The Plant Cell, Vol. 5, 215–226, February 1993 © 1993 American Society of Plant Physiologists

Molecular Characterization of NADH-Dependent Glutamate Synthase from Alfalfa Nodules

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Alfalfa NADH-dependent glutamate synthase (NADH-GOGAT), together with glutamine synthetase, plays a central role in the assimilation of symbiotically fixed nitrogen into amino acids in root nodules. Antibodies previously raised against purified NADH-GOGAT were employed to screen a cDNA library prepared using RNA isolated from nodules of 20-day-old alfalfa plants. A 7.2-kb cDNA clone was obtained that contained the entire protein coding region of NADH-GOGAT. Analysis of this cDNA and determination of the amino-terminal amino acids of the purified protein revealed that NADH-GOGAT is synthesized as a 2194–amino acid protein that includes a 101–amino acid presequence. The deduced amino acid sequence shares significant identity with maize ferredoxin-dependent GOGAT, and with both large and small subunits of *Escherichia coli* NADPH-GOGAT. DNA gel blot analysis of alfalfa genomic DNA suggests the presence of a single NADH-GOGAT gene or a small gene family. The expression of NADH-GOGAT mRNA, enzyme protein, and enzyme activity was developmentally regulated in root nodules. A dramatic increase in gene expression occurred coincidentally with the onset of nitrogen fixation in the bacteroid, and was absent in both ineffective plants that were nodulated with effective *Rhizobium meliloti* and effective plants that had been nodulated with ineffective *R. meliloti* strains. Maximum NADH-GOGAT expression, therefore, appears to require an effective, nitrogen-fixing symbiosis.

INTRODUCTION

In most plants, assimilation of ammonia into organic nitrogen is a result of the collaborative activity of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT) (Lea et al., 1990). GS catalyzes the incorporation of ammonia 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 (Benny and Boland, 1977). In the legume root nodule, ammonia is exported from nitrogen-fixing bacteroids to the host plant cytoplasm, where it is rapidly assimilated into amino acids via this GS/GOGAT cycle.

In higher plants, GOGAT occurs as three distinct forms that differ in molecular mass, kinetics, location within the plant, and reductant specificity (Suzuki and Gadal, 1984): NADH-GOGAT (EC 1.4.1.14), NADPH-GOGAT (EC 1.4.1.13), and ferredoxin-GOGAT (Fd-GOGAT, EC 1.4.7.1). In most cases, it is unclear whether the NADH- and NADPH-dependent GOGAT enzyme activities are a result of the presence of distinct proteins or a single protein that can utilize either pyridine nucleotide cofactor. Therefore, we will refer to these two GOGAT types together as NAD(P)H-GOGATs.

Fd-GOGATs range in estimated mass from 130 to 180 kD, are predominantly localized in chloroplasts, and are involved in assimilation of ammonia derived from the light-dependent reduction of nitrate and from photorespiration (Suzuki and Gadal, 1984; Kendall et al., 1986; Lea et al., 1990). The presence of Fd-GOGAT has also been demonstrated in soybean roots and nodules (Suzuki et al., 1984), where its physiological role is not understood. Antibodies prepared to rice and maize leaf Fd-GOGATs were used to demonstrate that monocot root Fd-GOGAT is antigenically quite different from the leaf enzyme, which suggests that they are encoded by different genes (Suzuki et al., 1982, 1984; Suzuki and Gadal, 1984). These leaf Fd-GOGAT antibodies also do not recognize either NAD(P)H-GOGATs, suggesting that these enzymes are also structurally distinct proteins and are probably encoded by separate genes (Suzuki and Gadal, 1984; Sakakibara et al., 1991). In contrast, antibodies to rice leaf Fd-GOGAT readily recognize soybean nodule Fd-GOGAT (Suzuki et al., 1984), indicating that some antigenic epitopes have been conserved between monocot and dicot ferredoxin-dependent forms of the enzyme. Recently, Sakakibara et al. (1991) characterized a full-length 5.6-kb cDNA that encodes maize leaf Fd-GOGAT. The maize Fd-GOGAT cDNA encodes a 1616–amino acid protein and hybridizes to a 5.5-kb mRNA, which increases in abundance upon illumination. The deduced amino acid sequence of maize

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Fd-GOGAT is ~42% identical to that of the large subunit of *Escherichia coli* NADPH-GOGAT; however, the maize amino acid sequence bears no similarity to the small subunit of the *E. coli* NADPH-GOGAT.

In comparison to the Fd-GOGATs in photosynthetic tissues, the NAD(P)H-GOGATs of nongreen tissues have received less attention. This is in part because root and nodule forms of NAD(P)H-GOGAT are highly unstable and occur in low abundance. Root nodule NADH-GOGAT from lupine (Benny and Boland, 1977), common bean (Chen and Cullimore, 1988, 1989), and alfalfa (Anderson et al., 1989) has been isolated and characterized, and its activity has been found to increase markedly during development of N_2-fixing (effective) nodules (Benny and Boland, 1977; Chen and Cullimore, 1988; Anderson et al., 1989). Plant-controlled, non-N_2-fixing (ineffective) nodules and nodules with reduced N_2 fixation, however, have very low to nondetectable NADH-GOGAT activities (Groat and Vance, 1982; Vance and Johnson, 1983; Egli et al., 1989). In alfalfa and lupine nodules, NADH-GOGAT activity is associated with a single form of the enzyme having an estimated native molecular mass of ~225 kD and a subunit molecular mass in excess of 200 kD (Benny and Boland, 1977; Anderson et al., 1989). In contrast, bean nodule NADH-GOGAT occurs as two isoforms (I and II), each with a molecular mass of ~200 kD (Chen and Cullimore, 1988). Increased NADH-GOGAT activity during development of bean nodules results primarily from an increase in isozyme II activity (Chen and Cullimore, 1988).

High-titer monospecific polyclonal antibodies to alfalfa nodule NADH-GOGAT recognize a protein of greater than 200 kD from nodules of alfalfa and other legume species and immunoprecipitate nodule NADH-GOGAT activity (Anderson et al., 1989). Alfalfa leaves and roots have low to nondetectable NADH-GOGAT activity and lack a cross-reacting polypeptide, suggesting that nodule NADH-GOGAT is either nodule specific or nodule enhanced (Anderson et al., 1989).

Because legume nodule NADH-GOGAT plays such a significant role in assimilation of biologically fixed N_2, it is imperative to gain a thorough understanding of the mechanisms that regulate its activity. To initiate such studies, we have isolated an NADH-GOGAT cDNA clone, deduced the enzyme's primary structure, and defined the amino terminus of the mature protein by amino acid sequence determination. In addition, we have evaluated expression of enzyme activity, enzyme protein, and mRNA accumulation as they relate to N_2 fixation and nodule development.

