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Characterization of a Nodule-Enhanced Glutamine Synthetase from Alfalfa: Nucleotide Sequence, *In Situ* Localization, and Transcript Analysis

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We have characterized two glutamine synthetase (GS) cDNA clones (pGS13 and pGS100) representing mRNA from root nodules of alfalfa. pGS13 is a full-length version of a previously isolated partial cDNA from an alfalfa nodule cDNA library, while pGS100 was previously isolated from an alfalfa suspension culture cDNA library. Using the 3′ untranslated region of the two cDNAs as gene-specific probes, we have shown that the GS genes represented by pGS100 and pGS13 are expressed in all organs tested, although at varying levels. pGS13, however, represents the nodule-enhanced GS gene class. Genomic Southern blot analysis using gene-specific probes shows multiple hybridizing bands, in each case suggesting multiple genes and/or alleles for each class of cytoplasmic GS genes. *In situ* hybridization of alfalfa nodule sections with gene-specific antisense RNA probes has shown that the nodule-enhanced GS genes are induced in the invasion zone and that their expression is limited to the symbiotic zone, while the GS genes represented by pGS100 are induced in the early symbiotic zone and are expressed throughout the symbiotic and senescent zones. Transcripts for both sets of GS genes are localized in the infected cells and based on the spatial expression pattern it would appear that the two gene classes are induced independently of the onset of nitrogen fixation.

Additional keywords: cDNA sequence, gene family, symbiosis.

Glutamine synthetase (GS; EC 6.3.1.2) is the key enzyme responsible for the initial step in ammonia assimilation (Lea *et al.* 1990; McGrath and Coruzzi 1991; Hirel *et al.* 1993). GS catalyzes the ATP-dependent condensation of ammonia with glutamate, to yield glutamine (Lea *et al.* 1990). GS is the major enzyme involved in the reassimilation of ammonia released from a variety of metabolic pathways such as photorespiration, catabolism of amino acids, and metabolism of phenylpropanoids (Lea *et al.* 1990; Marsolier *et al.* 1993). GS is also involved in nitrate (or nitrite) assimilation and in the assimilation of products of dinitrogen fixation in the root nodules of legumes (Miflin and Lea 1980). In higher plants, GS is an octameric enzyme of 320–380 kDa, which exists as a number of isoenzymes (Stewart *et al.* 1980).

In plants, GS is encoded by a small multigene family, whose members exhibit organ specific patterns of expression (Gebhardt *et al.* 1986; Tingey *et al.* 1987, 1988; Lightfoot *et al.* 1988; Bennett *et al.* 1989; Walker and Coruzzi 1989; Peterman and Goodman 1991; Roche *et al.* 1993), with separate genes encoding cytoplasmic and chloroplastic/plastid GS isoforms (GS_1 and GS_2, respectively) (Cullimore and Bennett 1992). Based on the site of location (cell type), the different GS isoforms assimilate or reassimilate ammonia derived from different metabolic processes (Hirel *et al.* 1993). In root nodules, the GS_1 isoform predominates, its primary function being the rapid assimilation of ammonia excreted into the plant cytosol of infected cells by the nitrogen-fixing bacteroids (Atkins 1987). Plastid-located GS expression has been observed in nodules (Tingey *et al.* 1988; Bennett *et al.* 1989), but its physiological role has not yet been determined.

The increase in GS activity associated with nodule development (Robertson *et al.* 1975) can be attributed to either the increased expression of the root form of GS_1 or in some legumes it may also be due to the expression of members not expressed in the roots. Nodule-specific GS_1 gene expression has been reported for *Phaseolus vulgaris* (Bennett *et al.* 1989), Lupin (Grant *et al.* 1989; Boron *et al.* 1989), soybean (Sengupta-Gopalan and Pitas 1986; Roche *et al.* 1993) and *Medicago sativa* (Dunn *et al.* 1988). In pea (Tingey *et al.* 1987; Walker and Coruzzi 1989) and *Medicago truncatula* (Stanford *et al.* 1993), a nodule-specific form is not present; however, there is a significant increase in the transcriptional activity of the root GS_1 genes (Tingey *et al.* 1987; Walker *et al.* 1989; Stanford *et al.* 1993).

The spatial expression of a number of GS_1 genes whose expression is elevated or specific to the nodule have been investigated in transgenic legume and nonlegume species using promoter fusions to the reporter gene β-glucuronidase (GUS). These include: the pea GS3A gene, the *P. vulgaris* glnγ, and soybean pGS20 gene which were all found to be expressed predominantly in the symbiotic region of the nodule of transgenic plants (Forde *et al.* 1989; Brears *et al.* 1991; Miao *et al.* 1991). The nodule-enhanced pea GS3A gene promoter showed expression in the nodule primordia and in the meristem, symbiotic zone, and nodule vasculature of transgenic alfalfa. The GS3A promoter also showed activity

in the vasculature of leaves, roots, and stem (Brears *et al.* 1991). The glnγ promoter of *P. vulgaris* showed activity specifically in the infected cells, while expression of another GS gene of *P. vulgaris*, glnβ, was detected in the roots and the vascular tissue of mature nodules of transgenic *L. corniculatus* (Forde *et al.* 1989). The promoter of a nodule enhanced soybean GS_1 gene, pGS20, showed activity in the infection zone, including uninfected cells and the inner cortex of nodules of transgenic *Lotus corniculatus* (Miao *et al.* 1991). A nodule-specific GS gene has now been identified in soybean (Roche *et al.* 1993); however, its cellular site of expression has not yet been determined. Immunocytochemical localization of GS has been carried out for *P. vulgaris* and soybean nodules (Brangeon *et al.* 1989; Datta *et al.* 1991). In both reports, the labeling was more intense in the infected cells than in the uninfected cells, with label detected in both cytosol and plastids (Brangeon *et al.* 1989; Datta *et al.* 1991). However, there have been no reports of *in situ* localization of GS transcripts in the native plant.

In this paper, we have extended our preliminary studies on the expression of GS_1 genes in nodules of alfalfa (Dunn *et al.* 1988; Temple *et al.* 1993). We present here the sequence of a full-length GS_1 cDNA isolated from an alfalfa cDNA library by its homology to the previously published short sequence pGSAn1 (Dunn *et al.* 1988). We have used gene-specific probes from this gene and a previously characterized alfalfa GS_1 gene (Tischer *et al.* 1986; Temple *et al.* 1993) to determine the complexity of the GS_1 gene family in alfalfa and their pattern of expression in nodules and other plant organs. Our study shows that there are at least two distinct classes of GS_1 genes and that the newly isolated GS_1 gene class is expressed in a nodule-enhanced manner. Using an *in situ* hybridization approach, we have shown that the expression of the nodule-enhanced GS_1 gene class is localized in the symbiotic zone of the nodule, while transcripts corresponding to the other class are uniformly distributed over the entire nodule behind the meristem.

