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Alfalfa NADH-dependent glutamate synthase: structure of the gene and importance in symbiotic N_2 fixation

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Summary

Glutamate synthase (GOGAT), a key enzyme in ammonia (NH_4^+) assimilation, occurs as two forms in plants: a ferredoxin-dependent form (Fd-GOGAT) and an NADH-dependent form (NADH-GOGAT). These enzymes are encoded by distinct genes as evidenced by their cDNA and deduced amino acid sequences. This paper reports the isolation and characterization of a *NADH-GOGAT* gene from alfalfa (*Medicago sativa* L.), the first GOGAT gene to be isolated from a eukaryote. RNase protection and primer extension experiments map the transcription start site of *NADH-GOGAT* to nearly identical positions. The transcribed region of this gene, 12 214 bp, is comprised of 22 exons separated by 21 introns. The 2.7 kbp region 5′ from the translation initiation site confers nodule-specific reporter gene activity when used in a chimeric β-glucuronidase (GUS) construct and transformed into *Lotus corniculatus* and *Medicago sativa*. Both infected and uninfected cells display GUS activity. The abundance of NADH-GOGAT transcripts increases substantially in developing nodules of plants infected with effective rhizobia. However, this increase is not observed when nodules are induced by a variety of ineffective rhizobial strains. Thus, unlike many other plant genes involved in root nodule NH_4^+ assimilation, high levels of *NADH-GOGAT* expression are strictly associated with effective nodules indicating that *NADH-GOGAT* plays a central role in the functioning of effective root nodules. An alfalfa Fd-GOGAT PCR product showing greater than 85% identity to maize Fd-GOGAT was isolated and used to investigate the contribution of this enzyme to NH_4^+ assimilation in nodules. Fd-GOGAT mRNA was abundant in leaves and cotyledons but was not detected in alfalfa root nodules. Fd-GOGAT in alfalfa does not appear to play a significant role in symbiotic N_2 fixation.

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Introduction

Nitrogen (N) is the major limiting nutrient for most plant species (Greenwood, 1982). Acquisition and assimilation of N is second in importance only to photosynthesis for plant growth and development (Greenwood, 1982; Vance and Gantt, 1992). Plants acquire N from two principal sources: (i) the soil, through commercial fertilizer, manure, and/or mineralization of indigenous organic matter; and (ii) the atmosphere, through symbiotic N_2 fixation. Irrespective of the source, in higher plants the reduced form of N ultimately available for direct assimilation is NH_4^+ (Lea *et al.*, 1990). Because NH_4^+ is toxic to plant cells, it must be rapidly incorporated into non-toxic amino acids. In most plants NH_4^+ is assimilated into amino acids through the cooperative activity of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT). GS catalyzes the incorporation of NH_4^+ into the amide position of glutamate, producing glutamine. GOGAT catalyzes the reductive transfer of the amido group of glutamine to the α-keto position of 2-oxoglutarate, resulting in the formation of two molecules of glutamate. While numerous studies have examined the genetic and metabolic regulation of GS (Brears *et al.*, 1991; Cock *et al.*, 1990; McGrath and Coruzzi, 1991; Miao *et al.*, 1991), much less is known about the control of GOGAT.

In higher plants, GOGAT occurs as two distinct forms that differ in molecular mass, kinetics, location within the plant, and reductant specificity (Lea *et al.*, 1990; Suzuki and Gadal, 1984): NADH-GOGAT (EC 1.4.1.14) and ferredoxin-GOGAT (Fd-GOGAT, EC 1.4.7.1). Another form of GOGAT (NADPH-GOGAT, EC 1.4.1.13) occurs in bacteria but there is little evidence, to date, for its occurrence in plants (Lea *et al.*, 1990). Isolation and characterization of Fd-GOGAT (Sakakibara *et al.*, 1991) and NADH-GOGAT (Gregerson *et al.*, 1993b) proteins and cDNAs have unequivocally proven that these enzymes are products of two distinct genes.

Fd-GOGAT is predominantly localized in chloroplasts and is involved in the assimilation of NH_4^+ derived from the light-dependent reduction of NO_3^- and from photorespiration (Lea *et al.*, 1990). Fd-GOGAT has also been implicated

in assimilation of NH_4^+ derived from NO_3^- in maize roots (Redinbaugh and Campbell, 1993). Legume root nodules are reported to contain substantial Fd-GOGAT activity, however, the role of this enzyme in NH_4^+ assimilation is not clear (Suzuki *et al.*, 1984, 1988). A complete cDNA encoding Fd-GOGAT has been isolated from maize (Sakakibara *et al.*, 1991) and incomplete clones for the enzyme have been isolated from tobacco (Zehnacker *et al.*, 1992) and barley (Avila *et al.*, 1993). The 5.6 kbp maize leaf Fd-GOGAT cDNA hybridizes to a 5.5 kb transcript which increases some eightfold when etiolated leaves are illuminated. The maize cDNA encodes a 1616 amino acid protein which includes a 97 amino acid presequence that appears to target the protein to plastids. The deduced amino acid sequence of maize Fd-GOGAT is 42% identical to that of the large subunit of *Escherichia coli* NADPH-GOGAT but bears no similarity to the small subunit of *E. coli* NADPH-GOGAT. The barley and tobacco Fd-GOGATs show greater than 85% identity to the maize enzyme and their mRNAs also increase upon illumination of plants. DNA blot analysis shows that *Fd-GOGAT* exists as a single gene in maize but tobacco appears to contain two *Fd-GOGAT* genes.

NADH-GOGAT, like Fd-GOGAT, is a flavoprotein containing an iron-sulfur cluster (Lea *et al.*, 1990). However, NADH-GOGAT is found primarily in non-green tissues. The enzyme has been well characterized from legume root nodules (Anderson *et al.*, 1989; Benny and Boland, 1977; Chen and Cullimore, 1988, 1989) and rice culture cells (Hayakawa *et al.*, 1992). The enzyme exists as a monomer with a native and subunit mass in excess of 200 kDa. The amount of NADH-GOGAT polypeptide in tissues is generally an order of magnitude less than that of GS and/or Fd-GOGAT (Anderson *et al.*, 1989; Chen and Cullimore, 1988, 1989; Egli *et al.*, 1989; Hayakawa *et al.*, 1992). Root nodule NADH-GOGAT activity increases markedly during the development of effective root nodules and this activity increase is accompanied by an increase in enzyme protein and mRNA (Egli *et al.*, 1989; Gregerson *et al.*, 1993b). By contrast, plant-gene-controlled ineffective root nodules have little NADH-GOGAT activity, protein, or mRNA. Moreover, little to no NADH-GOGAT activity and mRNA can be detected in leaves, stems, cotyledons, and roots of alfalfa plants.

We recently isolated a 7.2 kbp cDNA encoding the complete NADH-GOGAT enzyme which is expressed in effective root nodules of alfalfa (Gregerson *et al.*, 1993b). This cDNA contains a single long open reading frame of 2194 amino acids that corresponds to a 240 kDa protein. Amino acid sequence determination of the N-terminus of the mature protein showed that the primary translation product of the alfalfa nodule NADH-GOGAT contains a 101 amino acid presequence resulting in a processed protein of 229 kDa. Cell fractionation studies of root nodules show that the major portion of NADH-GOGAT activity resides in the plastid fraction. However, whether the presequence targets NADH-GOGAT to plastids is not clear. The deduced amino acid composition of the presequence more resembles a mitochondrial than plastid targeting sequence. When the deduced amino acid sequence of alfalfa NADH-GOGAT was aligned with maize Fd-GOGAT and *E. coli* NADPH-GOGAT, interesting similarities and unique differences were noted. The alfalfa protein contains domains that correspond to both the large (46% identity) and small (38% identity) subunits of *E. coli* NADPH-GOGAT. The alfalfa NADH-GOGAT contains a 57 amino acid highly charged domain that serves to connect the regions homologous to the prokaryotic large subunit C-terminus to the small subunit N-terminus. The region corresponding to the large subunit of alfalfa NADH-GOGAT also shows 48% identity to the maize Fd-GOGAT.

Because GOGAT is crucial to N assimilation, a fundamental understanding of the genes encoding this enzyme is requisite to any endeavor to improve and enhance N metabolism. Since no plant *GOGAT* genes have been isolated and since our primary efforts are directed toward understanding the assimilation pathway of symbiotically fixed N, we thought it imperative to isolate the *NADH-GOGAT* gene. Here we report the isolation and characterization of an alfalfa *NADH-GOGAT* gene. Furthermore, we show that the 5′ region upstream from the translation initiation site of this gene directs reporter gene activity specifically to root nodules. Lastly, we show that Fd-GOGAT has little or no role in alfalfa root nodule N assimilation.