RESULTS

Cloning and Sequence Analysis of an Alfalfa NADH-GOGAT cDNA

A cDNA library was constructed in λgt11 from poly(A)$^+$ RNA isolated from 20-day-old alfalfa nodules and screened with polyclonal antiserum prepared against purified NADH-GOGAT protein (Anderson et al., 1989). Two immunopositive bacteriophage, containing inserts of 1.0 and 1.7 kb, were identified from ~200,000 recombinants screened. Because the subunit molecular mass of alfalfa NADH-GOGAT exceeds 200 kD (Anderson et al., 1989), the length of the mRNA that would correspond to this protein must be greater than 6 kb. We therefore screened a second cDNA library by plaque hybridization using the 1.7-kb insert. A 765-bp BamHI fragment from the 5′ end of the longest resulting cDNA (2.7 kb) was then used as a hybridization probe to screen a third cDNA library that had been prepared using a reverse transcriptase lacking RNase H activity. This screening produced numerous hybridizing recombinant phage that contained inserts longer than those previously obtained, including one which was of a size that made it a candidate for a full-length cDNA clone (~7.2 kb).

The complete nucleotide sequence of this insert (7212 bp) was determined and can be obtained through GenBank as accession number L01660. A large open reading frame of 6582 bp capable of encoding a 2194–amino acid protein with a molecular mass of 240,390 D follows the first ATG codon found in the cDNA. Upstream of this ATG codon are translational stop codons in all three reading frames. A search of protein data bases with the deduced amino acid sequence revealed significant sequence identity of alfalfa NADH-GOGAT with maize Fd-GOGAT (Sakakibara et al., 1991) and with both the large and small subunits of *E. coli* NADPH-GOGAT, which are encoded by a polycistronic mRNA (Oliver et al., 1987). No other significant match was found in sequence data bases. Because the extreme length of the alfalfa NADH-GOGAT cDNA precludes publication in its entirety, a diagrammatic comparison of homologous regions found in the alfalfa, maize, and *E. coli* GOGAT proteins is shown in Figure 1.

Confirmation that the isolated cDNA corresponded to alfalfa nodule NADH-GOGAT was obtained by amino acid sequence determination of the amino terminus of the purified protein. A total of 13 amino-terminal amino acid residues was determined and found to match exactly amino acids 102 to 114 of

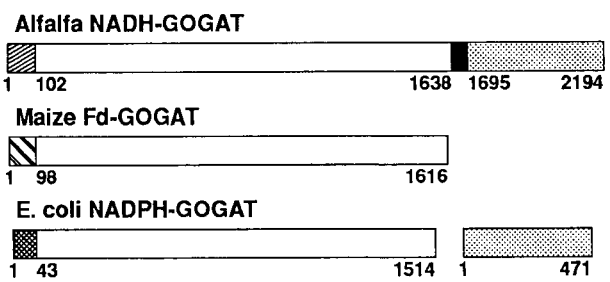

Figure 1. Diagrammatic Comparison of Known GOGAT Proteins.

Amino acid numbering starts with the initiator methionine of each polypeptide. Identically shaded areas indicate regions of sequence similarity. The hatched boxes denote the presequences of the enzyme precursors. The darkened portion of the alfalfa protein indicates a unique amino acid sequence that connects regions with sequence identity to the *E. coli* large and small subunits.

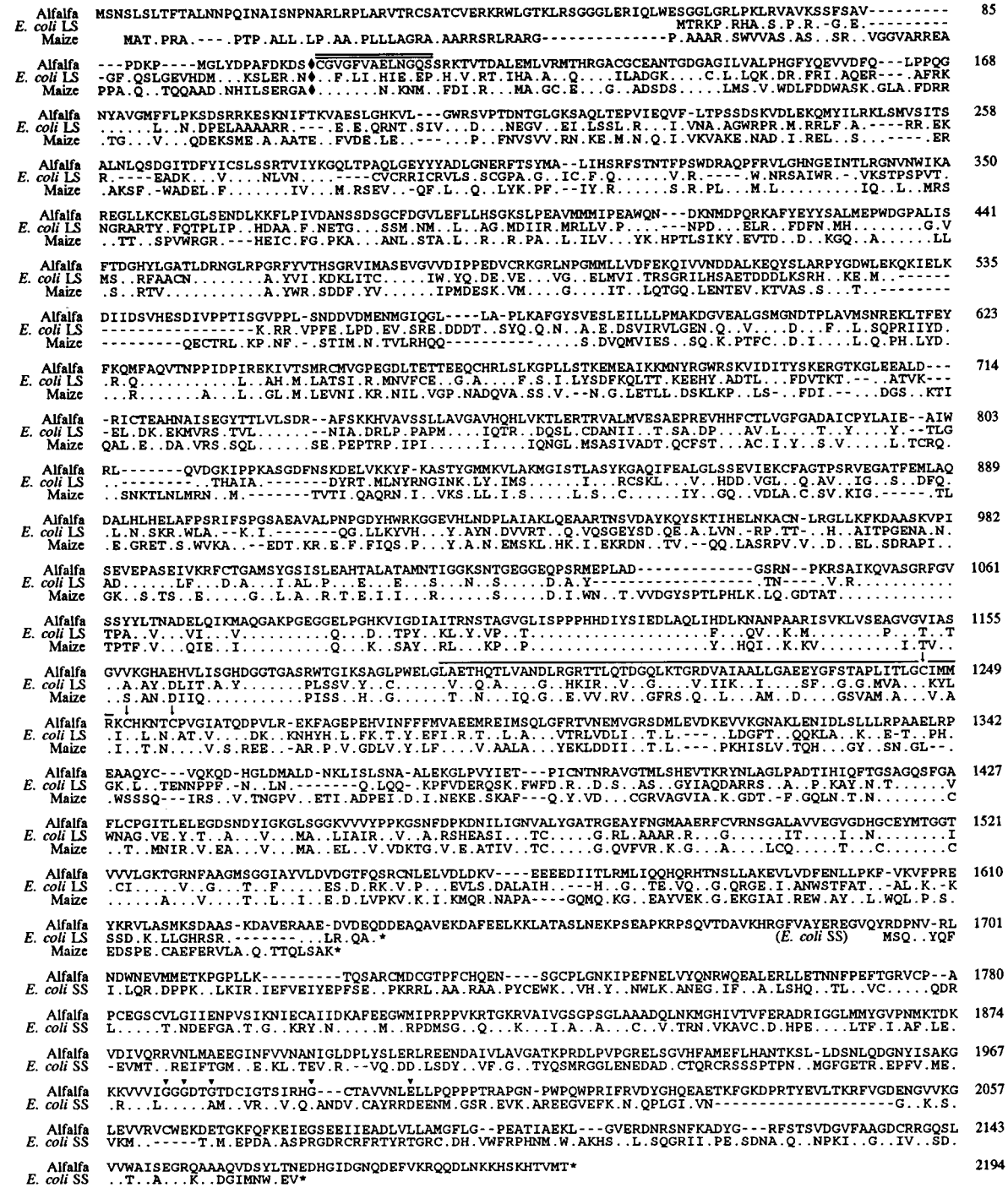

Figure 2. Amino Acid Sequence Alignment of Alfalfa NADH-GOGAT with Maize Fd-GOGAT and the Large and Small Subunits of *E. coli* NADPH-GOGAT.