RESULTS

The cDNA pGS13 shares high sequence identity to the nodule-enhanced GS gene of pea.

An alfalfa cDNA library prepared against 28-day alfalfa root nodule poly(A)-RNA was screened with a 390-bp GS cDNA clone (pAnGS1) that was originally characterized as a nodule-specific GS mRNA (Dunn *et al.* 1988). pGS13 was one of the cDNA clones that was isolated as a positively hybridizing clone and was found to contain an insert of approximately 1.4 kb. The entire cDNA insert was sequenced and the 3′ untranslated region and 3′ end of the coding region was shown to be 100% homologous to pAnGS1 (Dunn *et al.* 1988). Comparison of the pGS13 sequence with that of pGS100 (Tischer *et al.* 1986) established that pGS13 contains the entire coding sequence of 1,068 bp and a 231-bp 3′ untranslated region (Fig. 1). While the coding region showed 82% identity at the nucleotide level and 90% identity at the amino acid level to pGS100, there was no significant homology in the 3′ untranslated region. The possible polyadenylation site on pGS13 is underlined (Fig. 1). Sequence analysis of another partial cDNA clone (pGS7) with identity to pAnGS1, showed that the two cDNAs (pGS13 and pGS7) shared 100% identity, but that the latter had a slightly shorter 3′ untranslated region (by 10 bp). It is likely that pGS7 represents an allelic form of the gene represented by pGS13.

Comparison of the nucleotide sequence of the coding region of the cDNA clone pGS13 with the coding region of other known GS genes showed pGS13 to have the highest identity (89%) to pGS341, a nodule-enhanced GS_1 gene in pea. The homology between pGS13 and pGS100, another GS_1 gene in alfalfa is only 82% and suggests a higher GS

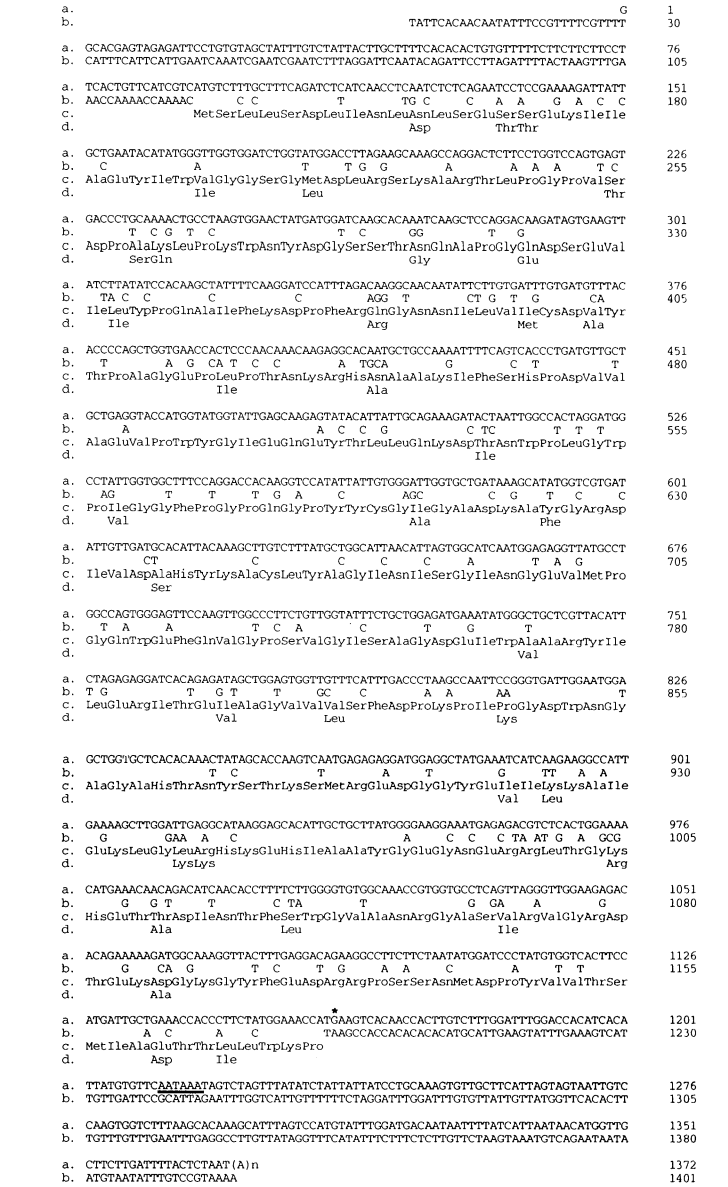

Fig. 1 Nucleotide sequence of the alfalfa glutamine synthetase (GS) cDNA clones pGS13 and its alignment with that of pGS100 (Tischer *et al.* 1986). The nucleotide sequence (numbered on the right) and the derived amino acid sequence of pGS13 are shown in full and are represented in rows a and c, respectively. The nucleotide and amino acid sequence of pGS100 (rows b and d, respectively) are only shown where they differ from pGS13. The translation termination codons are represented as asterisks. The putative polyadenylation site in the 3′ untranslated regions of pGS13 is underlined. The nucleotide sequence data reported will appear in the EMBL and GenBank Nucleotide Sequences Databases under the accession number U15591.

sequence conservation interspecifically than intraspecifically. In this context, it is interesting to point out that pGS100 shares 91% nucleotide sequence identity to the other class of nodule enhanced GS_1 gene of pea, pGS299. Comparison of the pGS13 nucleotide sequence with other GS genes showed higher overall sequence homology to GS_1 genes that show enhanced expression in nodules (Table 1). Furthermore, when the comparison was extended outside the legumes, the identity to other GS_1 gene members was still high (being higher in the dicots than in the monocots). Overall, the identity of both pGS13 and pGS100 to the plastid form of GS was consistently lower than to the cytosolic forms. Our conclusions that there is sequence conservation among GS genes with common functions is supported by the results of a more sophisticated phylogenetic study on GS genes (Doyle 1991).

pGS13 and pGS100 represent different GS_1 gene classes in alfalfa.