Results

Isolation and characterization of the NADH-GOGAT *gene*

An alfalfa genomic library (Gregerson *et al.*, 1994) was screened by hybridization with an alfalfa NADH-GOGAT cDNA, and with PCR amplification products corresponding to the 5′ and 3′ regions of the NADH-GOGAT cDNA. Six clones were obtained and the longest one, containing both the 5′ and 3′ domains, was sequenced from 2043 bp beyond the transcription initiation site to 51 bp beyond the polyA tail addition site. The complete nucleotide sequence of this gene (14 307 bp) can be obtained through EMBL and GenBank as accession number L37606. The nucleotide sequence of the *NADH-GOGAT* gene corresponding to the transcribed region is greater than 99% identical to that of the NADH-GOGAT cDNA. The sequence of the translated region differs from the previously reported cDNA at 16 bases, only two base pairs of which result in a change of charge of the coded amino acids. Nine of the base pair differences lie in the first two exons, with four of the first five differences lying in the region of the protein's presequence.

The positions of the exons and introns within the *NADH-*

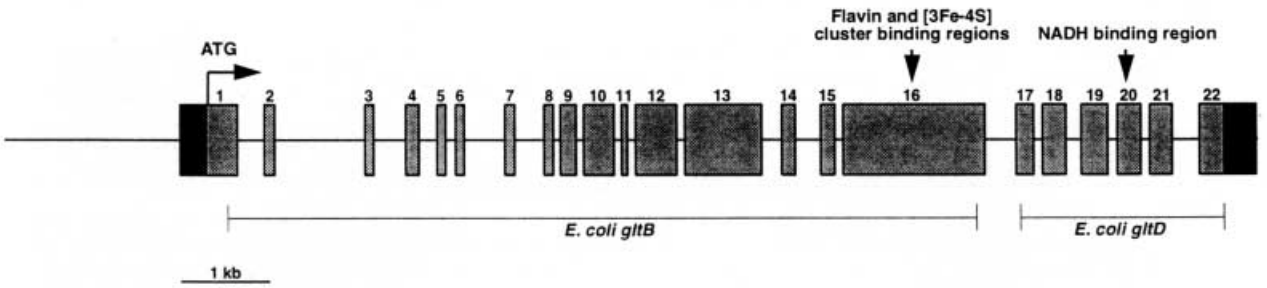

Figure 1. Diagrammatic representation of the structure of the alfalfa nodule *NADH-GOGAT* gene.
Exons are indicated by boxed regions while introns and the 5′ non-transcribed region are represented by lines. The blackened portion of exons 1 and 22 correspond to the untranslated sequences. Below the gene structure is shown the alignment of the *E. coli NADH-GOGAT* large and small subunit genes (Castano *et al.*, 1988) with the encoded homologous regions in the alfalfa gene.

GOGAT gene were determined by alignment of the NADH-GOGAT cDNA with that of the gene sequence. The exon–intron organization of *NADH-GOGAT* is shown in Figure 1. The gene is comprised of 22 exons interrupted by 21 introns that range in size from 82 to 1006 bp. Twenty of the introns conform to the splice site GT.... AG consensus boundary sequences, while intron two, the largest of the introns, begins with a GC dinucleotide. The coding region important for binding of flavin mononucleotide and insertion of the [3Fe–4S] cluster is located in exon 16, the largest of the exons. The five conserved residues important for NADH binding are located in exon 20.

We had previously shown (Gregerson *et al.*, 1993b) that *NADH-GOGAT* encodes a protein with a 101 amino acid presequence and has regions that are homologous with both the large and small subunits of *E. coli NADPH-GOGAT.* The regions homologous to the large and small subunits are connected by a highly charged, hydrophilic sequence of 57 amino acids. Within the *NADH-GOGAT* gene the presequence is located in the first exon. The region corresponding to the large subunit of *E. coli NADPH-GOGAT* was found to be encoded by exons 1–16, while the region corresponding to the *E. coli* small subunit is encoded by exons 17–22. The hydrophylic 'connector' region is encoded by exons 16 and 17 which are separated by a 353 bp intron. The fact that there is an intron in this 'connector' region is intriguing but unfortunately sheds little light on the probable evolutionary events from which this gene arose.

Both primer extension and RNase protection experiments were performed to identify the 5′ end of the *NADH-GOGAT* transcript which presumably identifies the site of transcription initiation. An oligonucleotide primer corresponding to nucleotides +198 to +182 was 5′-end labeled and hybridized to root nodule poly(A)$^+$ RNA. Two extension products (Figure 2) were observed that differed in size by eight nucleotides. RNase protection experiments also yielded two protected fragments that mapped within the nine nucleotide region shown in Figure 2 (data not shown). The data suggest that multiple transcriptional start sites

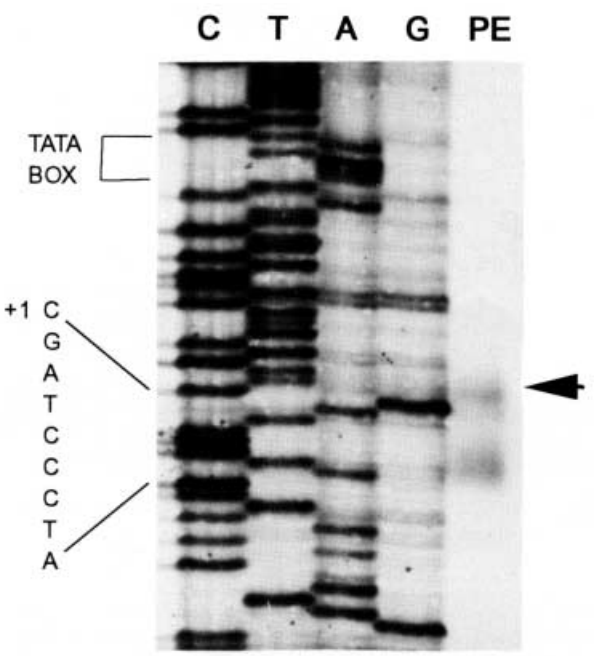

Figure 2. Primer extension analysis of the *NADH-GOGAT* transcripts in alfalfa nodules.
Alfalfa nodule poly(A)$^+$ RNA (5 μg) was hybridized to the end-labeled primer and used as a template for reverse transcription. The arrowhead indicates the longest transcript and the +1 designation at the C nucleotide shows the putative position of the transcriptional start site. A presumptive TATA box is indicated above at nucleotides –23 to –28. The sequencing ladder was made by using the unlabeled extension primer as a sequencing primer for the pGOGAT3E-2 clone.

are present in *NADH-GOGAT.* A consensus TATA box was identified 28 nt upstream from the longest primer extension product.

Expression of an NADH-GOGAT *promoter–*GUS *fusion in root nodules of transgenic* Lotus *and* Medicago *plants*

To assess whether sequences upstream of *NADH-GOGAT* confer gene expression to root nodules, a translational fusion was made with the putative promoter region of the *NADH-GOGAT* gene to the coding region of the