Residue numbering begins with the first amino acid in the alfalfa protein. Identical residues are indicated by dots. Dashes indicate gaps introduced to maximize sequence similarity. Asterisks denote the carboxy termini of the proteins. Diamonds indicate processing sites of the alfalfa and maize GOGATs and *E. coli* large subunit protein. The residues determined by amino acid sequencing of the mature alfalfa NADH-GOGAT protein are indicated by a double overline. Three cysteines putatively involved in binding a [3Fe-4S] cluster are denoted by arrows at positions 1246, 1252, and 1257. The region from amino acid 1193 to 1250 that may be important for binding a molecule of flavin mononucleotide is overlined. Five conserved residues that are important for NADH binding are indicated by arrowheads.

the deduced protein, as shown in Figure 2 (double-overlined residues). These data indicate that alfalfa NADH-GOGAT is proteolytically processed to its mature form (229,350 D) and define a presequence of 101 amino acids. Amino acid alignment of alfalfa NADH-GOGAT with maize Fd-GOGAT and *E. coli* NADPH-GOGAT sequences (Figures 1 and 2) revealed that the alfalfa protein, unlike maize Fd-GOGAT, contains sequences that correspond to both the large and small subunits of the prokaryotic NADPH-GOGAT protein. In addition to the regions of sequence identity shared with the *E. coli* enzyme subunits, alfalfa NADH-GOGAT contains a highly charged and hydrophilic sequence of ~60 amino acids that appears to link the regions corresponding to the prokaryotic large subunit's carboxy terminus to the region corresponding to the small subunit's amino terminus. The *E. coli* large subunit and the maize Fd-GOGAT amino acid sequences are ~46 and ~48% identical to the alfalfa NADH-GOGAT sequence, respectively. The *E. coli* small subunit is slightly less similar (~38%) to the alfalfa protein. The alfalfa and maize GOGATs and the *E. coli* large subunit polypeptide all are translated as preproteins, and show little sequence similarity preceding their mature amino termini (Figures 1 and 2). The alfalfa protein also has a carboxy-terminal sequence of 30 amino acids that has no corresponding region in the *E. coli* small subunit polypeptide.

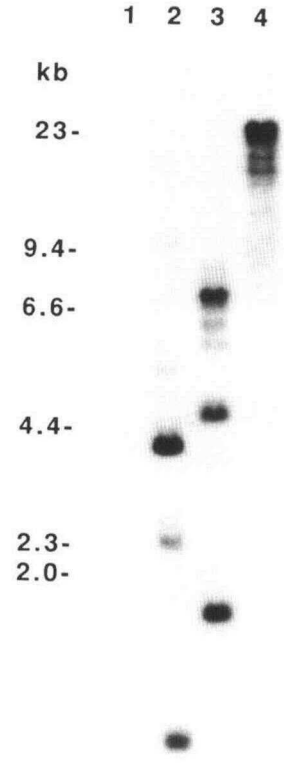

Figure 3. DNA Gel Blot Analysis of NADH-GOGAT Gene Sequences in the Alfalfa Genome.

Alfalfa genomic DNA (20 μg) was digested with BglII (lane 2), EcoRI (lane 3), or EcoRV (lane 4), and *R. meliloti* genomic DNA (0.02 μg) was digested with EcoRI (lane 1). The digested DNA was electrophoresed through a 0.8% agarose gel, denatured, transferred to a polyvinylidene difluoride membrane, and probed with the insert of pGOGAT7.2. The numbers at left refer to the positions of molecular length markers in kilobases.

DNA Gel Blot Analysis of NADH-GOGAT

To assess the genomic organization and gene copy number of NADH-GOGAT in alfalfa, a DNA gel blot analysis of genomic DNA was performed using highly stringent hybridization conditions. As shown in Figure 3, equimolar amounts of *Rhizobium meliloti* genomic DNA cut with EcoRI gave no hybridization signal (lane 1), whereas hybridizing fragments were seen in alfalfa genomic DNA cut with BglII (lane 2), EcoRI (lane 3), or EcoRV, which contains a single strongly hybridizing fragment (lane 4). This enzyme was the only one tested that gave a single hybridizing fragment, and although it is of high molecular weight, it is not the result of undigested DNA, as ethidium bromide staining of gels indicated that all enzymes cut the DNA efficiently (data not shown). The NADH-GOGAT cDNA sequence contains no EcoRV restriction site, three EcoRI sites, and four BglII sites, one of which is very close to the 5′ end of the 7212-bp GOGAT cDNA. Restriction mapping and DNA gel blot analysis of a recently isolated NADH-GOGAT genomic clone suggest that no additional site for these restriction enzymes is located within introns of the gene (data not shown). Therefore, a single hybridizing fragment would be expected for EcoRV-cut genomic DNA, and four and five hybridizing fragments would be expected for DNA cut with EcoRI and BglII, respectively. Although we see fewer bands than expected for DNA cut with the latter two enzymes, shorter exposures of DNA gel blot autoradiographs suggest that BglII-cut DNA contains two hybridizing fragments that are so close in size that they migrate to nearly the same position in gels (data not shown). In addition, the small size and AT richness of the 5′-most DNA fragment produced by digestion with BglII may not allow hybridization to occur, and thus one fewer hybridizing fragment would be observed. The DNA gel blot results suggest the presence of a single gene or small gene family; however, in all lanes numerous faintly hybridizing fragments were also present. Blots probed and washed under conditions of reduced stringency gave results similar to those shown in Figure 3 (data not shown).