Genomic Southern blot hybridization analysis was performed on DNA from a single alfalfa plant using both the coding region and the 3′ untranslated region of pGS13 and pGS100 as probes. A single plant was utilized for Southern analysis to minimize problems in the interpretation of the results arising from the polyploid and outcrossing nature of alfalfa. Genomic DNA was digested with various restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I), fractionated on a 0.8% agarose gel, blotted onto nitrocellulose, and probed under moderately stringent conditions with the different regions of the cDNAs as indicated (Fig. 2). Using replicate blots, the coding region of pGS100 and pGS13, which share 82% identity, hybridized to a set of restriction bands that are unique to each cDNA and a subset of these restriction bands hybridized to the corresponding 3′ untranslated region of the cDNAs (Fig. 3). The hybridization pattern with the coding region probes suggests that there are at least two classes of GS_1 genes in alfalfa. Furthermore, a comparison of the hybridization profile obtained with the coding region and the corresponding 3′ untranslated region as probes, on any particular restriction enzyme digest is consistent with all members of each gene class having the same 3′ untranslated region. Thus, for example the pGS13 gene with one *Xba*I site in the middle of the gene (Fig. 2) should produce two sets of bands that hybridize to the coding region probe and only one of each set should hybridize to the 3′ untranslated region. Four *Xba*I fragments (4.6, 3.2, 1.8, and 0.79 kb) hybridized to the pGS13 coding region probe and only the 3.2 and 1.8 kb fragments hybridized to the pGS13 3′ untranslated region probe suggesting that the alfalfa genome has at least two pGS13-related genes. The hybridization profile with the *Hin*dIII and *Eco*RI digests is however, consistent with there being only one pGS13 gene. The same argument holds for the pGS100 gene. However, there are inconsistencies and we attribute this to polymorphism among the different alleles and their flanking regions or to the presence of truncated gene

Table 1. Comparison of alfalfa nodule-enhanced glutamine synthetase (GS) gene sequences with other GS gene sequences

Plant species	Clone	Isoform	Subcellular location	Nucleotide comparison[a]	Amino acid comparison[a]	References
Medicago sativa	pGS13	GS1	Cytosol (Nod enhanc.)	...	...	This paper
	pGS100	GS1	Cytosol[b]	82	90	Tischer *et al.* 1986
Medicago truncatula	MtGSa	GS1	Cytosol (Nod enhanc.)[c]	97	100	Stanford *et al.* 1993
	MtGSb	GS1	Cytosol[c]	84	92	Stanford *et al.* 1993
Pisum sativum	pGS299	GS1	Cytosol (Nod enhanc.)	80	87	Tingey *et al.* 1988
	pGS341	GS1	Cytosol (Nod enhanc.)[d]	89	92	Tingey *et al.* 1987
	pGS185	GS2	Plastid (leaf, nodule)	72	77	Tingey *et al.* 1988
Lupinus angustifolius	pGS5	GS1	Cytosol (Nod specific)[e,f]	81	88	Grant *et al.* 1989
Lupinus luteus	pcLINGS	GS1	Cytosol (Nod specific)[g]	81	89	Boron *et al.* 1989
Phaseolus vulgaris	pcGS-α	GS1	Cytosol	80	84	Gebhardt *et al.* 1986
	pcGS-β	GS1	Cytosol	73	80	Gebhardt *et al.* 1986
	pcGS-γ	GS1	Cytosol (Nod specific)[f]	80	87	Bennett *et al.* 1989
	pcGS-δ	GS2	Plastid (leaf, nodule)	60	66	Lightfoot *et al.* 1988
Gylcine max	pGS20	GS1	Cytosol (Nod Enhanc.)	81	89	Miao *et al.* 1997
	pGSGmE	GS1	Cytosol (Nod specific)[g]	78	87	Roche *et al.* 1993
Nicotiana plumbaginifolia	pGS1/pGS15	GS1	Cytosol	82	89	Tingey and Coruzzi, 1987
Nicotiana tabacum	pcGS2-17	GS2	Plastid (leaf)	70	74	Becker *et al.* 1992
Lactuca sativa	pLGS15	GS1	Cytosol	79	85	Sakamoto *et al.* 1990
Arabidopsis thaliana	λAtgsr1	GS1	Cytosol (root, germ. seed)	77	82	Peterman and Goodman 1991
	λAtgsr2	GS1	Cytosol (root)	79	90	Peterman and Goodman 1991
	λAtgsKb6	GS1	Cytosol (root, germ. seed)	77	85	Peterman and Goodman 1991
	λAtgsl1	GS2	Plastid (leaf)	70	76	Peterman and Goodman 1991
Oryza sativa	λGS8	GS1	Cytosol	73	84	Sakamoto *et al.* 1989
	λGS28	GS1	Cytosol	76	88	Sakamoto *et al.* 1989
	λGS31	GS2	Plastid	71	76	Sakamoto *et al.* 1989
Hordeum vulgare	pcHvGS6	GS2	Plastid	68	75	Freeman *et al.* 1990

[a] The numbers (nucleotide and amino acid comparison) represent the percentage identities of the coding sequence.
[b] Subsequent transcriptional analysis in this paper and Temple *et al.* 1993.
[c] Only the last150 bp of the coding region are available for comparison. MtGSc is not included as it does not appear to be expressed (Stanford *et al.* 1993).
[d] A truncated cDNA (pGS132) which is nearly identical to pGS341 and referred to as a "twin gene" that also exibits nodule enhanced expression has been described (Tingey *et al.* 1987)
[e] The 3′ end of the coding region of this cDNA is missing.
[f] This gene also appears to be expressed at much lower levels in other tissues.
[g] The 5′ end of the coding region of this cDNA is missing.

members in the genome. Moreover, the genomic clone for pGS13 has not been characterized and the position of the introns and their nucleotide sequence is not known. Taken together our results suggest that there are at least two GS_1 gene classes in alfalfa with distinct 3′ untranslated regions.

pGS13 represents a nodule enhanced form of GS gene(s).

The expression of the two classes of GS_1 genes was examined in leaves, roots, and nodules of alfalfa. Poly(A)RNA from the different organs was fractionated on agarose/formaldehyde gels, in triplicate. Two of the panels were probed with the 3′ untranslated region of pGS13 (pGS13G) or pGS100 (pGS304), while the third panel was probed with a 28S rRNA gene probe as an internal standard for RNA loads (Fig. 4). For a quantitative comparison between the two transcript classes, the specific activities of the two GS_1 gene probes were kept identical as were the hybridization and washing conditions along with the exposure time on the X-ray film. As seen in Figure 4, both pGS13G and pGS304 hybridized to a 1.5- to 1.6-kb RNA species in all organs tested; however, the relative abundance of the two mRNA classes showed major variance among the different organs. In general, pGS13G showed a higher level of hybridization than pGS304 to RNA from all organs tested. Specifically pGS13G showed >20-fold higher level of hybridization in the nodules compared to roots, while pGS304 showed only a slight increase in hybridization in nodules compared to roots. Both classes of transcript were at relatively low levels in the leaves (Fig. 4). When compared to the roots, the leaves showed 10-fold and 3-fold lower level of hybridization with pGS304 and pGS13G probes, respectively. These results suggest that pGS13/pAnGS1 represent a nodule enhanced form of GS and not a nodule-specific form as had been proposed earlier (Dunn *et al.* 1988).

pGS13 and pGS100 genes show distinct patterns of expression in alfalfa nodules.