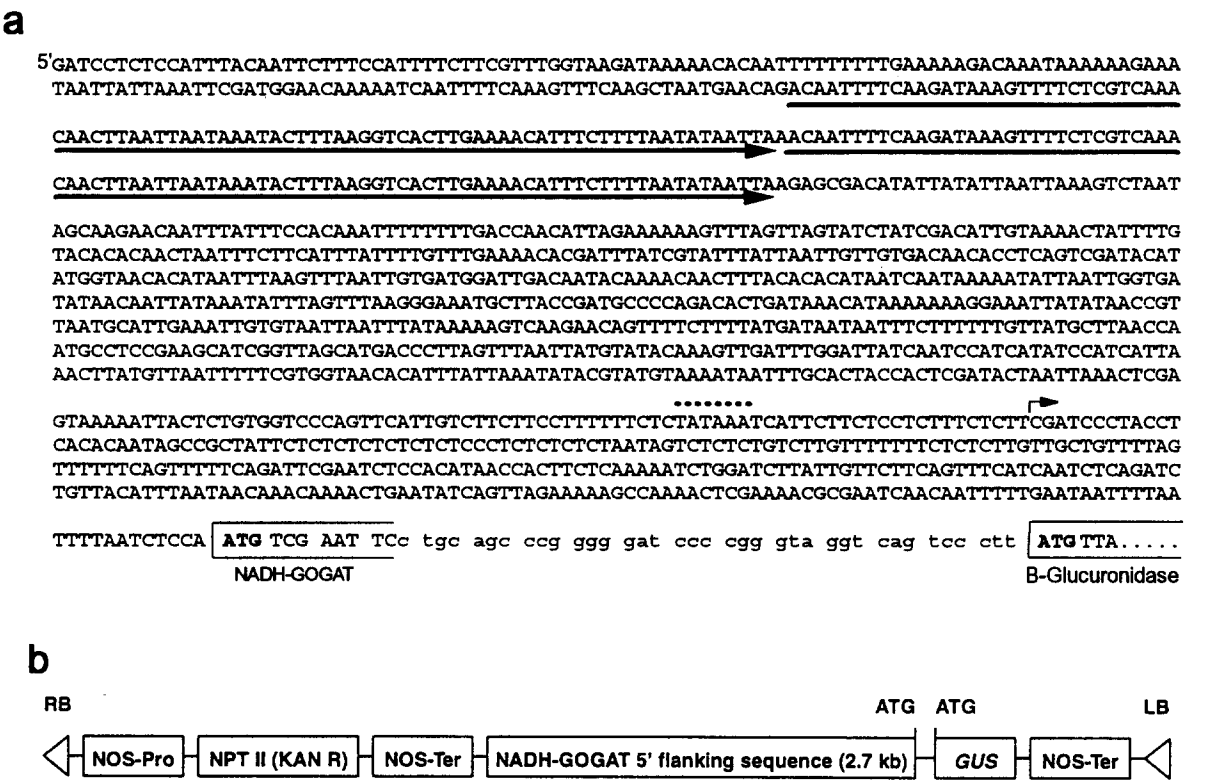

Figure 3. Sequence of the *NADH-GOGAT* promoter element and structure of the chimeric *NADH-GOGAT* / β-glucuronidase (*GUS*) reporter gene.
(a) Partial sequence of the 2.7 kb *NADH-GOGAT* 5' region (upper case) fused to the GUS reporter gene in pBI101.2. This construct (pBICGG-GUS) encodes a translational fusion consisting of the first three amino acids in the NADH-GOGAT primary translation product, an open reading frame encoded by the vector-derived linker region (lower case letters), followed by the *E. coli gusA* gene. The bent arrow indicates the putative start site of gene transcription. Two 88 bp direct repeats are indicated by horizontal arrows.
(b) Chimeric *NADH-GOGAT* promoter-*GUS* construct showing the translational fusion of the 2.7 kb 5' flanking sequence to the *GUS* reporter gene contained in the binary vector pBI101.2.

β-glucuronidase (*GUS*) gene. A portion of the construct's sequence is shown in Figure 3(a). The chimeric promoter-*GUS* construct was used to transform *Lotus corniculatus* via *Agrobacterium rhizogenes* and *Medicago sativa* via *A. tumefaciens* (Figure 3b). Nodules from fully transformed, regenerated plants were assayed histochemically for GUS activity. They were also sectioned and evaluated by dark-field microscopy for cellular localization of GUS activity. Figure 4(a)–(d) show the typical expression observed in root nodules of fully transformed *L. corniculatus* and *M. sativa*. GUS staining was detected in the interior of both the determinate, spherical *Lotus* (Figure 4(a and b) and indeterminate, elongate alfalfa (Figure 4c and d) nodules. Within the interior zone of *Lotus* nodules both infected and uninfected cells stained with near equal intensity. In alfalfa nodules all cells in the meristem (Zone I, Vasse *et al.*, 1990), invasion and prefixing region (Zone II), and the region of active N_2 fixation (Zone III) stained for GUS activity. Dark-field microscopy of nodule thin sections (Figure 4a and c) showed that GUS staining occurred rather uniformly in both infected and uninfected cell types of both *Lotus* and *Medicago*. The blue X-Gluc precipitate observed in the interior of sliced nodules (Figure 4b and d) using bright-field illumination appears red under dark-field conditions (Figure 4a and c). In transformed *Lotus* plants GUS staining did not occur in stems and leaves but slight staining was occasionally observed in roots. In contrast, alfalfa root tips, but not stems and leaves, occasionally showed GUS staining. No staining occurred in nodules from untransformed *Lotus* or alfalfa plants.

Accumulation of mRNAs encoding enzymes involved in nitrogen assimilation in effective and bacterial-controlled ineffective alfalfa nodules

In previous studies (Egli *et al.*, 1991; Gantt *et al.*, 1992; Gregerson *et al.*, 1993b; Pathirana *et al.*, 1992) we showed that there was little to no NADH-GOGAT mRNA accumulation in plant-controlled ineffective root nodules, yet RNAs of nodule-enhanced forms of GS, phosphoenolpyruvate carboxylase (PEPC), aspartate aminotransferase (AAT-2), and leghemoglobin (Lb) accumulated to significant, albeit reduced, levels during development of these nodules.

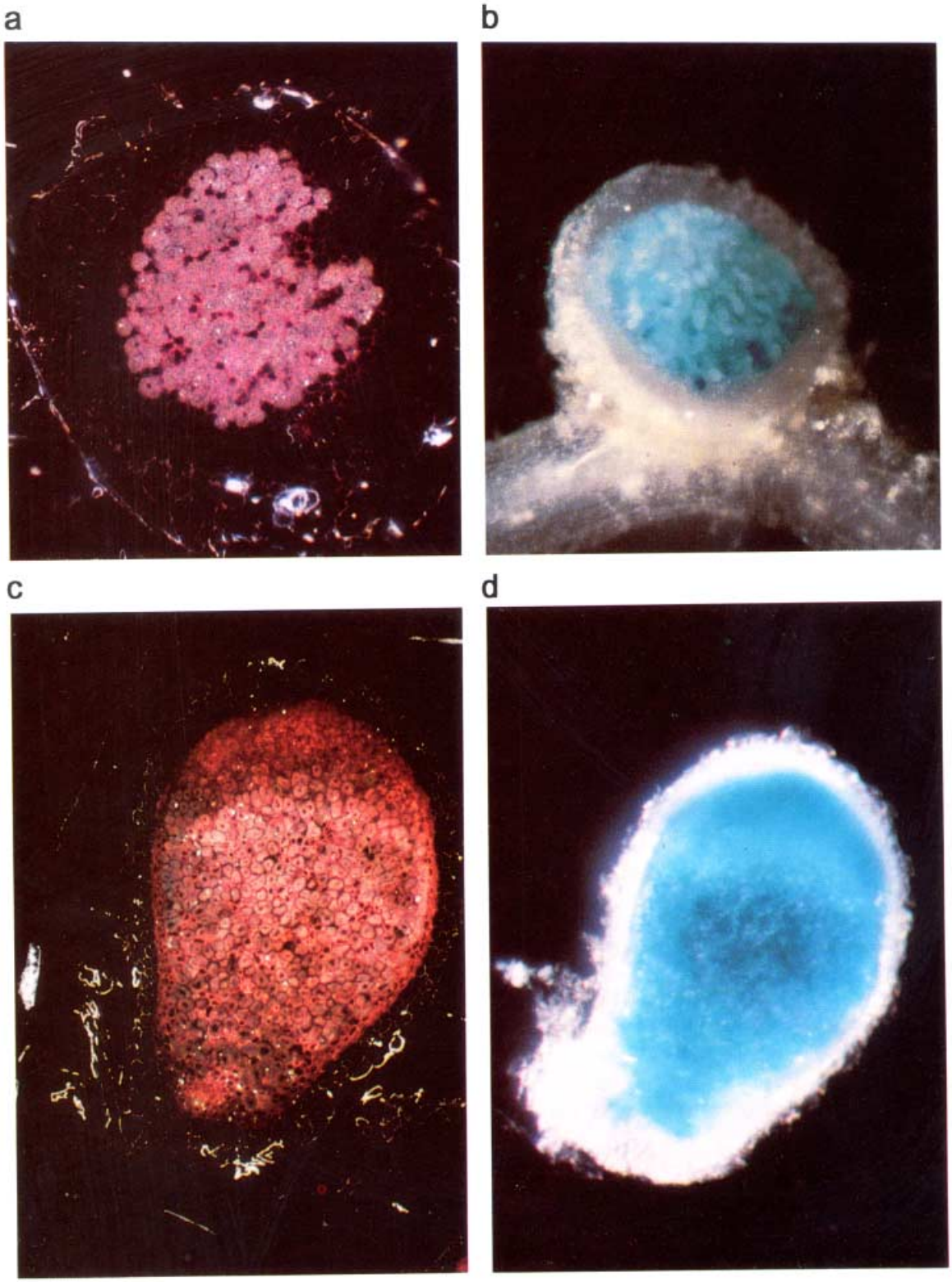

Figure 4. β-glucuronidase (*GUS*) staining in transgenic *Lotus* and alfalfa nodules generated by transformation with either *A. rhizogenes* (for *Lotus*) or *A. tumefaciens* (for alfalfa) containing the pBICGG-GUS construct.
GUS activity (seen as pink or red) in 4 μm sections of transgenic (a) *Lotus* and (c) alfalfa nodules as viewed by dark-field microscopy. *GUS* activity in the interior of (b) *Lotus* and (d) alfalfa nodules. Nodules were sliced after 12 to 24 h of staining.