NADH-GOGAT mRNA Accumulation in Effective and Ineffective Nodules

NADH-GOGAT mRNA levels in total RNA isolated from developing nodules of effective alfalfa (cultivar Saranac) and from

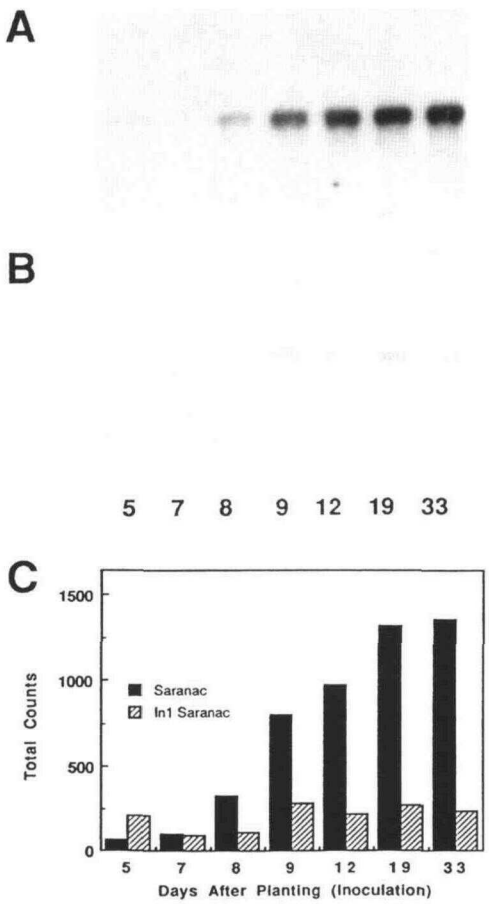

Figure 4. RNA Gel Blot Analysis of Alfalfa NADH-GOGAT mRNA Levels in Developing Alfalfa Nodules.

Ten micrograms of total RNA from roots (day 5) or nodules (days 7, 8, 9, 12, 19, and 33) was applied to each lane, electrophoresed on a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane, and probed with the insert of pGOGAT7.2.
(A) RNA extracted from effective Saranac.
(B) RNA extracted from ineffective *in*$_1$Sa.
(C) Mean counts averaged for three experiments determined by direct counting of radioactivity in the 7.2-kb band.

plant gene–controlled ineffective plants (*in*$_1$Sa) were determined by RNA gel blot analysis, as shown in Figure 4. During the course of this experiment, nitrogen fixation is detected at day 9 and maximum nitrogenase activity is achieved by day 12, as estimated by acetylene reduction (Gantt et al., 1992). RNA from effective nodules contained a single hybridizing species of ~7.2 kb, which increased in abundance from 5 to 33 days after planting. In comparison, RNA extracted from *in*$_1$Sa nodules at the same time points contained only small amounts of hybridizing GOGAT mRNA (Figure 4B). Shown in Figure 4C are mean counts per minute in the 7.2-kb band determined by direct counting of radioactivity on three blots each of RNA extracted from effective and ineffective nodules. To control for differences in RNA loading, blots were stripped and reprobed with radiolabeled polyuridine and the amount of hybridizing counts was determined (data not shown). Relative to the basal expression at day 5 (arbitrarily set to a value of 1), radioactive counts in GOGAT mRNA of effective Saranac at succeeding time points increased to 1.6, 4.9, 12.0, 14.6, 19.7, and 20.2 at days 7, 8, 9, 12, 19, and 33, respectively. For *in*$_1$Sa nodule mRNA, with the average counts at day 5 again set to a value of 1, the means were 0.43, 0.53, 1.39, 1.04, 1.30, and 1.14 at the same time points as given above. The decrease in counts from day 5 to days 7 and 8 in *in*$_1$Sa was reproduced in several experiments.

To determine whether the low-level expression of NADH-GOGAT mRNA in non-N_2-fixing nodules is unique to the *in*$_1$Sa genotype, NADH-GOGAT mRNA levels in nodule RNA from effective Saranac plants inoculated with effective *R. meliloti* 102F51 or with five mutant *R. meliloti* strains that produce ineffective nodules on Saranac plants were evaluated by RNA gel blot hybridization, as shown in Figure 5. In the effective symbiosis (Figure 5, first three lanes), an increase in NADH-GOGAT mRNA accumulation similar to that shown in Figure 4A was seen. However, all plants inoculated with ineffective *R. meliloti* strains contained low and relatively constant levels of hybridizing NADH-GOGAT mRNA (Figure 5, all subsequent lanes). Weak hybridization to ribosomal RNAs was occasionally observed in blots where total RNA was probed (Figure 5,

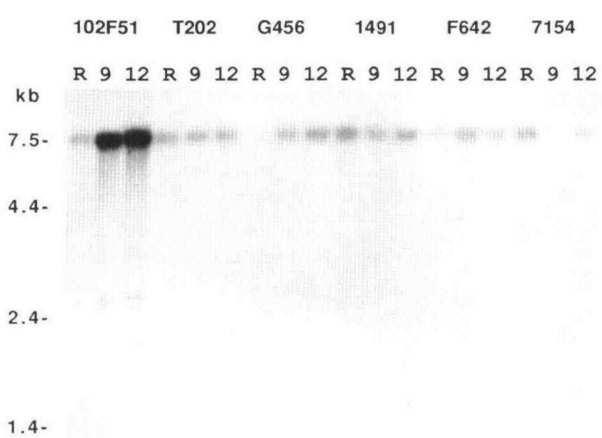

Figure 5. RNA Gel Blot Analysis of Alfalfa NADH-GOGAT mRNA Levels in Effective Saranac Nodules Induced by Effective or Ineffective *R. Meliloti* Strains.

RNA was isolated from roots (days 4, 5, or 6) and nodules (days 9 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 9 nodules (9), and day 12 nodules (12). No material was available for day 9 of 7154-infected plants. Total RNA (10 μg) was applied per lane, separated on a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane, and probed with the insert of pGOGAT7.2. Length markers at left are given in kilobases.

lower two bands). We have also hybridized this blot and duplicate blots with probes for nodule-enhanced aspartate aminotransferase (AAT2), phosphoenolpyruvate carboxylase (PEPC), and GS. In none of these cases was the level of mRNA in ineffective nodules reduced as dramatically as was the case for GOGAT (S. S. Miller and C. P. Vance, unpublished results). To examine possible differences in RNA loading, blots were hybridized with a constitutively expressed alfalfa fructose 1,6-diphosphate aldolase cDNA (V. Sangwan and C. P. Vance, unpublished results); all lanes were shown to contain nearly the same amount of this mRNA (data not shown).

The pattern of expression of NADH-GOGAT mRNA in different alfalfa tissues was characterized by gel blot analysis of RNA extracted from roots, stems, cotyledons, leaves, and nodules of effective Saranac plants, as shown in Figure 6. The highest expression level was seen in nodules, with much smaller amounts in root and stem. No NADH-GOGAT mRNA was detected in cotyledons or true leaves. To control for differences in the amount of RNA loaded, blots were stripped and reprobed with radiolabeled polyuridine.