A longitudinal section of a mature alfalfa nodule is made up of four zones (Fig. 5A): The apical meristematic zone/zone I, the invasion zone/prefixing zone II, the symbiotic/nitrogen-fixing zone III, and the distal senescent zone/zone IV (Vasse *et al.* 1990). With the exception of the apical meristem, the other three zones are surrounded by the endodermis and the cortex. Staining alfalfa root nodules with iodine which stains starch grains demarcates a region between the invasion zone and the nitrogen-fixing zone, where the cells are filled with amyloplasts (interzone II–III, Fig. 6D). The amyloplast rich interzone II–III has been shown to be the initial site of expression of leghemoglobin genes (de Billy *et al.* 1991).

In situ hybridization was performed on alfalfa nodule sections to localize the cellular site of expression of the two sets of GS_1 genes. Longitudinal sections of 28-day-old nitrogen-fixing alfalfa nodules were hybridized with ^{35}S-labeled sense and antisense RNA probes spanning the 3′ untranslated regions of the two genes (Figs. 5–7). The RNA probes were

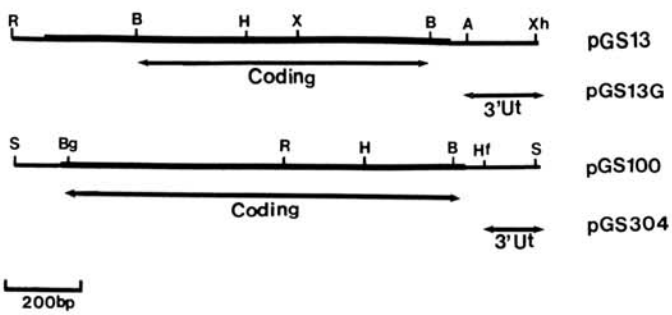

Fig. 2. Description of the probes, pGS13 (coding), pGS13 3′ untranslated region (3′ Ut, pGS13G), pGS100 (coding) and pGS100 3′ untranslated region (3′ Ut, pGS304). The translated region of the two cDNA clones (pGS13 and pGS100) are represented as thick lines, while the 5′ and 3′ untranslated regions are represented as thin lines on the linear maps. pGS13 (coding) was a 777-bp *Bam*HI-*Bam*HI fragment, pGS13G 3′Ut was a 201-bp *AvaII-XhoI* fragment. pGS100 (coding) was a 999-bp*Bgl*II-*Bam*HI fragment and pGS304 3′Ut was a 153-bp *Hin*FI-*Ssp*I fragment. The region of coding sequence and of the 3′ untranslated region used as probes are represented as lines within the arrowheads under the linear maps of the plasmids. The abbreviations for the different restriction sites are: *Ava*II (A), *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (R), *Hind*III (H), *Hin*FI (Hf), *Ssp*I (S), *Xba*I (X), *Xho*I (Xh).

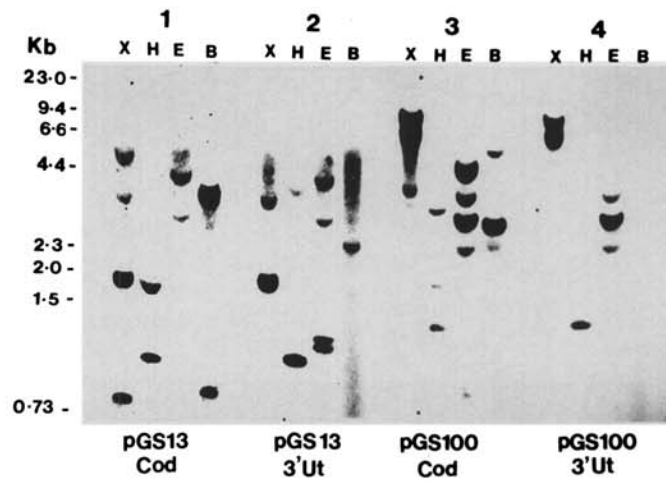

Fig. 3. Genomic Southern blot analysis. Identical Southern blots of total alfalfa genomic DNA (12.5 µg lane) digested with *Xba*I (X), *Hind*III (H), *Eco*RI (E), and *Bam*HI (B) were probed with ^{32}P-labeled probes consisting of the following: pGS13 coding region (1); pGS13 3′ untranslated region (2); pGS100 coding region (3); pGS100 3′ untranslated region (4). Complete details of the probes is given in Figure 2. The position of the size standards are indicated on the left in Kb.

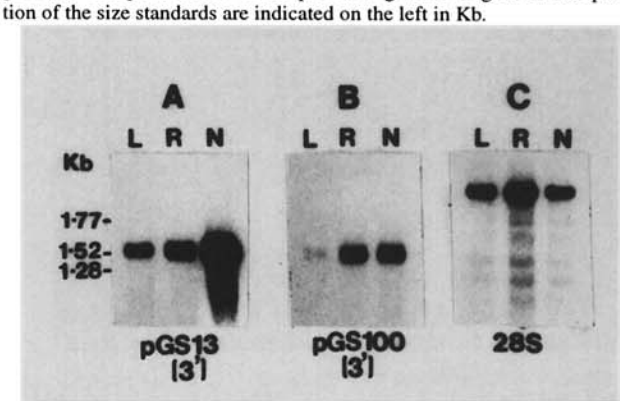

Fig. 4. Analysis of the glutamine synthetase mRNA species in the leaves, roots, and nodules of alfalfa. Poly(A) RNA (1.5 µg lane) isolated from leaves (L), roots (R), and nodules (N) formed by *Rhizobium meliloti* strain 1021 were subjected to Northern analysis using the 3′ untranslated region of pGS13 **(A)**, the untranslated region of pGS100 **(B)**, and the 28S rRNA gene fragment (a kind gift from F. Ausubel) **(C)**. The position of RNA standards of known molecular weight are indicated. Complete details of the probes is given in Figure 2.

made by transcribing the inserts in clones pGS13G and pGS304, using either the SP6 polymerase or the T7 polymerase to obtain the sense or the antisense probe as the case may be. Both the sense probes showed no significant hybridization and as such the data has not been shown.