Table 1. Properties of *R. meliloti* strains

Strain designation	Nitrogenase	Mutation	Bacteroids in nodules	NADH-GOGAT activity (nmol min^{-1} mg $protein^{-1}$)	Leghemoglobin in nodules	Reference
102F51	Fix^+	Wild-type	Yes	47±7	+++[c]	Groat and Vance (1981)
T202	Fix^-	oxr^-[a]	Yes[b]	0	+	Virts *et al.* (1988)
G456	Fix^-	dme^-	Yes[b]	0	++	Driscoll and Finan (1993)
1491	Fix^-	$nifH^-$	Yes[b]	0	+	Hirsch *et al.* (1983)
F642	Fix^-	$dctA^-$	Yes[b]	0	+	Yarosh *et al.* (1989)
7154	Fix^-	$exoH^-$	No	0	-	Leigh *et al.*, (1987)

[a]Mutation designation: oxr^-, oxygen regulation; dme^-, NAD-malic enzyme; $nifH^-$, iron protein; $dctA^-$, organic acid transport; and $exoH^-$, exopolysaccharide lacks succinate.
[b]Bacteroids senesce prematurely.
[c]Relative amount of leghemoglobin as visualized in nodules.

However, comparable experiments with a normally effective alfalfa genotype inoculated with ineffective *Rhizobium meliloti* mutants had not been performed. We therefore determined the levels of RNAs encoding enzymes involved in N assimilation in ineffective nodules induced by several *R. meliloti* mutants. Properties of the *R. meliloti* strains used in this study as well as characteristics of nodules induced by these strains are given in Table 1. Total RNA was extracted from day six roots (R), and day nine (9) and day 12 (12) nodules from normally effective Saranac plants inoculated with either effective *R. meliloti* strain 102F51 or with five mutant strains of *R. meliloti* that produce ineffective nodules on Saranac plants.

Figure 5 shows representative RNA blots probed for NADH-GOGAT, AAT-2, PEPC, GS, Lb, AAT-1, and aldolase (ALD). Expression of NADH-GOGAT in effective nodules induced by *R. meliloti* 102F51 was similar to that reported earlier (Gregerson *et al.*, 1993b). Roots had basal levels of expression, while expression increased significantly in nodules on day nine and again on day 12. It should be noted that nitrogenase activity in effective nodules is first detected on day nine and reaches a maximum on day 12 (Gantt *et al.*, 1992; Gregerson *et al.*, 1993b). The expression patterns of AAT-2, GS, PEPC, and Lb RNAs during development of these effective nodules were very similar to that observed for NADH-GOGAT. Radioanalytic image analysis showed that RNA levels increased six-fold for GS and PEPC, 10-fold for AAT-2 and NADH-GOGAT, and 25-fold for Lb between days six and 12. The amounts of ALD and AAT-1 RNAs, in comparison with the nodule-enhanced enzymes, were relatively constant during development of plants inoculated with wild-type *R. meliloti* 102F51.

In contrast to RNA accumulation patterns seen in effective nodules, NADH-GOGAT RNA did not accumulate beyond root (R) levels in nodules induced by any of the ineffective *R. meliloti* mutants. However, by day 12, AAT-2, GS, PEPC, and Lb RNAs accumulated to elevated levels in nodules induced by most of the ineffective *R. meliloti* mutants (Figure 5). The range of increase as measured by radioanalytic image analysis varied from two- to threefold for AAT-2, GS, and PEPC; with a two- to sixfold increase for Lb. Nodules induced by *R. meliloti* mutant strain 7154 were an exception in that PEPC and GS RNAs were expressed only at root levels, while Lb and AAT-2 RNAs were not detectable. Nodules induced by *R. meliloti* strain 7154 were unique, as compared with nodules induced by the other mutant strains, in that they were empty tumor-like nodules containing no bacteroids (Leigh *et al.*, 1987). As seen for effective nodules, the amounts of ALD and AAT-1 RNA were relatively constant during development of ineffective nodules.

Fd-GOGAT in Alfalfa

Although it is generally agreed that NADH-GOGAT is the form of GOGAT most associated with the assimilation of symbiotically fixed N in root nodules (Anderson *et al.*, 1989; Chen and Cullimore, 1988), Fd-GOGAT enzyme activity has been reported to be two-fold greater than that of NADH-GOGAT in soybean root nodules (Suzuki *et al.*, 1984) and the predominant form of GOGAT in alfalfa nodules (Suzuki *et al.*, 1988). To determine whether Fd-GOGAT is important in nodulation and N_2 fixation in alfalfa, we evaluated the expression of Fd-GOGAT RNA in various tissues of alfalfa and during root nodule development.

Using degenerate primers designed from conserved sequences of the maize (zmfdg) and tobacco (ntfdg) Fd-GOGAT proteins (see methods), a 1203 bp PCR product was obtained from alfalfa leaf cDNA. No corresponding PCR product was obtained from root nodule cDNA. Figure 6 shows the deduced amino acid sequence of the alfalfa Fd-GOGAT PCR product aligned with homologous sequences from maize and tobacco Fd-GOGAT. The alfalfa Fd-GOGAT PCR product corresponded to amino acid residues 1096–1497 of the maize Fd-GOGAT. The deduced amino acid sequence of the alfalfa Fd-GOGAT PCR product is greater

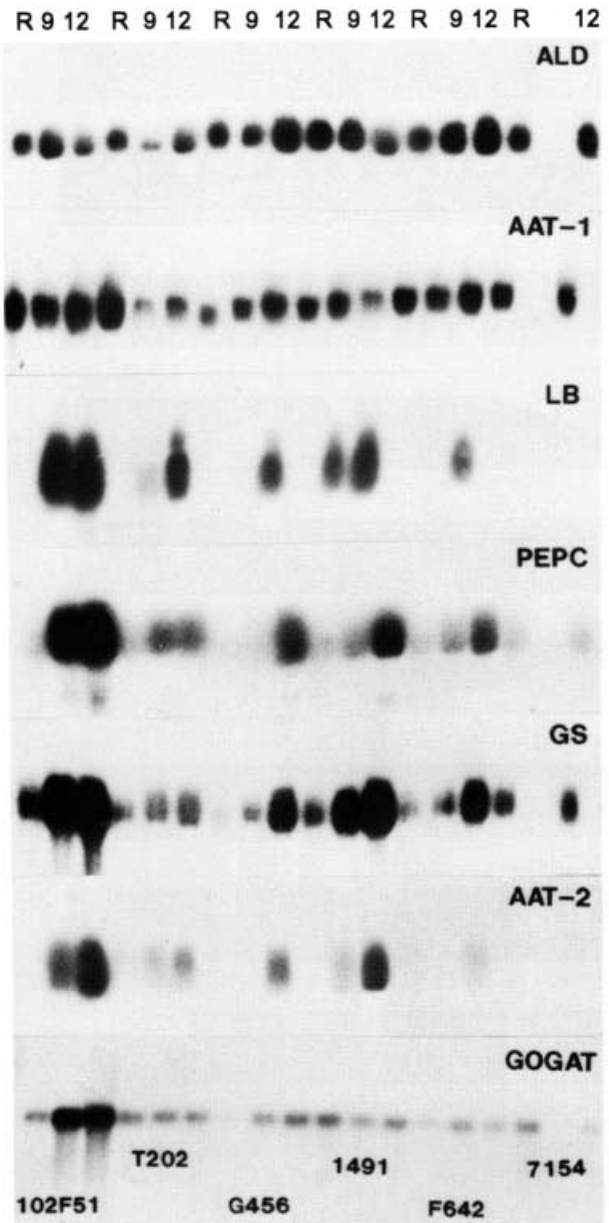

Figure 5. Gel blot analysis of RNA levels for several enzymes of nitrogen and carbon metabolism in alfalfa roots and in nodules induced by effective or ineffective *R. meliloti* strains.
RNA was isolated from roots (day six) and nodules (days nine and 12) of plants inoculated with either effective *R. meliloti* 102F51 or with ineffective strains T202, G456, 1491, F642 and 7154, as indicated. Each group of three lanes contains RNA from roots (R), day nine nodules (9), and day 12 nodules (12). No material was available for 7154-infected plants on day nine. Total RNA (10 μg) was fractionated by electrophoresis through a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane, and probed for the presence of transcripts encoding aldolase (ALD) pALD47 (ALD), phosphoenolpyruvate carboxylase (PEPC), NADH-glutamate synthase (GOGAT),aspartate aminotransferase-2 (AAT-2), the PCR product of alfalfa AAT-1 or the cDNAs for alfalfa leghemoglobin (LB) or glutamine synthetase (GS).

than 85% identical to that of the maize and tobacco Fd-GOGAT cDNAs.

