Journal of Experimental Botany, Vol. 51, No. 342
MP Special Issue, pp. 29–39, January 2000

Journal of Experimental Botany

Decreased NADH glutamate synthase activity in nodules and flowers of alfalfa (*Medicago sativa* L.) transformed with an antisense glutamate synthase transgene

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Received 19 January 1999; Accepted 29 April 1999

Abstract

Legumes obtain a substantial portion of their nitrogen (N) from symbiotic N_2 fixation in root nodules. The glutamine synthetase (GS, EC 6.3.1.2)/glutamate synthase (GOGAT) cycle is responsible for the initial N assimilation. This report describes the analysis of a transgenic alfalfa (*Medicago sativa* L.) line containing an antisense NADH-GOGAT (EC 1.4.1.14) under the control of the nodule-enhanced aspartate aminotransferase (AAT-2) promoter. In one transgenic line, NADH-GOGAT enzyme activity was reduced to approximately 50%, with a corresponding reduction in protein and mRNA. The transcript abundance for cytosolic GS, ferredoxin-dependent GOGAT (EC 1.4.7.1), AAT-2 (EC 2.6.1.1), asparagine synthase (EC 6.3.5.4), and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) were unaffected, as were enzyme activities for AAT, PEPC and GS. Antisense NADH-GOGAT plants grown under symbiotic conditions were moderately chlorotic and reduced in growth and N content, even though symbiotic N_2 fixation was not significantly reduced. The addition of nitrate relieved the chlorosis and restored growth and N content. Surprisingly, the antisense NADH-GOGAT plants were male sterile resulting from inviable pollen. A reduction in NADH-GOGAT enzyme activity and transcript abundance in the antisense plants was measured during the early stages of flower development. Inheritance of the transgene was stable and resulted in progeny with a range of NADH-GOGAT activity. These data indicate that NADH-GOGAT plays a critical role in the assimilation of symbiotically fixed N and during pollen development.

Key words: Amino acid, nitrogen assimilation, pollen.

Introduction

A significant fraction of the nitrogen (N) needed for the growth of alfalfa (*Medicago sativa* L.) is derived from atmospheric N_2 reduced by the microsymbiont *Sinorhizobium meliloti* in root nodules (Heichel *et al.*, 1981). The N_2 reduced by *S. melilloti* is released into the cytoplasm of the host cell as ammonia (O'Gara and Shanmugam, 1976; Ta *et al.*, 1986; Kaiser *et al.*, 1998). It is then assimilated into the amino acid pools by the combined activities of glutamine synthetase (GS, EC 6.3.1.2) and NADH-dependent glutamate synthase (NADH-GOGAT, EC 1.4.1.14). GS catalyses the ATP-dependent amination

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Published by Oxford University Press

of glutamate to form glutamine. GOGAT then catalyses the transfer of the amide group from glutamine to α-ketoglutarate to yield two molecules of glutamate. These two reactions, collectively referred to as the GS/GOGAT cycle, provide the primary route of nitrogen assimilation in plants (for a review see Temple *et al.*, 1998*b*). The synthesized glutamate can be used either to replenish the pool of glutamate for subsequent GS catalysed assimilation of ammonia, or may donate its amino group to form other nitrogen-containing compounds including aspartate and asparagine, through the activities of aspartate aminotransferase (AAT, EC 2.6.1.1) and asparagine synthetase (AS, EC 6.3.5.4) respectively. The activity of AAT and AS as well as cytosolic GS (GS_1), NADH-GOGAT, and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), which provides a source of C skeletons for nitrogen assimilation, increase during the course of alfalfa root nodule development (Egli *et al.*, 1989; Vance *et al.*, 1994). Biochemical and molecular analysis indicates that the nodule-specific or nodule-enhanced forms of these enzymes are responsible for the majority of the increased enzymatic activity (Anderson *et al.*, 1989; Farnham *et al.*, 1989; Miller *et al.*, 1987; Temple *et al.*, 1995).