The two antisense RNA probes showed hybridization throughout the nodule sections behind the meristem (Fig. 5). No hybridization was observed with either probe in the peripheral cortical tissue. However, the two probes showed differences in the level of hybridization and the pattern of distribution of the silver grains. Overall, the pGS13 probe showed a higher hybridization signal, with the intensity being highest in the symbiotic zone proximal to the meristem and decreased progressively towards the senescent zone (Fig. 5B). The pGS100 antisense RNA hybridized more uniformly to transcripts all over the symbiotic and senescent zones (Fig. 5C)

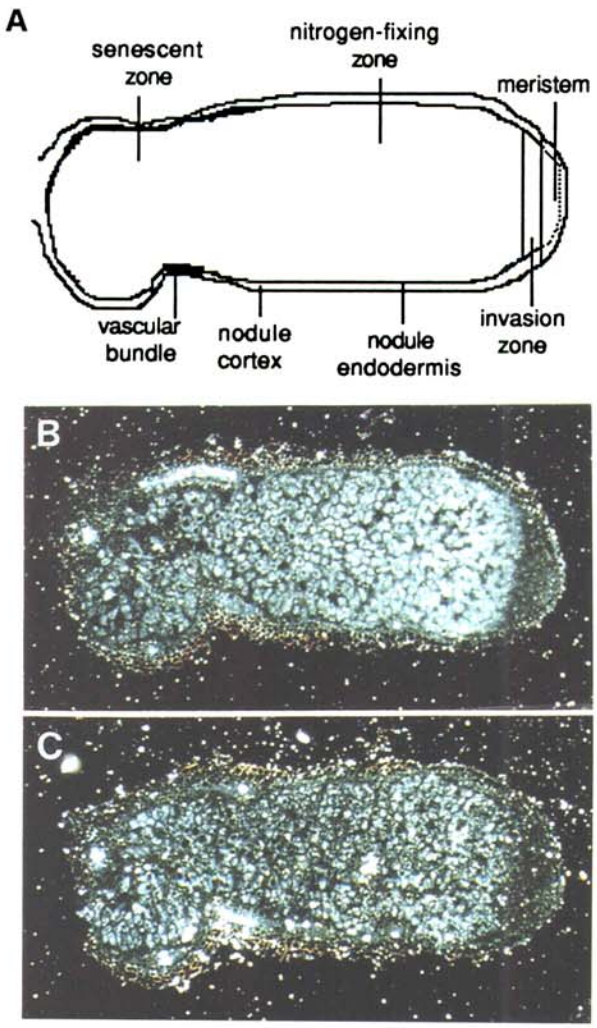

Fig. 5. Localization of the two classes of GS_1 transcripts by *in situ* hybridization in alfalfa root nodules. **A,** Diagrammatic representation of the different zones and tissues in a longitudinal section through a mature alfalfa nodule. **B** and **C** are aligned dark field micrographs of serial longitudinal sections through a 28-day-old wild-type alfalfa nodule hybridized with the pGS13G ^{35}S-labeled antisense RNA probe (**B**) or the pGS304 ^{35}S-labeled antisense RNA probe (**C**). The autoradiographic signal appears as white silver grains. Both probes were labeled to have the same specific activity. The hybridization conditions, washing conditions and exposure times to film were kept constant. Magnification ×36.

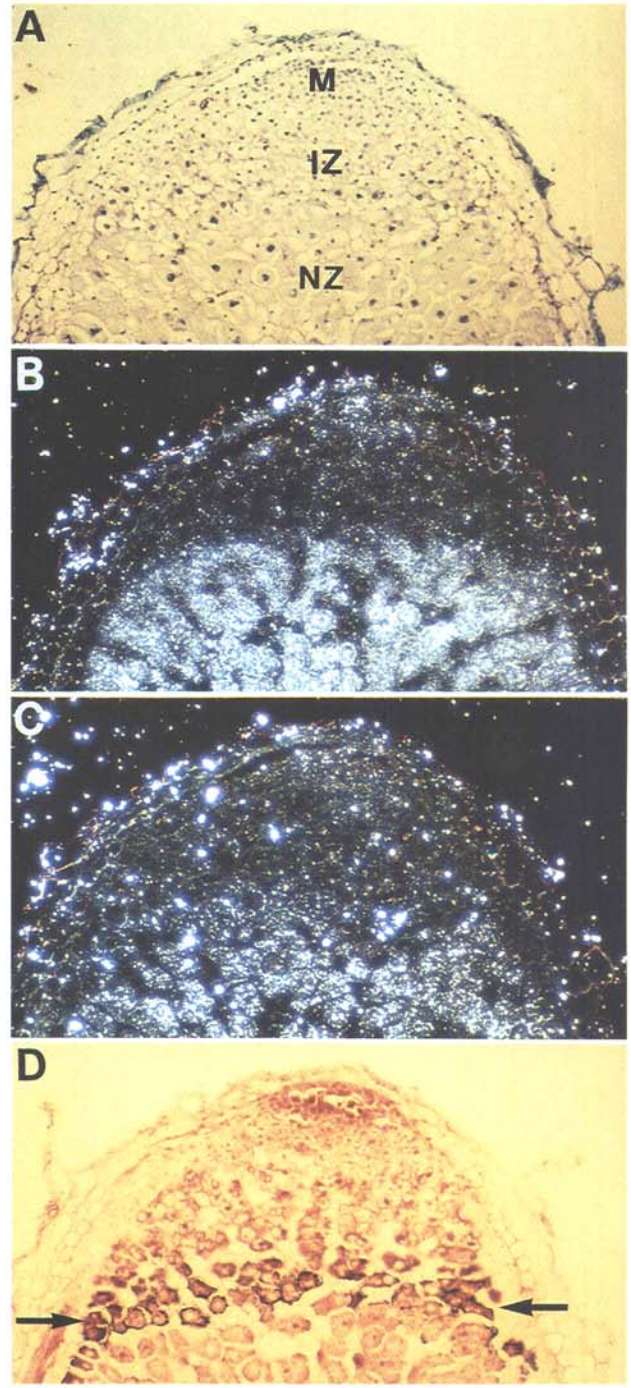

Fig. 6. *In situ* localization of the GS_1 transcripts in the meristem, invasion zone and early symbiotic zone of alfalfa nodules. **A** is a bright field of the anterior region of a 28-day-old wild-type alfalfa nodule stained with toluidine blue. The position of the meristem (M), invasion zone (IZ), and early symbiotic zone (NZ) is indicated. **B** and **C** are aligned dark field micrographs from longitudinal serial sections of the same 28-day-old nodule shown in **A**. The sections were hybridized with the antisense RNA probe to pGS13G (**B**) and pGS304 (**C**). Silver grains representing hybridization signals are visible as white spots. The hybridization signals represent the relative transcript levels for the two GS_1 classes. **D** is a section of the same nodule stained with neutral red stain for 5 min and counter-stained for starch with an iodine-potassium iodide solution. The arrows point to the layer of cells rich in amyloplasts. Magnification = ×195.