When the alfalfa Fd-GOGAT PCR product was used to probe poly(A)$^+$ RNA blots as shown in Figure 7, using high-stringency conditions, it hybridized to an RNA of the predicted size (5.6 kb) in both leaves (L) and cotyledons (C). No Fd-GOGAT transcripts were detected in roots (R), nodules (N), or stems (S). By comparison, when the identical RNA blot was hybridized with the NADH-GOGAT cDNA, effective nodule RNA contained high levels of the 7.2 kb NADH-GOGAT transcript. Roots also displayed a small quantity of NADH-GOGAT message. Moreover, when the RNA blots shown in Figure 5 were hybridized with the alfalfa Fd-GOGAT PCR product, no hybridizing bands were detectable (data not shown), thus providing additional evidence that Fd-GOGAT mRNA is not detectable in alfalfa root nodules.

To determine the genomic organization and gene copy number of Fd-GOGAT in alfalfa, DNA gel blot analysis of genomic DNA was performed using highly stringent hybridization conditions. As shown in Figure 8, equimolar amounts of *R. meliloti* genomic DNA cut with *Eco*RI gave no hybridization signal (lane 1), whereas hybridizing fragments were seen in alfalfa genomic DNA cut with *Xba*I (lane 2), *Eco*RI (lane 3), or *Eco*RV (lane 4). All restriction digests of alfalfa genomic DNA contained multiple fragments that hybridized to the probe, indicating that Fd-GOGAT is encoded by a small multigene family or that the *Fd-GOGAT* gene contains several large introns. Alternatively, the hybridization pattern could reflect allelic variation at a single genetic locus in this tetraploid species. The presence of additional, significantly divergent *Fd-GOGAT* genes cannot be ruled out since hybridization was performed at a relatively high stringency.

Discussion

Glutamate synthase (GOGAT) in collaboration with GS catalyzes the initial incorporation of NH_4^+ into amino acids in plants (Lea *et al.*, 1990; McGrath and Coruzzi, 1991). In leaves and cotyledons Fd-GOGAT is the major form of GOGAT involved in N assimilation, while in N_2-fixing root nodules and in roots NADH-GOGAT is thought to be the predominant form of the enzyme (Anderson *et al.*, 1989; Chen and Cullimore, 1988). Reported here, for the first time, is the isolation and characterization of a plant *GOGAT* gene. This alfalfa gene encodes a nodule enhanced form of NADH-GOGAT. The protein encoded by this *NADH-GOGAT* gene is 99% identical to that deduced from the originally characterized nodule enhanced NADH-GOGAT cDNA. Of the 17 bp differences between the *NADH-GOGAT* gene and cDNA, eight have no effect on the encoded amino acid (one occurs in the 5′ untranslated region), seven result in an amino acid change but have no effect on charge, and two result in amino acid substitutions that have altered charge. However, because the charge differences are compensatory, the net charge of the protein encoded by this

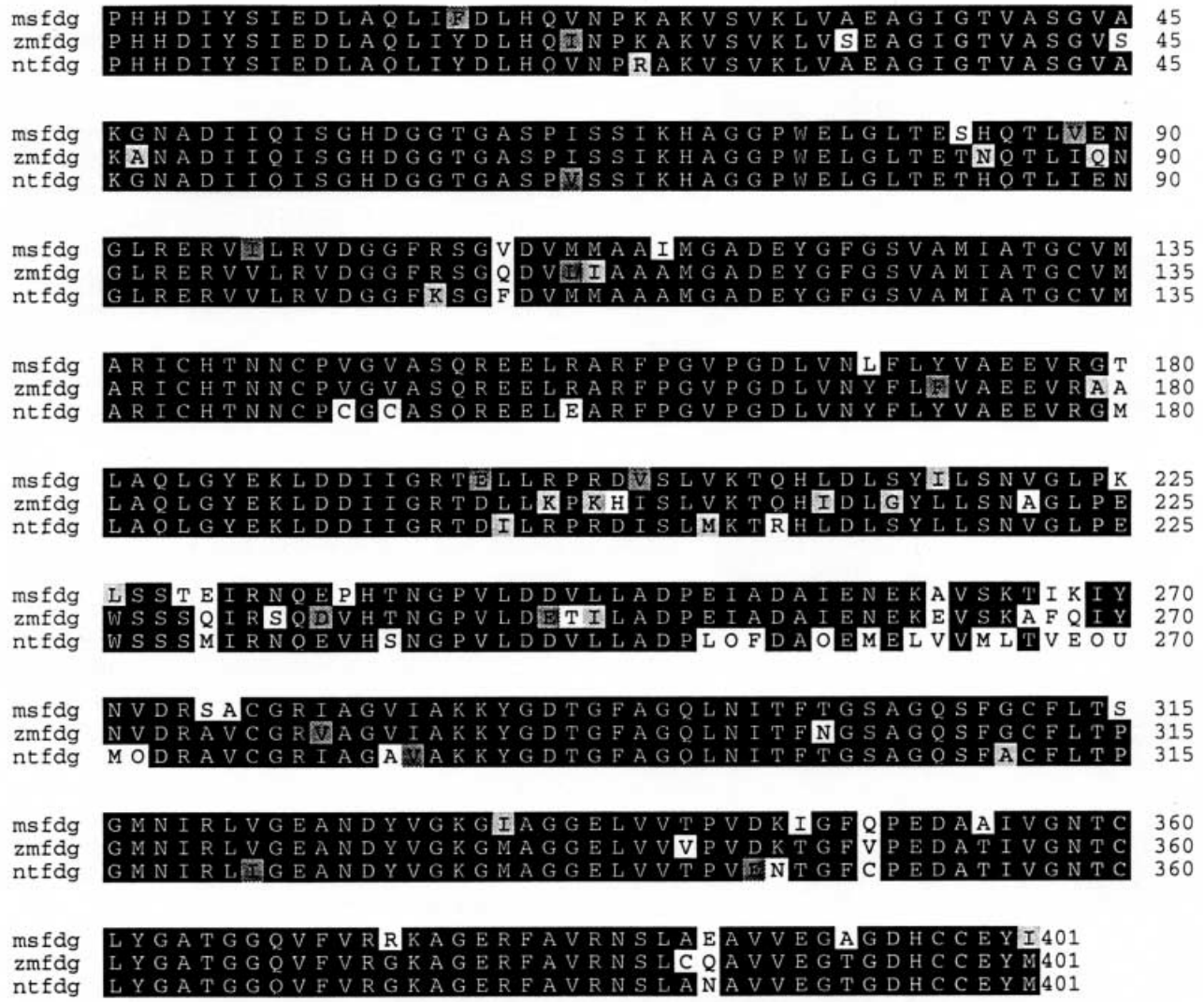

Figure 6. Amino acid comparison of known Fd-GOGAT proteins with that of the deduced amino acid sequence of an alfalfa Fd-GOGAT PCR product. The amino acids shown correspond to the translation of a 1203 bp PCR product generated from alfalfa cDNA (msfdg) aligned with the deduced amino acid sequences of the maize (zmfdg) and tobacco (ntfdg) Fd-GOGAT proteins. The blackened areas represent complete identity and the shaded areas conservative substitutions.