Attempts to improve alfalfa performance with respect to N_2 fixation by selecting for increased rates of nitrogenase activity and higher nodule assimilatory enzyme activities have met with mixed results. While selection for higher nitrogenase activity results in an increase in nodule NADH-GOGAT and PEPC activities (Jessen *et al.*, 1987), selection for increased nodule assimilatory enzyme activity does not necessarily increase the performance of the plant (Jessen *et al.*, 1988). Selection for lower nodule ammonia assimilatory enzyme activity resulted in lower N_2 fixation and dry matter (Jessen *et al.*, 1988). One or more steps in a biochemical pathway may exert control over the rate of product formation (Kacser, 1987). Plants low in a single enzyme activity would be useful in determining which enzyme(s) might catalyse the rate-limiting step in the overall process of N_2 fixation and assimilation.

Traditional breeding studies are limited by the variability within the germplasm and are complicated by the pleiotropic effects of accompanying traits. Mutant studies are limited by the availability of suitable mutants and the ability to generate and screen for them. *Arabidopsis*, barley and pea mutants lacking Fd-GOGAT activity have been extensively characterized (Somerville and Ogren, 1980; Avila *et al.*, 1993; Lea and Forde, 1994; Oliveira *et al.*, 1997) and a recent report describes the preliminary analysis of constitutive antisense Fd-GOGAT tobacco transformants (Hirel *et al.*, 1997). However, there are no reports of plant mutants lacking or with reduced levels of NADH-GOGAT activity. The tetraploid nature of alfalfa precludes the generation of such mutants using established mutagenesis techniques. Therefore, this study undertook to reduce specifically the level of NADH-GOGAT activity in alfalfa nodules using an antisense transgene. The effectiveness of using antisense technology to impair nodule function has been reported for GS_1, (Hirel *et al.*, 1997), PEPC (Schulze *et al.*, 1998), AAT-2 (Mett *et al.*, 1996), and uricase (Lee *et al.*, 1993). The objectives of this study were to determine whether it is possible to modify the activity of NADH-GOGAT in alfalfa root nodules and to assess if the reduction in activity has a proportional impact on the rate and amount of N_2 fixation and N assimilation in the plant.

Materials and methods

Construction of the antisense NADH-GOGAT transgene

A *Hind*III-*Bgl*II fragment of the plasmid pMON969 (Monsanto Co., St Louis, MO) containing the cauliflower mosaic virus 35S promoter (CaMV35S) was excised and replaced with a 1.5 kbp *Hind*III-*Bam*HI fragment containing the promoter region of the alfalfa *AAT-2* gene and the start codon (Gregerson *et al.*, 1994). A 2.5 kbp *Bgl*II fragment from the middle of the GOGAT cDNA (Gregerson *et al.*, 1993) was inserted into the *Bam*HI site of the modified pMON969 in antisense orientation with respect to the AAT-2 promoter. The antisense orientation was confirmed by restriction analysis, and the promoter-GOGAT junction was confirmed by sequence analysis. The resulting 4.6 kbp *Not*I cassette, containing the AAT-2 promoter-antisense NADH-GOGAT fusion and the E9 transcription terminator was cloned into the *Not*I site of the plasmid pMON886 (Monsanto Co., St Louis, MO). The vector pMON886 contains the T-DNA right border sequence, spectinomycin resistance marker and the *NPTII* gene driven by the CaMV35S promoter for kanamycin selection of transformed plants. The resulting construct, pA2TAGA, was mobilized from *E. coli* strain DH5α into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating with helper *E. coli* 2013 (Rogers *et al.*, 1987).

Plant transformation

Transgenic *Medicago sativa* L. cv. Regen SY plants were generated essentially as described previously (Austin *et al.*, 1995). Embryos were recovered from callus cultured on media containing 50 $\mu g\ ml^{-1}$ kanamycin. Rooted plantlets were transplanted first to vermiculite and subsequently to sand in pots in a glasshouse, and inoculated with *Sinorhizobium meliloti* strain 102F51. The presence of the antisense NADH-GOGAT transgene in regenerated plants was determined by PCR using a pair of primers flanking the junction of the NADH-GOGAT cDNA insert (5′-TGTGCATATCCTGTCTA-3′) and the E9 terminator (5′-CTTGACGAACGTTGTCG-3′). This pair of primers amplifies an approximately 800 bp region from the control template pA2TAGA. Plants testing positive for the transgene were propagated clonally through stem cuttings. Glasshouse plants were maintained in sand supplemented with K, P, and lime, and grown under Na-vapour lamps to provide a 16 h daylength, with a midday photon flux density of approximately 1500 $\mu E\ m^{-2}\ s^{-1}$.