Closer examination of the apical end of the nodules (Fig. 6), showed that transcription of the pGS13 gene is triggered in the invasion zone ahead of the interzone II–III and that the peak of transcription activity is in the symbiotic zone just behind interzone II–III (Fig. 6B). The peak of transcription of the pGS100 genes appears to be in the interzone II–III, a few cell layers behind the induction site of pGS13 (Fig. 6C). Higher magnification of the region of hybridization showed that the labeling was limited to only the infected cells with both probes (Fig. 7).

DISCUSSION

In this paper, we have clearly established that there are at least two GS_1 gene classes that are expressed in the indeterminate nodules of alfalfa. Genes represented by pGS13, which—although not nodule specific—are expressed at a level approximately 20-fold higher in nodules than in roots. The expression level of pGS13 genes in leaves is approximately equivalent to that found in roots, while genes represented by pGS100 are expressed at a slightly elevated level in nodules when compared to roots and at a much lower level in leaves. Our data cannot, however, rule out the possibility that other GS_1 genes are also expressed in the alfalfa nodule. In the related plant, *M. truncatula*, there are only two functional GS_1 genes and both are expressed in the nodule (Stanford *et al.* 1993), while in pea, which also produces indeterminate nodule, three GS_1 genes are expressed in the nodule (Tingey *et al.* 1987; Walker *et al.* 1989).

Southern blot analysis of alfalfa genomic DNA, while suggesting that there are multiple genes for GS_1, cannot determine the exact number of genes. We have ruled out the possibility that any of the major hybridizing bands on genomic Southern blots represent GS_2 genes, since GS_1 genes share only limited homology to the GS_2 genes (Table 1). Furthermore, we had shown earlier that the pGS100 cDNA did not hybridize to GS_2 mRNA from alfalfa leaves (Temple *et al.* 1993) and a cDNA representing the pea GS_2 (Tingey *et al.* 1988) showed a hybridization pattern on alfalfa genomic DNA that was different from that seen with either pGS100 or pGS13 (K. L. Bemis, S. J. Temple, and C. Sengupta-Gopalan, unpublished data). Since the coding regions of the two repre-

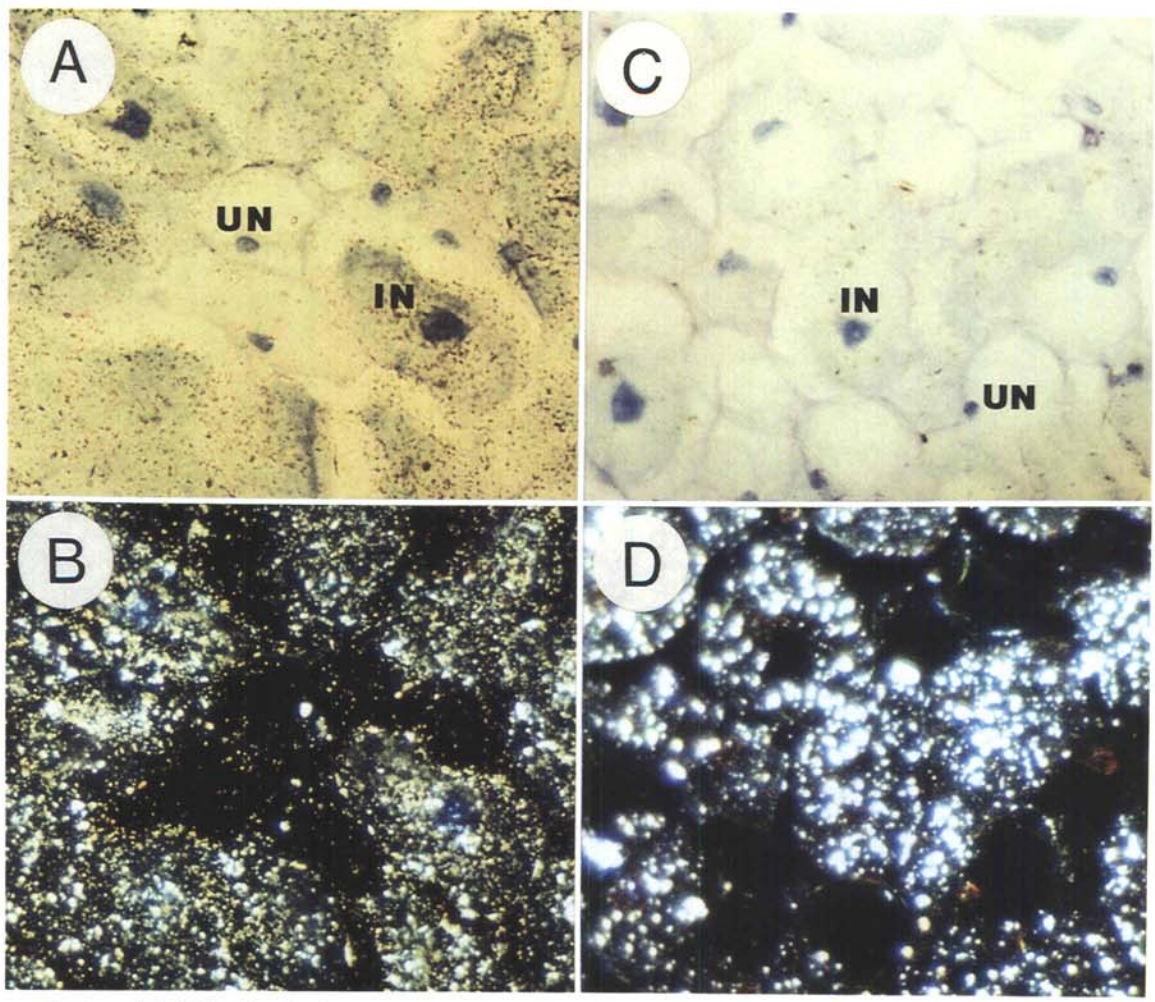

Fig. 7 A magnification of the infected region from Figure 5A and C. Bright field micrograph of a section of the early symbiotic region from Figure 5 showing infected cells (IN) and uninfected cells (UN), hybridized with either pGS13G (**A** and **C**) or pGS304 (**B** and **D**). **B** and **D**, are the dark field micrographs of the same sections used in **A** and **C**, respectively. The autoradiographic signals appear as white silver grains. The exposure time for the two sections was adjusted for the best resolution and does not reflect the relative transcript levels. Magnification = ×300.

sentative GS_1 genes showed a different genomic DNA hybridization profile, it would follow that other GS_1 genes that do not share very high homology (<82%) to either pGS13 or pGS100, remain unaccounted for in this study. Our data also cannot distinguish between unique genes or allelic forms of the same gene member. Since alfalfa is an outcrossing tetraploid, any one plant could have four different allelic members for any gene. The complex genomic pattern for GS_1 genes, however, does not imply that all of the GS_1 genes in alfalfa are functional. Some members could be pseudogenes or truncated genes as has been shown for the gene represented by MtGSc in *M. truncatula* (Stanford *et al.* 1993) and glnε from *P. vulgaris* (Forde *et al.* 1989).