Analysis of NADH-GOGAT Enzyme Activity and Protein Levels

Although we have previously published a study evaluating NADH-GOGAT activity and GOGAT enzyme protein in developing alfalfa nodules (Egli et al., 1989), we thought it important to repeat those experiments sampling nodules more frequently and at early time points for this study. We also wanted to ensure a direct comparison of nitrogenase activity, NADH-GOGAT activity, and NADH-GOGAT protein with NADH-GOGAT mRNA levels. Nodule initiation and visible emergence of nodules from roots occurs between days 7 and 10, a period during which nitrogenase activity increases from zero to near maximal specific activity (Egli et al., 1989; Gantt et al., 1992). Because in our previous study we sampled on days 7 and 10, we did not know the sequence of events during the important intervening days.

As shown in Figure 7C, total NADH-GOGAT enzyme activity was determined in both effective Saranac and ineffective *in*$_1$Sa nodule extracts at the same time points at which NADH-GOGAT mRNA was evaluated (Figure 4). In the effective symbiosis, NADH-GOGAT activity increased from nearly undetectable levels at days 5 and 7 to ~200 nmol min^{-1} (g fresh weight)$^{-1}$ at days 8 and 9 (Figure 7C). This initial increase was also seen with ineffective *in*$_1$Sa nodules. On day 9, when nitrogenase activity is first detectable in effective Saranac nodules, and, subsequently through day 19, NADH-GOGAT activity continued to increase in effective nodules. From day 19 to day 33, NADH-GOGAT activity remained high in effective Saranac nodules, at ~1000 nmol min^{-1} (g fresh weight)$^{-1}$. In contrast, NADH-GOGAT activity of ineffective *in*$_1$Sa nodules decreased after day 8 and was nearly undetectable on days 12, 19, and 33.

Immunoblots of soluble protein extracts from effective Saranac and ineffective *in*$_1$Sa nodules probed with monospecific NADH-GOGAT antibodies (Anderson et al., 1989) are shown in Figures 7A and 7B, respectively. In both genotypes, levels of NADH-GOGAT enzyme protein appeared to correlate well with enzyme activity (Figure 7C) and NADH-GOGAT mRNA levels (Figure 4). A very faint NADH-GOGAT protein band was detected in extracts of effective Saranac nodules on days 7 and 8. The intensity of the NADH-GOGAT band increased from days 9 to 19 and thereafter remained relatively constant. Undetectable or very small amounts of NADH-GOGAT were found in *in*$_1$Sa nodules on all days. These data not only confirm those of Egli et al. (1989), but also extend their findings by showing that NADH-GOGAT activity and protein reflect mRNA levels in both effective and ineffective nodules. We also evaluated NADH-GOGAT activity and enzyme protein during development of ineffective nodules induced by *R. meliloti* mutants at the same time points shown in Figure 5, and detected neither enzyme activity nor enzyme protein (data not shown).

DISCUSSION

Nitrogen is the major growth-limiting nutrient for most plant species (Greenwood, 1982). The majority of plants acquire nitrogen from the soil in the form of nitrate. This nitrate is derived either from the atmosphere, from decaying organic matter,

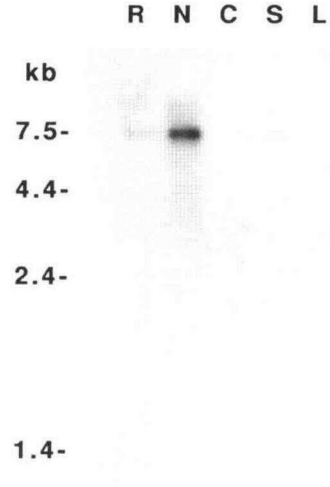

Figure 6. RNA Gel Blot Analysis of Alfalfa NADH-GOGAT mRNA in Various Plant Tissues.

A total of 0.5 μg of poly(A)$^+$ RNA from root (R), nodule (N), cotyledon (C), stem (S), and leaf (L) was applied per lane, separated through a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with the insert of pG30. Numbers at left indicate positions of molecular length markers in kilobases.

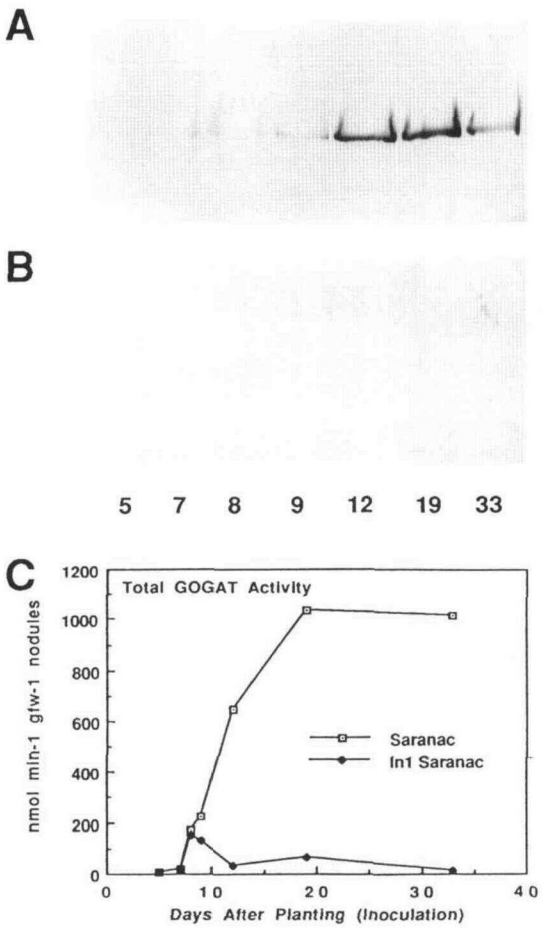

Figure 7. NADH-GOGAT Enzyme Activity and Protein Levels.

(A) Immunoblot detection of NADH-GOGAT protein from effective roots (day 5) or nodules (days 7, 8, 9, 12, 19, and 33).

(B) Immunoblot of in_1Sa total nodule protein from identical time points as given for **(A)**.

(C) Plot of enzyme activity for effective Saranac (open squares) and ineffective in_1Sa (filled squares).

or from applied fertilizers. Prior to its incorporation into organic compounds, nitrate must be reduced to ammonia through the sequential action of two plant enzymes, nitrate reductase and nitrite reductase. In comparison, most legumes obtain fixed nitrogen from atmospheric dinitrogen through symbiosis with nitrogen-reducing bacteria, which convert dinitrogen directly to ammonia. Incorporation of ammonia into organic compounds, irrespective of source, is accomplished by the GS/GOGAT pathway, which converts 2-oxoglutarate and ammonia into glutamate. Glutamine synthetase has been well studied biochemically, and GS genes have been cloned and their expression characterized in a variety of plants (Cullimore et al., 1984; Gebhardt et al., 1986; Brears et al., 1991). Similar molecular studies have been lacking for NADH-GOGAT, the predominant form of the enzyme in root nodules. We have recently characterized the expression of alfalfa genes encoding nodule-enhanced AAT2 (Gantt et al., 1992) and PEPC (Pathirana et al., 1992), two other key enzymes in nodule nitrogen assimilation. To complement these studies and further our understanding of the processes of nitrogen fixation, ammonia assimilation, and nodule function and development, we have begun the characterization of alfalfa NADH-GOGAT gene structure and expression.