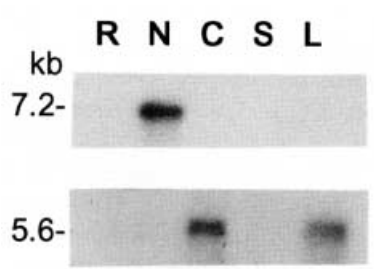

Figure 7. RNA gel blot analysis of Fd- and NADH-GOGAT mRNA expression in various alfalfa organs.
Poly(A)$^+$ RNA (2 μg) from root (R), nodule (N), cotyledon (C), stem (S) and leaf (L) was electrophoresed through a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized to either the cDNA insert of pGOGAT7.2 (upper half) or the insert of pFdGOGAT (lower half). The molecular length of the hybridizing bands are given on the left in kilobases.

NADH-GOGAT gene is identical to that of the originally sequenced cDNA. It is possible that this gene and that represented by the pGOGAT 7.2 cDNA correspond to different alleles of *NADH-GOGAT*. This would not be unusual in alfalfa since it is an outcrossing tetraploid species. The occurrence of multiple allelic forms of nodule-enhanced genes has been documented for alfalfa aspartate aminotransferase-2 (AAT-2) where four alleles occur and all are expressed in root nodules (Gregerson *et al.*, 1993a).

Promoter–*GUS* reporter gene results support the idea that *NADH-GOGAT* expression is related to root nodulation. The 5′ untranslated region of 291 bp and approximately 2.7 kb of the 5′ flanking region upstream to the transcription start site were subcloned into the pBI 101.2 plasmid creating a translational fusion with the reporter *gusA* gene

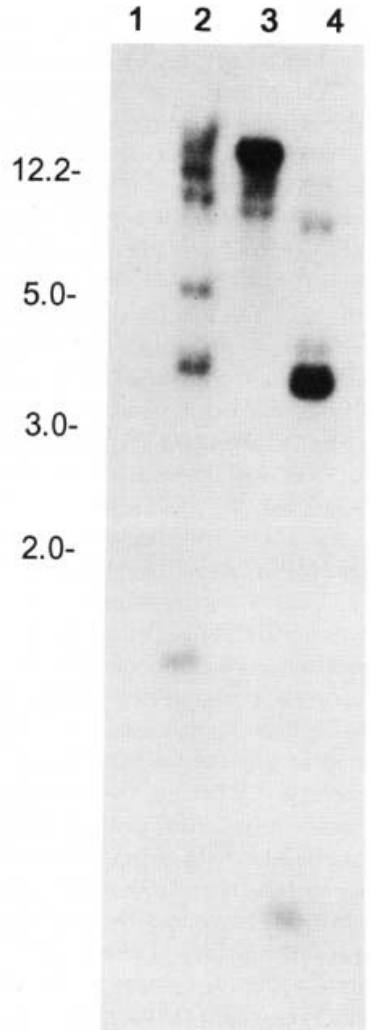

Figure 8. DNA gel blot analysis of *Fd-GOGAT* gene sequences in the alfalfa genome.
Alfalfa genomic DNA (18 μg) was digested with *Xba*I (lane 2), *Eco*RI (lane 3) or *Eco*RV (lane 4), and *R. meliloti* genomic DNA (0.02 μg) was digested with *Eco*RI (lane 1). The digested DNA was electrophoresed through a 0.75% agarose gel, denatured, transferred to a polyvinylidene difluoride membrane, and probed with the insert of pFdGOGAT. The numbers at the left refer to the positions of molecular length markers in kilobases.

which encodes β-glucuronidase (Figure 4a–d). GUS staining results indicated that the *NADH-GOGAT* promoter is active primarily in nodules of both *Lotus* and *Medicago*, is not active in leaves, and only slightly so in roots and stems. These data are consistent with previous studies which showed that NADH-GOGAT activity and mRNA are high in effective nodules but low to non-detectable in other tissues (Anderson *et al.*, 1989; Gregerson *et al.*, 1993b). The fact that the alfalfa *NADH-GOGAT* promoter confers high-level GUS activity to determinate *Lotus* nodules further confirms previous studies showing that common control mechanisms may regulate nodulin gene expression across legume species (Jorgensen *et al.*, 1991; Metz *et al.*, 1988; Stougaard *et al.*, 1987). The staining patterns conferred by the *NADH-GOGAT* promoter were very similar to those seen when the promoter of the soybean cytosolic *GS15* gene was fused to *GUS* and used to transform *L. corniculatus* (Miao *et al.*, 1991) and when the promoter from the nodule-enhanced cytosolic *GS3A* of pea was used to drive *GUS* in alfalfa (Brears *et al.*, 1991). In *Lotus*, *GS15–GUS* activity was localized to the interior of the nodule and occurred in both infected and uninfected cells. Visually, infected cells appeared to have more GUS staining than uninfected cells. Miao *et al.* attributed this staining pattern to the rapid diffusion of NH_4^+, which appears to induce *GS15* expression, in the infection zone and inner cortex. Lack of staining in the outer cortex of the nodule was hypothesized to result from the inhibition of NH_4^+ diffusion through the nodule endodermis. In alfalfa nodules, *GS3A–GUS* activity occurred in all cell types except the outer cortex. The intensity of staining was relatively uniform across cell types. However, in older nodules the inefficient fixing region and/or senescent zone had reduced staining. By contrast, the promoter from the nodule-enhanced cytosolic GS(*gln*-γ) of *Phaseolus vulgaris* confers high levels of GUS activity to only the infected cells of *L. corniculatus* nodules, while the *gln*-β promoter directs GUS expression to cortical and infected cells of young nodules and is limited to vascular bundles in older nodules (Forde *et al.*, 1989). The various patterns of GUS activity regulated by *GS* promoters should be viewed with caution since all have been evaluated in heterologous systems. Clearly, addition data from *in situ* hybridization and immunolocalization studies are needed to localize definitively the sites of *NADH-GOGAT* expression.

Primer extension and RNase protection experiments mapped the transcription start site of *NADH-GOGAT* to nearly identical sites approximately 291 bp upstream of the ATG start codon (Figure 2). The transcribed portion of the gene is 12 214 bp. In many legume nodule-enhanced genes, two sequence motifs located upstream from the transcription start site, CTCTT and AAAGAT, have been conserved. These motifs have been implicated as *cis*-acting elements involved in appropriate expression of leghemoglobin genes of soybean (Stougaard *et al.*, 1987) and *Sesbania* (Metz *et al.*, 1988), and in nodulins 20, 22, 23, and 44 from soybean (Sandal *et al.*, 1987). As can be seen in Figure 3(a) (and in additional sequence entered into EMBL accession number L37606), these conserved sequences are not found within the 2000 bp upstream of the *NADH-GOGAT* transcriptional initiation site. Thus, other sequences must be important as *cis*-acting elements in this gene. A potentially interesting candidate for a *cis*-acting element is an 88 bp direct repeat found between positions −909 and −733. Currently, deletion constructs fused to GUS are being evaluated in transgenic *Lotus* and alfalfa to define the elements which contribute to nodule enhanced expression of NADH-GOGAT.

The root nodules evaluated in Figure 5 of this study can be divided into three phenotypes:

(i) effective nodules induced by strain 102F51;
(ii) ineffective nodules containing bacteria in infected host plant cells as exemplified by those induced by mutant strains T202, G456, 1491, and F642 (Driscoll and Finan, 1993; Hirsch *et al.*, 1983; Virts *et al.*, 1988; Yarosh *et al.*, 1989);
(iii) ineffective tumor-like nodules with no bacteria released into plant cells as occurs in those induced by mutant strain 7154 (Leigh *et al.*, 1987).

By contrasting the levels of NADH-GOGAT, AAT-2, PEPC, and GS RNAs among these phenotypes additional insight into regulation of these genes becomes evident. Of these enzymes, all of which are involved in a common pathway for NH_4^+ assimilation (Vance and Gantt, 1992), only increased NADH-GOGAT mRNA expression was strictly associated with the development of effective nodules (Figure 5). The other enzymes and the oxygen-binding protein Lb showed significant increases in RNA accumulation in non-N_2-fixing nodules which contain bacteria released into host plant cells (phenotype 2 above). Moreover, even though significant RNA accumulates for AAT-2, PEPC, and GS in most of these ineffective nodules by day 12, little to no enzyme activity and protein can be detected (data not shown), which suggests that translational and post-translational events may play an additional role in the expression of these genes. These observations are consistent with our earlier studies of plant-controlled ineffective nodules (Egli *et al.*, 1989; Gantt *et al.*, 1992; Gregerson *et al.*, 1993b; Pathirana *et al.*, 1992) and show that expression of these genes is reduced in ineffective nodules irrespective of whether the plant or bacteria control the ineffective nodule phenotype. The data also provide added support for our earlier suggestion that *NADH-GOGAT* expression appears to be regulated somewhat differently than AAT-2, GS, PEPC, and Lb.