The nodule-enhanced GS_1 gene represented by pGS13 in alfalfa has an expression pattern similar to the GS_1 gene represented by MtGSa in *M. truncatula* (Stanford *et al.* 1993) and pGS341 in pea (Tingey *et al.* 1987). Not only do these GS_1 genes show similarity in the expression pattern, they also share high sequence identity in the coding and 3′ untranslated regions (Table 1, [Stanford *et al.* 1993]). Similarity in the expression pattern may also imply commonality in some of the promoter sequences or elements. The other GS_1 gene that is expressed in alfalfa nodules, represented by pGS100, is most closely related to MtGSb of *M. truncatula* (Stanford *et al.* 1993) and pGS299 of pea (Tingey *et al.* 1988), in DNA sequence. However, the expression pattern of pGS100 differs from that of MtGSb of *M. truncatula* and pGS299 of pea. The pea GS_1 gene represented by pGS299 is expressed at highly enhanced levels in nodules (Tingey *et al.* 1988), while the homologs in alfalfa and *M. truncatula* are either expressed at the same or depressed level in nodules compared to roots. Furthermore, while the alfalfa pGS100 gene is expressed at very low levels in leaves, its homolog in *M. truncatula* (MtGSb), is expressed in leaves at levels equivalent to that in nodules (Stanford *et al.* 1993). The differences in the expression pattern between pGS100 and its homologs, could however, be due to differences in the nodule age or growth conditions of the plants used in the different studies.

In this context it is important to point out that the nodule-specific/enhanced GS_1 genes in soybean (Roche *et al.* 1993) and *P. vulgaris* (Bennett *et al.* 1989) are more similar to each other than to other GS_1 members in the same plant. This observation that the nodule-enhanced/specific GS_1 genes are more highly conserved interspecifically, suggests some functional or physiological relevance related to where the particular enzyme functions.

In this paper we have used *in situ* hybridization to study the distribution of GS_1 gene-specific transcripts within the nodule of alfalfa. A longitudinal section through an indeterminate-type nodule with a persistent meristem reveals the entire developmental pathway along the axis. Thus, cellular localization of a transcript in the nodule section should allow us to relate gene expression with a particular stage in nodule development. *In situ* hybridization has been used to study the developmental pattern of expression of genes encoding leghemoglobin (Lb) (de Billy *et al.* 1991), some late nodulins (de Billy *et al.* 1991; Allen *et al.* 1992), and early nodulins (Allen *et al.* 1991; Scheres *et al.* 1990; Vande de Wiel *et al.* 1990). While there have been a few reports on the cellular localization of GS gene activity in transgenic plants transformed with GS promoter-GUS fusions (Forde *et al.* 1989; Brears *et al.* 1991; Miao *et al.* 1991; Marsolier *et al.* 1993), this is the first report of cellular localization of GS transcripts by *in situ* hybridization. Using gene-specific regions of the two GS_1 genes as probes for *in situ* hybridization of nodule sections, we have investigated the precise stage at which the two classes of GS_1 gene transcripts begin to accumulate during nodule development. The pGS13 gene is induced immediately behind the apical meristem, in the invasion zone or the preinfection zone where cell differentiation begins just prior to the release of bacteria from the infection thread (Vasse *et al.* 1990). Based on the intensity of hybridization, it appears that expression of this class of GS_1 genes is the highest in the invasion zone, the interzone II–III and the proximal region of the symbiotic zone. Lb genes are not expressed in the invasion zone (de Billy *et al.* 1991; Allen *et al.* 1992). Its expression begins at the interzone II–III. The induction of pGS13 and the Lb genes at two distinct cell layers suggest that the two sets of genes are differentially regulated. The pGS13 gene transcript like the Lb transcript is present throughout the symbiotic zone, although the intensity of hybridization gradually decreases towards the senescent zone. This decrease cannot be attributed to a reduction in transcriptional activity or transcript stability of all mRNAs being associated with senescence since transcripts for the Nms22 gene are distributed uniformly throughout the symbiotic and senescent zones (Allen *et al.* 1992). The uniform distribution of hybridization signal with the pGS100 gene probe all over the nodule behind the invasion zone suggests that this GS_1 gene is regulated by a different mechanism from that represented by pGS13.

It is interesting to note that while the GS_1 gene represented by pGS13 is not expressed in the meristem of alfalfa nodules, the pea homolog of this gene is expressed at a relatively high level in the meristem of transgenic alfalfa nodules (Brears *et al.* 1991). Promoter activity of the pea GS_1 gene was monitored by measuring the activity of β-glucuronidase (GUS) in transgenic alfalfa plants transformed with the promoter-GUS fusion (Brears *et al.* 1991). This discrepancy in the expression pattern could be attributed to basic differences in the promoter activity of the two genes or the availability of trans factors that interact with promoter elements or differences in the stability of the GS and GUS transcripts. Moreover, it is also possible that the expression of pea GS3A promoter-GUS gene fusion in alfalfa does not accurately reflect the normal expression of this promoter in the pea plant. In that respect, we view *in situ* localization of transcripts as a much more accurate reflection of promoter activity.