A 7212-bp cDNA capable of encoding a 240-kD protein was isolated and characterized. The identity of the 7.2-kb cDNA clone was verified by a perfect match of 13 residues of the deduced amino acid sequence with the sequenced amino-terminal 13 amino acids of purified alfalfa nodule NADH-GOGAT (Figure 2, amino acids 102 to 114, double-overlined). Furthermore, this protein shares significant sequence identity with maize Fd-GOGAT and with both the large and small subunit of *E. coli* NADPH-GOGAT (Figures 1 and 2). The presequences of the alfalfa and maize GOGAT proteins and the *E. coli* NADPH-GOGAT large subunit protein share very little similarity. Within the mature proteins, conserved residues are found throughout the length of the sequence, excluding two regions unique to the alfalfa protein: a "connector" region of some 60 amino acids that separates domains homologous to the *E. coli* large and small subunits, and a carboxy-terminal region of 30 amino acid residues (Figures 1 and 2). Of particular interest are three regions involved in cofactor binding (Figure 2). Conserved cysteines indicated by arrows at amino acid positions 1246, 1252, and 1257 are likely to be involved in binding to the [3Fe-4S] cluster (Knaff et al., 1991; Sakakibara et al., 1991). Sequences similar to those implicated in flavin mononucleotide binding in yeast flavocytochrome b_2 (Lederer et al., 1985; Sakakibara et al., 1991) are found in the alfalfa, maize, and *E. coli* GOGAT proteins between amino acids 1193 and 1250 (Figure 2, single-overline). Five highly conserved amino acids at positions 1974, 1976, 1979, 1990, and 1998 (Figure 2, arrowheads) have been shown to be critical for NADH binding in a number of enzymes that utilize this cofactor (Scrutton et al., 1990).

The similarity among alfalfa, maize, and *E. coli* GOGATs suggests that the genes encoding these enzymes share a common evolutionary origin. Bacterial NADPH-GOGATs (Trotta et al., 1974; Adachi and Suzuki, 1977; Hemmilä and Mäntsälä, 1978; Oliver et al., 1987) and yeast (*Saccharomyces cerevisiae*) NADH-GOGAT (Masters and Meister, 1982) are comprised of two nonidentical subunits. In contrast, all higher plants studied thus far (Suzuki and Gadal, 1984; Anderson et al., 1989; Chen and Cullimore, 1989), as well as *Chlamydomonas reinhardtii* (Cullimore and Sims, 1981) and *Neurospora crassa* (Hummelt and Mora, 1980), contain NAD(P)H-GOGATs consisting of a single polypeptide. A comparison of the deduced alfalfa and *E. coli* GOGATs (Figures 1 and 2) indicates that the alfalfa protein is comprised of sequences related to both prokaryotic subunits. Whether during the course of evolution an event occurred that resulted in the combining of two GOGAT subunit-encoding genes or whether a single ancestral GOGAT gene was split into two separate coding regions is unknown. Another question complicating an interpretation of the evolutionary relationships between GOGATs is whether plant and

fungal NAD(P)H-GOGATs, at least some of which appear to be localized to organelles, evolved from a single ancestral eukayotic gene or from a gene transferred to the nucleus from a prokaryotic endosymbiont. Also clouding this issue is the relatedness of NAD(P)H-GOGATs to Fd-GOGATs, the latter of which are found in cyanobacteria, green algae, and plants (Stewart et al., 1980) and contain sequences similar only to the bacterial large subunit.

In bean, evidence was presented indicating that NADH-GOGAT is localized in the nodule plastid and cytoplasm (Chen and Cullimore, 1989). Similarly, preliminary evidence from subcellular fractionation experiments indicates that alfalfa NADH-GOGAT is found in the nodule amyloplast (D. Robinson and C. P. Vance, unpublished results). However, the amino acid content of the alfalfa NADH-GOGAT presequence, from amino acids 1 to 101, predicts a mitochondrial localization for this enzyme, based upon the serine-to-arginine ratio. This ratio has been shown to be able to discriminate between 90% of mitochondrial and chloroplastic targeting peptides (von Heijne et al., 1989). Another unusual feature of this presequence is that it contains a relatively high percentage of acidic residues (Asp+Glu content of 7% versus an Asp+Glu content of 1 and 1.5%) for compiled mitochondrial and chloroplast transit peptides, respectively (von Heijne et al., 1989). Similar to alfalfa NADH-GOGAT, maize UDP-glucose starch glycosyltransferase (encoded by the *waxy* gene) contains a transit peptide whose amino acid composition predicts a mitochondrial localization, yet the protein is found in amyloplasts of the endosperm, pollen, and embryo sac (Klösgen et al., 1986). Whereas it is possible that sequence or structural information required for import into amyloplasts is quite different from that which directs precursor proteins into chloroplasts, the maize waxy transit peptide directs transport into both isolated amyloplasts and chloroplasts (Klösgen et al., 1989). Thus, it appears that common import mechanisms may be utilized by these two types of plastids, and control of protein localization is determined by which plastid is present in the cell where the particular gene is expressed. Another explanation that might account for the uniqueness of the NADH-GOGAT transit sequence would be multistep processing of the preprotein, including a cleavage upon translocation across the amyloplast membrane and a second cleavage, perhaps similar to that which occurs to produce the mature *E. coli* enzyme, to yield the mature amino terminus. The fact that the *E. coli* protein, which is not transported, is processed to the same conserved cysteine residue as maize Fd-GOGAT and alfalfa NADH-GOGAT suggests that a portion of the presequence may be unrelated to organelle targeting. Another possible explanation for the amino acid composition of the alfalfa NADH-GOGAT presequence is simply that great variation exists in amino acid sequences that effectively direct precursor proteins into plastids (von Heijne et al., 1991).

DNA gel blot analysis of alfalfa and *Rhizobium* genomic DNA (Figure 3) indicated that a plant gene rather than a bacterial gene had been isolated, and suggested that NADH-GOGAT is encoded by a single gene or small gene family. The presence of a single gene would be consistent with previous results from enzyme purifications, which did not yield any chromatographically separable enzyme peaks, and native activity gels, which did not show any apparent isozymes (Anderson et al., 1989). In all lanes of the DNA gel blot containing alfalfa DNA, minor bands were observed. These might be the result of hybridization to distantly related GOGAT genes or may be due to minor allelic variation that one would expect to find in a highly heterozygous outcrossing tetraploid such as alfalfa.

