background was observed for all of the mutant strains except in the case of RNA isolated from nodules elicited by *exo* mutants (data not shown). Again, hybridization to the nodules from the *ntrA* mutant was reduced. This latter experiment, although only preliminary, suggested that the nodule-specific GS gene is coordinately regulated with the other nodulins tested and does not require an effective nitrogen-fixing symbiont for expression.

GS activity in effective and ineffective nodules. To determine whether the increase in nodule-specific GS mRNA was also accompanied by a corresponding increase in GS enzyme specific activity, GS enzyme assays were made on protein extracts from wild-type and mutant nodules (Table 2). All of the mutant nodules that expressed the nodule GS mRNA had a corresponding level of GS activity. In contrast, nodules elicited by the *exoA* and *ntrA* mutants, which had undetectable or very low levels of nodule GS mRNA, had a GS activity level similar to the basal level found in roots.

DISCUSSION

In this paper we have described the isolation of five alfalfa cDNA clones, each representing a gene that is specifically expressed in symbiotic nitrogen-fixing root nodules elicited by *R. meliloti*. One of these clones corresponds to Lb, an oxygen-binding protein found in all *Rhizobium*-elicited nodules. A second nodulin cDNA clone corresponds to a GS gene, which is transcribed exclusively in root nodules. The other three cDNA clones correspond to nodulins of unknown functions with molecular masses of 14 kDa (N-14; clone 2B), 22 kDa (N-22; clone 6D), and 32–34 kDa (N-32/34; clone 9A). As demonstrated by *in vitro* translation of total nodule RNA (Fig. 2B), Lb, GS, N-14, N-22, and N-32/34 represent only a few of many nodulins that are expressed during the nodulation process.

Are the nodulins coded by unique genes or small gene families? In corroboration of previous work demonstrating that a variety of legume nodules, including alfalfa, contain several Lb species, our work shows that alfalfa has at least four Lb proteins, all of similar molecular weight but

differing in their molecular charges. In addition, our data on the nodule-specific GS gene that we identified corroborates the accumulating evidence that GS is encoded by a small gene family showing organ-specific expression (Cullimore *et al.* 1984). The alfalfa nodule-specific GS gene shares considerable coding sequence homology to another alfalfa GS gene (Tischer *et al.* 1986), which was found to be amplified in tissue culture cells selected to be resistant to the herbicide PPT, a mixed competitive inhibitor of GS (Donn *et al.* 1984). However, the nodule GS gene is highly divergent from the amplified GS gene in the 3′-untranslated region.

Nodulin N-14 migrated as a single polypeptide on 2-D gel electrophoresis; however, N-22 was too basic to be resolved on our 2-D gel system but migrated as a single band on a 1-D gel. Both N-14 and N-22 appeared to be coded by single-copy genes. In contrast, N-32/34 was resolved as five polypeptides and could represent a small gene family.

Nodulin mRNA. Northern blot analysis of nodule RNA revealed that the mRNA corresponding to Lb, GS, N-14, and N-32/34 migrated as single bands but that mRNA corresponding to the N-22 clone (6D) was heterogeneous in size despite the fact that *in vitro* translation of the latter message indicated a single-sized polypeptide. The nature of this heterogeneity is not known but could be the result of improper or incomplete processing of the mRNA. Alternatively, the N-22 message may be selectively degraded as suggested by the presence of the 22-kDa polypeptide, which corresponds to the largest of the mRNA species. This would also explain the relatively low levels of *in vitro* translation with the N-22 message despite the presence of seemingly adequate amounts of mRNA.