Analysis of the different zones of alfalfa nodules have shown that nitrogenase activity is associated with the proximal region (to the meristem) of the symbiotic zone (Vasse *et al.* 1990). Since the pGS13 and pGS100 gene transcripts first appear in a region ahead of the region active in nitrogenase activity, it would imply that these GS_1 genes in alfalfa are induced independent of nitrogen fixation. The GS_1 gene represented by pGS100, appears to be expressed maximally in the region of nitrogenase activity. Thus it is possible that this gene may be induced slightly by N_2 fixation. Stanford *et al.* (1993) concluded from their study that the plant's nitrogen supply is not the key effector of GS gene expression in *M. truncatula*. However, the expression of both the GS_1 genes in their system appears to be influenced to a small extent by the exogenous nitrogen supply. The effect of exogenously sup-

plied nitrogen and ammonia produced by the symbiont, however, has a very significant role in the induction of a GS_1 gene in soybean (Miao *et al.* 1991; Roche *et al.* 1993). For *P. vulgaris*, none of the GS_1 gene members are affected by ammonium (Cock *et al.* 1990). Thus, what emerges from all these studies is that there is no consensus regarding ammonium-mediated regulation of GS_1 genes in legumes.

Furthermore, our results also suggest that both the GS_1 gene transcripts are located only in the infected cells. Based on the pea GS3A promoter-GUS expression pattern in transgenic alfalfa nodules, it appears that in the symbiotic region, GUS activity is localized to the infected cells (Brears *et al.* 1991). Similarly the nodule enhanced Glnγ of *P. vulgaris* is expressed only in the infected cells of transgenic *L. corniculatus* nodules (Forde *et al.* 1989). However, the soybean pGS20 gene promoter in transgenic *L. corniculatus* nodules, shows activity in both infected and uninfected cells (Miao *et al.* 1991). However, the pGS20 gene, being ammonia inducible is likely to behave differently from other nodule-specific/enhanced GS genes. With respect to our results with the two GS_1 genes from alfalfa, no absolute conclusions can be drawn concerning the absence of transcript in the uninfected cells of alfalfa nodules, because amide-producing plants have few uninfected cells and they are vacuolate.

It is important to point out that in an earlier paper Northern analysis using an oligonucleotide covering a region of the 3′ untranslated region of pAnGS1 (partial clone of pGS13) as a probe, suggested the presence of a nodule-specific GS in alfalfa (Dunn *et al.* 1988). However, in the present paper using a 201 bp of 3' untranslated region of pGS13 as probe, we have very clearly demonstrated the nodule-enhanced, instead of nodule-specific, expression pattern of this GS_1 gene. This discrepancy between our earlier and present studies can be attributed to the greatly increased sensitivity obtained when using poly(A) RNA for the RNA gel and the higher specific activity of a probe prepared by random priming compared to end labeling an oligonucleotide.

We are presently trying to determine if there are additional functional alfalfa GS_1 genes not accounted for by the two GS_1 classes and the functional significance of multiple GS_1 genes in alfalfa and other legumes.

MATERIALS AND METHODS

Growth of plant tissue.

Alfalfa (*Medicago sativa* L.) seeds were germinated in sand and inoculated with a late log phase culture of *Rhizobium meliloti* 1021 at 7 days after planting and were supplied with modified one-quarter strength Hoagland's solution (Hoagland and Arnon 1938) with the nitrogen omitted, twice weekly. Nodules and leaf tissue were harvested in liquid N_2 and stored at –80° C. Uninoculated root tissue was harvested from aseptically grown seedlings.

DNA manipulations.

Standard techniques (Sambrook *et al.* 1989) were used unless otherwise stated. An alfalfa nodule cDNA library was constructed in lambda Uni-ZAP XP (Stratagene, La Jolla, CA) according to the manufacturer's instructions. cDNA was prepared against poly(A) RNA isolated from *Medicago sativa* 'Iroquois' root nodules harvested 28 days after inoculation. A full-length nodule enhanced GS cDNA clone (pGS13) was isolated by hybridization to a ^{32}P-labeled probe prepared from the 390-bp GS cDNA clone 237C (pAnGS1) (Dunn *et al.* 1988). Double-stranded plasmids were sequenced with Sequenase version 2 (United States Biochemical, Cleveland, OH) according to the instructions of the manufacturer. Fragments were subcloned into pGEM3Zf (–) (Promega, Madison, WI). The DNA sequence data were analyzed using DNAlysis (W. Buikema, University of Chicago) and PC Gene (Inteligenetics). The second GS cDNA clone from alfalfa (pGS100) was obtained from Goodman (Das Sarma *et al.* 1986). Previous partial characterization has established that it codes for a GS_1 gene which is expressed in roots and nodules of alfalfa (Temple *et al.* 1993).

Nucleic acid isolation and analysis.

Total RNA was isolated from alfalfa leaves, roots, and nodules using the LiCl precipitation method (De Vries *et al.* 1982). Poly(A) RNA was isolated by subjecting total RNA to poly(U) Sephadex chromatography as described earlier (Murray *et al.* 1981). Poly(A) RNA was fractionated on 1% agarose/formaldehyde gels and transferred to nitrocellulose (Sambrook *et al.* 1989). Genomic DNA was isolated using a modified hexadecyltrimethyl ammonium bromide procedure (Richter *et al.* 1991). RNA was removed by precipitation with 2 M LiCl and the DNA was recovered by precipitation with 2 volumes of ethanol. The DNA was digested with the appropriate restriction enzymes using a threefold excess of enzyme, ethanol precipitated, and redigested to ensure complete digestion. The restricted DNA was ethanol precipitated, washed with 70% ethanol, fractionated on a 0.8% agarose gel, and transferred to nitrocellulose (Sambrook *et al.* 1989). Probes were prepared from inserts isolated from agarose by the ground-glass procedure (Vogelstein and Gillespie 1979), using the random priming method (Feinberg and Vogelstein 1983). Due to the AT-rich sequence of the 3′ untranslated regions, particularly of the nodule-enhanced GS cDNA, the probes used for Southern hybridization were prepared using a combination of both [α^{32}P]dCTP and [α^{32}P]dATP. All filters containing RNA and DNA were prehybridized overnight and hybridized for 20–24 hr in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 5 mM sodium phosphate (pH 7), 0.1% SDS, 0.1 mg/ml denatured calf thymus DNA, and 0.04 mg/ml poly(A) at 42° C. All filters were washed 3 times with 2× SSC, 0.1% SDS at 42° C for 15 min followed by two washes with 0.1× SSC, 0.1% SDS at 42° C for 20 min, and exposed to X-ray film.

In situ hybridization.

In situ hybridization was carried out using the procedure essentially as described by Allen *et al.* (1991). RNA probes were transcribed in the sense and antisense direction from pGS13G and pGS304 which contain the 3′ untranslated region of pGS13 and pGS100, respectively (Fig. 2). Longitudinal sections (7 μm) from 28-day-old alfalfa nodules infected with *R. meliloti* were hybridized with each probe. All sections shown are from the same experiment in which all slides were treated in an identical manner. Emulsion-coated slides were developed after a 2-wk exposure at 4° C.

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