The availability of an NADH-GOGAT cDNA clone provided the means to characterize mRNA levels on RNA gel blots. GOGAT mRNA was most abundant in mature effective nodules, with much lower levels present in roots and stems (Figure 6). Within effective nodules, GOGAT mRNA increased prior to the onset of detectable nitrogenase activity (Figure 4A). The requirement of effective nodulation for the maximum expression of this gene is demonstrated by results from blots of RNA extracted from either plant gene–controlled or bacterially controlled ineffective nodules (Figures 4B and 5). In both cases, GOGAT mRNA levels stayed at or below levels in roots. In plant-controlled ineffective nodules, early stages of nodule organogenesis proceed normally and slight nitrogenase activity is detectable (Vance and Johnson, 1983; Egli et al., 1989). The *Rhizobium* mutants employed for this study have different genetic lesions and are arrested at various developmental stages of the symbiotic process, from infection thread release to bacteroid development within the nodule (Hirsch et al., 1983; Leigh et al., 1987; Virts et al., 1988; Yarosh et al., 1989; Driscoll and Finan, 1993), and all are unable to fix nitrogen. This suggests that early events in nodule development, including bacterial release from infection threads, are not directly involved in induction of NADH-GOGAT gene expression. It is obvious that some signal present in effective nodules around the time of onset of nitrogen fixation is required for induction of NADH-GOGAT gene expression.

At this point, we cannot differentiate whether ammonia itself secreted from the bacteroid provides a primary signal for inducing maximum NADH-GOGAT expression or if some other bacterial- or plant-derived factor associated with nodule effectiveness is necessary for maximum expression of this gene. Attempts to address the role of ammonia in expression of enzymes involved in ammonia assimilation have yielded conflicting results. Studies with alfalfa (Groat and Vance, 1981), soybean (Hirel et al., 1987), common bean (Cock et al., 1990), and pea (Walker and Coruzzi, 1989) measuring GOGAT or GS gene expression in response to applied nitrogen have shown unchanged, decreased, or increased expression.

The developmental increase in GOGAT enzyme activity correlates well with that of its mRNA (Figures 7C and 4A) and appears to occur in two phases. The initial increase coincides with nodule emergence from the root at days 7 and 8, and is also present in ineffective nodules. This increase may be induced by some signal related to early nodule development. A second and more dramatic increase in GOGAT enzyme activity that is only observed in effective nodules occurs following the onset of N_2 fixation at day 9 and is consistent with a further increase in NADH-GOGAT mRNA levels. Enzyme protein levels, as detected by protein immunoblotting (Figure 7), are

also consistent with mRNA levels and enzyme activity. In ineffective nodules (Figure 7B), no GOGAT enzyme protein was detected, consistent with low mRNA levels (Figure 4B) and enzyme activity (Figure 7C) found in these plants. In effective Saranac nodules, enzyme protein was detectable after day 5 and was highest at days 12, 19, and 33, the same days at which enzyme activity was greatest.

In contrast to the correlation between GOGAT mRNA abundance and enzyme activity in effective and ineffective nodules, GS (Egli et al., 1991), AAT2 (Gantt et al., 1992), and PEPC (Pathirana et al., 1992) mRNAs are found at moderately high levels in ineffective nodules at day 12 and later when little or no enzyme protein or activity is detected, suggesting that translational control or differential protein turnover of these enzymes occurs in effective and ineffective nodules. In addition, unlike NADH-GOGAT, mRNAs for AAT2, PEPC, and GS increase in Saranac nodules inoculated with ineffective strains of *Rhizobium*. Thus, these enzymes, which are part of a common metabolic pathway, appear to be regulated differently. The fact that expression of the NADH-GOGAT gene shows the strictest requirement for effective nodulation, as well as the fact that NADH-GOGAT is present in effective nodules in much lower amounts than the other three enzymes (Groat and Schrader, 1982; Vance and Stade, 1984; Anderson et al., 1989; Griffith and Vance, 1989), leads us to consider the possibility that it may be the rate-limiting or regulated step of nitrogen assimilation. We are currently in the process of characterizing genomic clones for NADH-GOGAT, AAT2, and nodule-enhanced PEPC to elucidate the regulatory mechanisms involved in the expression of these enzymes, each of which plays an important role in the nitrogen assimilatory pathway in root nodules.

METHODS

Plant Material and Bacterial Strains

Seeds of alfalfa (*Medicago sativa*) cultivar Saranac and a single gene recessive genotype ineffective Saranac (*in*$_1$Sa), which produces early-senescing ineffective nodules (Peterson and Barnes, 1981), were obtained from Dr. D. K. Barnes (U.S. Department of Agriculture–Agricultural Research Service, St. Paul, MN). The fact that alfalfa is an outcrossing tetraploid species precludes the formation of isogenic lines; however, more than 90% of the *in*$_1$Sa genotype is from the Saranac background. Plants of these two genotypes were maintained in glasshouse sand benches and were inoculated with effective *Rhizobium meliloti* 102F51, as described previously (Egli et al., 1989). For analysis of GOGAT expression in bacterially conditioned ineffective nodules, Saranac seeds were planted in sterilized sand that was inoculated with the ineffective strains. The *R. meliloti* strains and their relevant genotypes are as follows: T202, *oxr* (oxygen regulation deficient; Virts et al., 1988); 1491, *nifH* (nitrogenase subunit mutant; Hirsch et al., 1983); F642, *dctA* (dicarboxylic acid uptake deficient; Yarosh et al., 1989); 7154, *exoH* (acid exopolysaccharide succinylation deficient; Leigh et al., 1987); and G456, *dme* (malic enzyme deficient; Driscoll and Finan, 1993). These strains were generous gifts of Don Helinski (University of California, San Diego), Ann Hirsch (University of California, Los Angeles), and Brian Driscoll and Turlough Finan (McMaster University, Hamilton, Ontario, Canada). In all experiments, the date that seeds were planted was the date of inoculation and was designated as day 0. For experiments analyzing developmental expression of GOGAT, roots (day 5) or nodules (days 7, 8, 9, 12, 19, and 33) were collected by hand, placed on ice, weighed, and used immediately for either protein or RNA extraction. Leaves, stems, and cotyledons were also collected and placed on ice for subsequent RNA extraction.