The increased accumulation of AAT-2, GS, PEPC, and Lb RNAs seen in day 12 effective nodules combined with the noticeable lack of enhanced accumulation of these RNAs in empty nodules induced by *R. meliloti* strain 7154 and the intermediate levels induced by other ineffective strains (T202, G456, 1491, and F642) bolster the hypothesis that two steps or signals are required for maximum expression of these genes. The first step leading to increased expression does not require nitrogenase. The second step, leading to maximum expression, however, requires a product associated with effective bacteroids. Whether this latter step involves NH_4^+ release from bacteroids or whether other products are involved is not known. Attempts to demonstrate that NH_4^+ is a signal for enhanced expression of GS and other nodule enzymes have been contradictory (Cock *et al.*, 1990; Groat and Vance, 1982; Miao *et al.*, 1991; Walker and Coruzzi, 1989). Recently, however, the promoter of the nodule-enhanced cytosolic *GS15* gene from soybean was shown to be responsive to NH_4^+ (Marsolier *et al.*, 1993) and the responsive element was located between positions -3.5 and -1.3 kbp relative to the transcription start site. Evidence has also been recently obtained which shows that an AT-rich element between positions -690 and -666 in the *Sesbania rostrata lb3* gene is important for regulation of nodule enhanced expression of lb3 (Welters *et al.*, 1993) and this region reacts with a DNA-binding protein from *Azorhizobium caulinodans*.

Isolation of an alfalfa Fd-GOGAT PCR product which corresponds to a highly conserved region of the maize and tobacco Fd-GOGAT cDNAs (Figure 6) allowed us to determine that Fd-GOGAT RNA was not detectable in alfalfa nodules. The fact that the Fd-GOGAT PCR product does not hybridize to alfalfa root nodule poly(A)+ RNA (Figure 7) but does so readily to a 5.6 kb RNA in leaves and cotyledons shows that Fd-GOGAT gene expression is very low to absent in alfalfa nodules. In addition, the fact that no PCR product was obtained when nodule cDNA was used as template supports this interpretation. Moreover, the lack of any detectable hybridization when the Fd-GOGAT PCR product is used as a probe for blots shown in Figure 4 further corroborates this finding. These data provide convincing evidence that Fd-GOGAT plays very little or no role in either nodule development or assimilation of symbiotically fixed N in alfalfa and is consistent with a lack of detectable Fd-GOGAT enzyme activity in alfalfa nodules (Anderson *et al.*, 1989; Groat and Vance, 1981). Our results, however, contrast with the presence of substantial Fd-GOGAT activity in nodules of soybean (Suzuki *et al.*, 1984) and alfalfa (Suzuki *et al.*, 1988). The high level of Fd-GOGAT activity reported for alfalfa was attributed to a 68.2 kDa polypeptide (Suzuki *et al.*, 1988). However, it seems improbable that this reported alfalfa nodule Fd-GOGAT activity could be attributed to the plant form of the enzyme because the cDNAs encoding plant Fd-GOGATs encode a 166 kDa polypeptide (Avila *et al.*, 1993; Sakakibara *et al.*, 1991; Zehnacker *et al.*, 1992). Furthermore, Chen and Cullimore's (1988, 1989) studies of bean nodule GOGAT support our interpretation, in that while they found detectable Fd-GOGAT activity it was only 30% that of NADH-GOGAT.

Fd-GOGAT is a plastid-localized enzyme considered to be involved in assimilation of NH_4^+ derived from the light-dependent reduction of NO_3^- and from photorespiration (Lea *et al.*, 1990). In maize (Sakakibara *et al.*, 1991), tobacco (Zehnacker *et al.*, 1992) and barley (Avila *et al.*, 1993) levels of Fd-GOGAT mRNA and protein are much higher in photosynthetic organs than in non-photosynthetic organs. Consistent with the proposed role of Fd-GOGAT and data from other species, Fd-GOGAT mRNA is abundant in both leaves and cotyledons of alfalfa (Figure 7). Although we did not detect Fd-GOGAT mRNA in alfalfa roots, it may

accumulate under certain conditions. In maize roots, Fd-GOGAT mRNA is very low but increases when plants are exposed to NO_3^- (Redinbaugh and Campbell, 1993).

Several lines of evidence presented in this current study and in previous studies (Anderson *et al.*, 1989; Egli *et al.*, 1989; Gregerson *et al.*, 1993b; Groat and Vance, 1981) support the hypothesis that *NADH-GOGAT* may be a rate-limiting step in NH_4^+ assimilation of alfalfa root nodules. Unlike PEPC, GS, and AAT-2, expression of alfalfa NADH-GOGAT is strictly correlated with effective nodules and the enzyme occurs as a single isoform. *NADH-GOGAT* appears to be regulated differently than the other enzymes examined and the abundance of protein and enzyme activity is several-fold less than that of GS, PEPC, and AAT-2. A region corresponding to the promoter of *NADH-GOGAT* when fused to a reporter gene and transformed into *Lotus* and alfalfa confers high levels of reporter gene enzyme activity to both infected and uninfected cells of the inner zone of nodules. Lastly, of the total GOGAT activity expressed in alfalfa nodules little if any can be attributed to Fd-GOGAT. Future studies with sense and antisense GOGAT constructs fused to either the *NADH-GOGAT* promoter or other nodule-specific promoters will allow us to test this hypothesis directly.

Experimental procedures

Genomic library construction and screening

A total of 1.5×10^5 recombinant bacteriophage from an amplified alfalfa (cv. Saranac) genomic library (Gregerson *et al.*, 1994) was initially screened at high stringency using the full-length cDNA probe pGOGAT7.2 (Gregerson *et al.*, 1993b). Replicate lifts of the nine hybridizing plaques were rescreened with a PCR product synthesized from the 5′ end of the pGOGAT7.2 plasmid using the T3 primer and a degenerate primer designed to hybridize to the last six amino acids of the mature N terminus of the protein (5′-ACRAANCCNACNCCRCA-3′). Replicate lifts of the five positively hybridizing plaques were purified through one final round and screened with the G3 probe used previously (Gregerson *et al.*, 1993b) which represents 1.7 kb of the 3′-most sequence of the GOGAT cDNA.

DNA was isolated from the putative *NADH-GOGAT* containing phage by a method described elsewhere (Grossberger, 1987) and digested with *Eco*RI. The largest clone contained four fragments of approximately 6.0, 4.5, 3.1 and 2.2 kb in length, the number of fragments predicted by the three *Eco*RI sites in the cDNA. Using the full-length and 3′ end of the cDNA as separate probes, it was determined that all four fragments were part of the GOGAT gene and that the 6.0 kb fragment contained the 3′ end of the gene. The 3.1 kb fragment hybridized very weakly to the full-length cDNA probe. It was found to represent the 5′ end of the clone which codes for only the first two amino acids of the immature protein, the 5′ untranslated sequence and the 5′ sequence upstream of the gene. The 6.0, 4.5 and 3.1 kb *Eco*RI fragments along with a 4.5kb *Bgl*II fragment (which contained the sequence found in the 2.2 kb *Eco*RI fragment) were subcloned into the plasmid vector pBluescript KS^+. Each subclone was sequenced from nested deletion fragments (Henikoff, 1984) using Sequenase 2.0 (US Biochemical, Cleveland, OH). In some cases internal primers were also used. Approximately 2000 bp of the 5′ flanking sequence upstream of the 5′ untranslated region was sequenced from the 3.1 kb *Eco*RI subclone (named pGOGAT3E-2). Sequence analysis was performed using the Intelligenetics software package (Palo Alto, CA).