Nodule-specific GS. Information about the GS proteins in alfalfa is limited, but two isoforms of the 40,000 M_r subunit have been identified (Groat and Schrader 1982). In *Phaseolus vulgaris*, there are four distinct forms of the holoenzyme: Two of these are found in leaf, one is found in roots and nodules, and the fourth appears to be nodule-specific (Lara *et al.* 1984; Gebhardt *et al.* 1986). The *Phaseolus* GS isoforms cannot be separated by molecular weight, but the octomeric holoenzymes have been differentiated by charge differences and immunological properties. In addition, the holoenzyme appears to be composed of subunits that have the same molecular weight but differ in their isoelectric points. Some of these subunits show organ specificity as determined by either analysis of protein extracts on two-dimensional gels or by hybridization of GS cDNA clones to RNA extracted from different tissues (Cullimore *et al.* 1984). Finally, recent data from pea (*Pisum sativum*) also suggests that several different isomeric subunits of GS are present in leaf and root, one of which is increased in nodules over the steady state level found in uninfected roots (Tingey *et al.* 1987). However, unlike in alfalfa and *P. vulgaris*, a nodule-specific GS has not been identified. Similar results have been reported by Hirel *et al.* (1987); that is, an increase in the expression of a root GS gene, rather than a nodule-specific gene, is responsible for the elevated levels of GS in soybean nodules. Interestingly, this soybean root GS gene appears to be expressed in response to ammonia. Finally, pea nodules also contain a 44-kDa form of GS that appears to correspond to a chloroplast-specific form; its presence in nodules is believed to be due to the large number of plastids that are found in nodule tissue (Tingey *et al.* 1987).

Developmental expression of alfalfa nodulins. The five nodulins examined in this study appeared to be coordinately expressed in that all five first become detectable at 10 days following inoculation with *R. meliloti* and all five attained a maximum level of expression at about 3 wk postinoculation. In soybean, the four Lb genes are induced in a sequential manner during nodule development and expression continues throughout nodulation. As yet, we have not determined whether they are also expressed sequentially in alfalfa. However, our results are consistent with those obtained with soybean and pea in that Lb mRNA increased simultaneously with a number of other nodulins, suggesting a common regulatory mechanism.

All five nodulins that we studied were present in a variety of defective Fix^- nodules which have in common the presence of infection threads and intracellular rhizobia. That is, all five nodulins were detected in Fix^- nodules in which the rhizobia have invaded the plant root via infection threads and developed into bacteroids, even though the bacteroids were defective in their ability to fix nitrogen and secrete ammonia. These findings are particularly interesting with respect to the nodule-specific GS because they demonstrate that expression of the GS gene is not dependent on the presence or accumulation of ammonia, one of the primary substrates of GS. The presence of elevated levels of GS activity in Fix^- mutants also suggests that ammonia or other by-products from an active nitrogen-fixing process are probably not involved in regulating the translation of the GS message or in modifying the primary GS polypeptide. On the other hand, although various Fix^- nodules expressed the five nodulins we studied, the amounts of these nodulins were reduced. Similar results have also been observed by Lang-Unnasch and Ausubel (1985); therefore, we cannot rule out the existence of an additional regulatory mechanism that monitors the secretion of ammonia by the bacteroids and regulates nodulin gene expression accordingly.

In contrast to Fix^- nodules that contain differentiated bacteroids, nodules elicited by Exo^- mutants do not contain intracellular bacteria. In these latter "empty" nodules there was no expression of Lb, the nodule-specific GS gene, or the three unidentified nodulins. These observations suggest that the five nodulins described in this study are not required for the formation of nodule structure but rather play a role in nodule function and/or maintenance. Apparently, expression of these nodulins requires an additional signal to the signal(s) generated by the bacterial nodulation genes that elicits nodule formation. The subsequent signal(s) may be produced by the plant as a consequence of bacterial invasion or, alternatively, may be produced directly by the developing bacteroids. The fact that $ntrA^-$ mutant nodules show no significant increase in GS activity and a much reduced amount of the GS and N-22 nodulin message suggests that the bacterial *ntrA* gene may be controlling additional symbiotic genes from those already identified. Although GS and N-22 may initially be activated by the same signals as LB, N-14, and N-32/34, additional signals, perhaps related to further development of the functioning bacteroid, may be required for a higher level of expression.

Thus, the results reported here are consistent with the model that nodulins can be divided into at least two major groups: One group of nodulins is expressed early during the nodulation process and is involved directly in the morphogenesis of the nodule structure. Such nodulins may include the N-75 (ENOD2) gene of pea (Govers *et al.* 1986), the N-30, N-60, N-64, N-85, N-90 and N-160 nodulins of alfalfa (Lang-Unnasch *et al.*). The second group of nodulins depends on a secondary signal(s) from the invading bacteria and includes Lb, GS, N-14, N-22, and N-32/34 in alfalfa.

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