Enzyme assays for nodule ammonia and carbon assimilatory enzymes

Glasshouse plants used for nodule enzyme activity measurements were unpotted, rinsed free of sand and packed in ice. The nodules of these plants were harvested and stored on ice. Soluble protein was extracted by grinding tissues in a ground

glass homogenizer with extraction buffer (Egli et al., 1989). The activities of nodule enzymes were measured as previously described for NADH-GOGAT (Groat and Vance, 1981), AAT (Sulebele and Silverstein, 1969), PEPC (Vance et al., 1983), and GS (Egli et al., 1989). Soluble protein in extracted samples was measured by a modification of the method of Bradford (Bradford, 1976) as described by Groat and Vance (Groat and Vance, 1981). The significance of difference in the population enzyme activity means was determined using the Student *t*-test.

Nitrogenase activity was measured as the representative H_2 evolution in an open-flow system using a Nitrogenase Activity Analysis System (Morgan Scientific, Haverhill, MA) as described earlier (Blumenthal *et al.*, 1997). Apparent nitrogenase activity (ANA) was a measure of H_2 evolution by the nodulated root system exposed to air (80% N_2 and 20% O_2). After stable values for ANA were obtained total nitrogenase activity (TNA) was measured. TNA was the peak H_2 production in 20% O_2 and 80% Ar. Clonally propagated nodulated crowns of antisense and control genotypes were potted into PVC pipe sections filled with coarse silica sand and grown under a 14 h daylength in a growth chamber. The photon flux density was approximately 500 $\mu E\ m^{-2}\ s^{-1}$, the light period temperature was 23.5 °C and the dark period temperature was 20 °C. These plants were watered twice daily to saturation with N-free half-strength Hoagland's solution. The plants were arranged as a randomized complete block with two factors.

NADH-GOGAT protein immunodetection

Nodule soluble protein extracted as described for the enzyme assays was loaded onto a 6% SDS-PAGE gel, separated electrophoretically, and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Levels of NADH-GOGAT protein were estimated by incubating the protein blots with rabbit-derived anti-GOGAT antiserum (Anderson *et al.*, 1989) and developed with horseradish peroxidase linked goat anti-rabbit secondary antibody. The relative density of the developed blot was compared using the densitometer function of the AMBIS Radioanalytic Imaging System (AMBIS Inc., San Diego, CA).

Analysis of N content

Mature glasshouse antisense and pARC100E control plants used in the +N and –N fertilizer regime experiment were uniformly trimmed (shoot and root), repotted and re-inoculated with *S. meliloti* strain 102F51. The alfalfa crowns were given either 20 mM KNO_3 or 10 mM K_2SO_4 at a rate of 65 ml every other day for 25 d. Plants also received tap water as needed to prevent wilting. For N content analysis, approximately 100 mg of dried, finely ground whole shoot was used for Lachat quikchem AE method Kjeldahl digestion and ammonia measurement (Lachat Instruments, Milwaukee, WI).

RNA isolation and gel blot analysis

Total RNA was isolated using the method described earlier (Strommer *et al.*, 1993). For RNA blot analysis, total RNA was separated in a 1.5% formaldehyde-agarose gel (Sambrook *et al.*, 1989) and transferred by capillary blotting to Zeta Probe membrane (BioRad, Hercules, CA). The DNA probes used were as previously described (Vance *et al.*, 1995) and were prepared from plasmid inserts isolated from agarose gels and labelled with ^{32}P by random priming (Sambrook *et al.*, 1989). Hybridization was performed at 42 °C in 50% deionized formamide, 0.12 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 7% SDS, 1 mM EDTA as recommended by the manufacturer and washed to a final washing stringency of 0.2× SSC, 0.1% SDS at 65 °C. Signal intensities were quantified with an AMBIS radioanalytic scanner.

Pollen germination and scanning electron microscopy

Pollen was suspended in water and spotted on to pollen germination medium containing 15% sucrose, 0.4 mM $CaCl_2$, 0.4 mM H_3BO_4 and 1% agar (Carpenter *et al.*, 1992). Low temperature, low voltage scanning electron microscopy (SEM) of uncoated pollen grains was performed as described previously (Ahlstrand, 1996).