RNA gel blot analysis

RNA was isolated from roots, nodules, cotyledons, stems and leaves of alfalfa cv. Saranac plants and from the nodules of alfalfa plants inoculated with ineffective *Rhizobium meliloti* strains T202, 1491, F642, G456, and 7154 described earlier (Gregerson *et al.*, 1993b) by the method of Strommer *et al.* (1993). For organ poly(A)$^+$ RNA blot analysis, 2 μg of RNA from each organ were hybridized with the pFdGOGAT insert (see below) or the pGOGAT7.2 insert as described previously (Gantt *et al.*, 1992). For total RNA blot analyses, 10 μg of RNA extracted from developing effective and ineffective nodules were hybridized with ^{32}P-labeled inserts from pGOGAT7.2 (GOGAT; Gregerson *et al.*, 1993b), pPEPC-61 (PEPC; Pathirana *et al.*, 1992), pCAAT2-3 (AAT-2; Gregerson *et al.*, 1993a), pALD47 (ALD; Vance and Gantt, unpublished data), and the cloned alfalfa AAT-1 PCR product (AAT-1; Gregerson *et al.*,1994). Alfalfa leghemoglobin (Lb) and glutamine synthetase (GS) cDNAs were kindly provided by A. Hirsch and K. Dunn, respectively, for ^{32}P labeling. Three separate blots were probed for each RNA tested. The amount of radioactivity that hybridized to each mRNA was quantitated by AMBIS radioanalytic image analysis and the average counts of the three blots calculated.

Primer extension analysis

Primer extension analysis was performed essentially as described previously (Sambrook *et al.*, 1989). An oligonucleotide primer of the sequence 5′-ATGTAACAGATCTGAGA-3′ which is complementary to the nucleotides +198 to +182 bp in the 5′ untranslated region of subclone pGOGAT3E-2 was end-labeled with ^{32}P using T4 polynucleotidyl kinase: 10 pmol of oligonucleotide were incubated in 1× T4 PNK buffer containing 50 μCi of [γ^{32}P]ATP and 7.5 units of T4 polynucleotide kinase (Pharmacia, Piscataway, NJ) for 45 min at 37°C. Following heat inactivation of the enzyme and ethanol precipitation, 5 μg of poly(A)$^+$ RNA were mixed with 100 000 c.p.m. of labeled primer then precipitated with ethanol. The pellet was resuspended in hybridization buffer (40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% formamide), incubated at 85°C for 10 min then placed at room temperature overnight. The primer:RNA hybrids were precipitated with ethanol, dissolved in 20 μl of RT buffer (50 mM Tris pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 1 mM DTT, and 1 unit μl^{-1} RNase inhibitor), and 50 units of murine reverse transcriptase (GibcoBRL, Gaithersburg, MD) were added and the reaction was incubated at 37°C for 1.5 h. The reaction was stopped and the RNA was degraded by the addition of EDTA to 25 mM and DNase-free RNase (5 μg ml^{-1}) followed by a 30 min incubation at 37°C. Following phenol extraction and ethanol precipitation, the extension products were dissolved in 3 μl of TE (pH 8.0) and 2 μl Sequenase stop solution was added. The entire sample was loaded on to a 6% DNA sequencing gel. The sequencing ladder control was made using the unlabeled oligonucleotide primer as the sequencing primer for the pGOGAT3E-2 genomic clone.

RNase protection analysis

RNase protection experiments were performed using the RPAII Assay Kit (Ambion, Austin, TX). A subclone of the pGOGAT3E-2

plasmid was used to synthesize the antisense RNA probe. The pGOGAT3E-2 plasmid was digested with *Bgl*II and *Xho*I generating a 267 bp fragment corresponding to nucleotides +188 to –79 of the genomic clone. The DNA fragment was purified from an agarose gel and subcloned into a *Bam*HI/*Xho*I-digested Bluescript KS^+ vector. The resulting plasmid was then linearized by digestion with *Xho*I and the linearized plasmid was purified from an agarose gel. The DNA was dissolved in RNase-free TE (pH 8.0), extracted with phenol:chloroform:IAA (25:24:1) then chloroform:IAA (24:1), precipitated with ethanol and resuspended at 1 mg ml^{-1} in RNase-free TE (pH 8.0). The radiolabeled probe was prepared as follows: 1 μg of linearized plasmid prepared as above was incubated in 1× T7 transcription buffer containing 30 mM DTT, 3 μM rTTP, 0.4 mM ATP, CTP and GTP, 50 μCi [α^{32}P]UTP, 20 units placental ribonuclease inhibitor and 5 units of T7 RNA polymerase (Stratagene, La Jolla, CA). The reaction was incubated at 37°C for 60 min followed by DNA digestion with 2 units of RQ1DNase (Promega) for 15 min. The probe was gel purified on a 6% acrylamide gel and eluted into 350 μl of probe elution buffer as per the manufacturer's instructions.

Hybridization of the radiolabeled probe (5×10^5 c.p.m.) to alfalfa cv. Saranac total RNA (prepared as described above; 20 μg) was carried out as per the manufacturer's directions at 45°C overnight. Following hybridization, single-stranded RNA was digested by the addition of 20 units of RNase T1 at 37°C for 30 min. The remaining hybrids were separated on a 6% DNA sequencing gel and the dried gel was exposed to X-ray film.

Polymerase chain reaction for Fd-GOGAT

For PCR amplification of Fd-GOGAT cDNA sequences, two degenerate oligonucleotide primers were synthesized, each of which are complementary to sequences conserved in the tobacco (Zehnacker *et al.*, 1992) and maize (Sakakibara *et al.*, 1991) cDNAs. The primer sequences are as follows: primer 1,5′-CATRTAYT-CRCARCARTGRTC-3′ and primer 2,5′-CCNCAYCAYGAYATHTA-3′. First strand cDNA was synthesized from 2 μg poly(A)$^+$ alfalfa leaf RNA using 1 μg of *Not*I primer-adapter (GibcoBRL) in 1× first strand buffer containing 10 mM DTT, 1 mM of each dNTP and 400 units of Superscript reverse transcriptase (GibcoBRL). The reaction was incubated at 37°C for 1 h then heated to 70°C for 5 min to inactivate the enzyme and finally diluted to 100 μl with DEPC-treated water. The PCR reaction contained 1 μl of the diluted cDNA, 2.5 mM $MgCl_2$, 1× Taq DNA polymerase buffer, 200 μM of each dNTP, 25 pmol of each primer and 5 units of Taq DNA polymerase (Promega). An amplification product of the size expected (1203 bp) was purified from an agarose gel and cloned directly into the pCRII plasmid (Invitrogen, San Diego, CA) vector creating the plasmid named pFdGOGAT. The entire PCR product was sequenced with a series of synthesized oligonucleotide primers using Sequenase 2.0. Sequence analysis was performed using the GCG programs (Devereux *et al.*,1984).

DNA extraction and DNA gel blot analysis

Genomic DNA was isolated from alfalfa cv. Saranac leaves, digested with restriction enzymes, electrophoresed, and transferred to Immobilon membrane (Millipore, Bedford, MA) as described earlier (Gregerson *et al.*,1993b). The membrane was hybridized overnight with the ^{32}P-labeled insert from pFdGOGAT and washed under high-stringency conditions as detailed previously (Gregerson *et al.*,1993b).

Construction of chimeric gene and plant transformation

The entire insert of pGOGAT3E-2-3 (opposite orientation clone of pGOGAT3E-2) was excised from the plasmid by digestion at the *Sal*I and *Bam*HI sites in the Bluescript vector DNA. This DNA fragment was purified and then inserted into the corresponding sites in plant transformation vector pBI101.2 (Jefferson, 1987) producing pBICGG–GUS. Sequence analysis of pBICGG–GUS demonstrated that the insert was correctly cloned and that the insertion created a translational fusion product.

pBICGG-GUS was introduced into *Agrobacterium rhizogenes* strain A4TC24 using the freeze–thaw method (Hofgen and Willmitzer, 1988) and into *A. tumefaciens* by electroporation. Transgenic *Lotus corniculatus* cv. Rodeo plants were obtained following the procedures of Petit *et al.* (1987). Following rooting, plants were inoculated with *Rhizobium loti* strain 95C11. Transgenic *Medicago sativa* cv. Regen SY plants were obtained essentially as described by Austin *et al.* (1995). Following rooting, plants were inoculated with *R. meliloti* strain 102F51. Histochemical detection of GUS activity in nodules was performed following the protocol of Jefferson (1987). Nodules were hand-sectioned then vacuum-infiltrated in staining solution.

Stained nodules were fixed, dehydrated, and embedded in LR White resin (Polysciences, Inc., Warrington, PA) as described by Peleman *et al.* (1989). Sections (4 μm) were cut using an MT-7000 Ultramicrotome (Research and Manufacturing Co., Tucson, AZ), mounted on slides, and observed using dark-field microscopy. The blue color seen with X-Gluc staining appears red in dark-field microscopy.

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