Isolation of cDNA Clones Encoding NADH-GOGAT

A cDNA synthesis kit (Pharmacia, Piscataway, NJ) was used to construct an oligo(dT)-primed alfalfa nodule cDNA library in the expression vector λgt11. The library was screened with antisera prepared against alfalfa GOGAT (Anderson et al., 1989) using horseradish peroxidase–conjugated goat anti–rabbit antibodies as a detection system. Approximately 200,000 recombinant bacteriophage were screened, and two clones were obtained that reacted with the antiserum. The cDNA inserts from these phage were subcloned into the plasmid vector pBluescript KS+ (Stratagene). The nucleotide sequence of the ends of these inserts (plasmids pG1 and pG3) was determined by the dideoxy termination method with Sequenase 2.0 (U.S. Biochemicals). In an attempt to isolate larger cDNA clones, the 1.7-kb insert of pG3 was used as a hybridization probe to screen an oligo(dT)-primed plasmid cDNA library prepared from nodule RNA using a cDNA synthesis kit (Invitrogen, San Diego, CA). Approximately 120,000 colonies were screened and 60 hybridizing colonies resulted. Of these, most contained inserts of 1.5 kb or less. One colony contained a plasmid (pG30) of larger insert size (2667 bp). The sequence of this insert corresponded to nucleotides 4533 to 7200 of the pGOGAT7.2 insert.

Because none of the isolated cDNA clones was large enough to encode the complete amino acid sequence of NADH-GOGAT, an alfalfa nodule cDNA library in λgt22A was constructed utilizing RNA isolated from 20-day-old nodules using a kit that employs a reverse transcriptase lacking RNase H activity (Superscript; GIBCO BRL). This library was screened with a 765-bp BamHI restriction fragment from the 5′ end of the pG30 insert. Approximately 50,000 recombinant bacteriophage plaques were screened. Of the hundreds of hybridizing plaques obtained, the inserts of 55 were characterized. Bacteriophage DNA was isolated from purified plaques as described elsewhere (Grossberger, 1987), and the insert sizes of these clones were determined. The longest insert (~7.2 kb) was subcloned into pBluescript KS+, and its sequence was determined from nested deletion fragments (Henikoff, 1987). This plasmid was designated pGOGAT7.2. Analysis of the nucleotide and deduced amino acid sequences was performed using the Intelligenetics (Palo Alto, CA) software package. Data base searches and amino acid sequence alignments were performed using the FASTDB program (Intelligenetics, Brutlag et al., 1990). The nucleotide sequence of the pGOGAT7.2 insert has been submitted to GenBank as accession number L01660.

DNA Extraction and DNA Gel Blot Analysis

Genomic DNA was isolated from alfalfa cultivar Saranac leaves by a method described elsewhere (Shure et al., 1983). For DNA gel blot analysis, approximately equimolar amounts of alfalfa (20 μg) and *R. meliloti* (0.02 μg) were digested to completion with restriction enzymes as recommended by the manufacturers (GIBCO BRL and Promega) and separated by electrophoresis on a 0.8% agarose gel. The DNA was depurinated by treatment with 0.25 M HCl for 15 min, denatured by soaking in a solution of 1 M NaOH, 1.5 M NaCl for 1 hr, neutralized

in 1 M Tris, pH 8.0, 1 M NaCl for 1 hr, and transferred to Immobilon membrane (Millipore, Bedford, MA) with 10 × SSC (1 × SSC is 150 mM sodium chloride, 15 mM sodium citrate). The resulting filter was hybridized overnight with the ^{32}P-labeled pGOGAT7.2 insert in a solution consisting of 5 × SSC, 5 × Denhardt's solution (1 × Denhardt's is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 100 μg/mL denatured salmon sperm DNA, and 0.5% SDS at 65°C. Three washes were performed in a solution of 0.1 × SSC, 0.1% SDS at the same temperature.

RNA Isolation and RNA Gel Blot Analysis

Total RNA was isolated from freshly collected root, nodule, leaf, cotyledon, and stem tissue from Saranac plants, and from roots and nodules of *in*$_1$Sa plants by methods described elsewhere (Gantt et al., 1992; Strommer et al., 1993). Poly(A)$^+$ RNA was obtained by one pass over an oligo(dT)-cellulose column (GIBCO BRL). RNA gel blotting was performed essentially as described previously (Gantt et al., 1992) using either 1 μg of poly(A)$^+$ RNA per lane for blots of different alfalfa tissues or 10 μg of total RNA for blots of nodule RNA. The resulting filters were hybridized with the ^{32}P-labeled insert of plasmid pG30 or pGOGAT7.2 prepared using the random primer method (Feinberg and Vogelstein, 1983). Hybridization was in a solution of 50% deionized formamide, 120 mM sodium phosphate, pH 6.8, 250 mM NaCl, 7% SDS, 1 mM EDTA at 42°C for 16 to 20 hr. Blots were washed three times in 0.1 × SSC, 0.1% SDS for 30 min each at 65°C. Radioactivity on blots was quantified with an AMBIS Radioanalytic Imaging System (San Diego, CA). The amount of radioactivity that hybridized to GOGAT mRNA was counted on multiple blots, and averages were determined. Following quantitation, filters were placed into x-ray film cassettes for autoradiography.

Protein Extraction, Enzyme Assays, and Protein Immunoblotting

Triplicate samples of roots or nodules were ground in extraction buffer (100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.8, 100 mM sucrose, 2% 2-mercaptoethanol, 15% ethylene glycol, 2 mM phenylmethylsulfonyl fluoride, and 0.2% antipain) using a ground-glass homogenizer and centrifuged 15 min at 15,500*g* to obtain the soluble protein fraction. GOGAT enzyme activity was measured in vitro by a spectrophotometric assay described by Groat and Vance (1981). For protein immunoblot analysis, 100 μg of total nodule protein, determined by the method of Lowry et al. (1951), was loaded onto lanes of an SDS–polyacrylamide gel (6%), electrophoresed, and transferred to nitrocellulose membranes. GOGAT protein levels were determined by reaction of these filters with anti-GOGAT antiserum as described previously (Anderson et al., 1989). The amino acid sequence of the amino terminus of the GOGAT protein, purified as described by Anderson et al. (1989), was determined by the University of Minnesota Institute of Human Genetics protein sequencing facility, Minneapolis.

ACKNOWLEDGMENTS

We thank Todd Lenvik and Ruby Larson for technical assistance, and the numerous students, postdoctoral scientists, and faculty who contributed substantial time to pick nodules for protein and RNA isolations. We would also like to thank Don Helinski, Ann Hirsch, Brian Driscoll, and Turlough Finan for generously sharing ineffective *Rhizobium* strains, and Dr. Mark Farnham, Dr. Burle Gengenbach, Dr. Neil Olszewski, Dr. Deborah Samac, and two anonymous reviewers for critical reading of the manuscript. This work was supported in part by U.S. Department of Agriculture (USDA) Grant No. 87-CRCR-1-2588 and National Science Foundation Grant No. IBN-9206890. This paper is a joint contribution from the Plant Science Research Unit, USDA, Agricultural Research Service, and Minnesota Agricultural Experiment Station (Paper No. 20,059, Scientific Journal Series). Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA, and does not imply its approval to the exclusion of other products or vendors that might also be suitable.

Received September 25, 1992; accepted December 3, 1992.

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