Results

Identification of transgenic plants

The antisense nodule-enhanced NADH-GOGAT transgene construct pA2TAGA contained a 1.5 kbp alfalfa nodule-enhanced AAT-2 gene promoter fragment fused to a 2.5 kbp fragment of the NADH-GOGAT cDNA in the antisense orientation. This 1.5 kbp 5′ promoter region of the *AAT-2* gene was previously demonstrated to direct significant expression of the GUS reporter gene in nodules of transgenic alfalfa and *Lotus corniculatus* (Gregerson *et al.*, 1994; Yoshioka *et al.*, 1998). From the approximate 1200 leaf explants induced to form callus following co-cultivation with *A. tumefaciens* containing the pA2TAGA antisense construct, 21 embryos were recovered following selection on kanamycin. Of these, 13 developed into plantlets that developed roots. Diagnostic PCR using primers flanking the recombinant junctions of the antisense construct showed that seven of these plants carried the transgene, among these, one subsequently senesced. The remaining six plants were propagated clonally as stem cuttings which when rooted were inoculated with *S. meliloti* strain 102F51.

Small clonal populations of each transformed line were assayed for root nodule NADH-GOGAT enzyme activity. As a control, plants from two regenerated lines carrying the CaMV35S promoter driving the GUS reporter gene (pARC100A and E) were assayed. The activity of AAT was also determined as an additional control to identify plants having a non-specific reduction in ammonia assimilatory enzyme activity. Of the six transgene genotypes, only the population derived from primary transformant 7-11-10 had significantly reduced nodule NADH-GOGAT activity (Fig. 1). Plants derived from 7-11-10 had NADH-GOGAT activities consistently reduced to 46–48% of the control mean. Transgene-containing plants having nodule NADH-GOGAT activity levels comparable to the controls (*c.* 70–80 nmol NADH mg^{-1} protein min^{-1}) were termed 'silent antisense' genotypes, and were maintained as additional control material.

Alfalfa transformants with depressed levels of NADH-GOGAT enzyme activity exhibit a corresponding reduction in enzyme protein and transcript

Polyclonal antiserum raised against the alfalfa root nodule NADH-GOGAT (Anderson *et al.*, 1989) was used to determine whether amounts of NADH-GOGAT protein

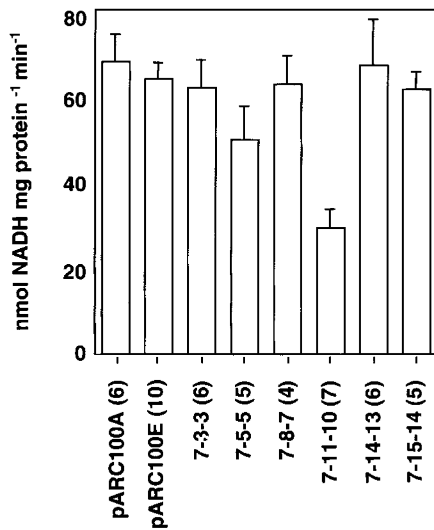

Fig. 1. Root nodule NADH-GOGAT activity in antisense transformants. Clonal populations of the six antisense NADH-GOGAT genotypes and two control lines (pARC100A and pARC100E) were assayed for root nodule NADH-GOGAT activity. The number of plants tested for each genotype is reported in parentheses. The error bars represent one standard deviation.

were reduced in the root nodules of antisense line 7-11-10 and to determine the correlation with the observed reduction in NADH-GOGAT activity. Soluble protein extracts from the nodules of line 7-11-10 and the control line pARC100E were subjected to SDS-PAGE immunoblot analysis (Fig. 2A). A significant reduction in the amount of NADH-GOGAT protein in extracts of the nodules from 7-11-10 plants was observed. Quantitation of multiple protein blots by image densitometry demonstrated a consistent reduction in NADH-GOGAT protein in the 7-11-10 plants to approximately 40% of control

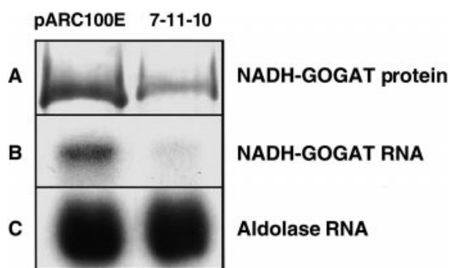

Fig. 2. Comparison of NADH-GOGAT protein and mRNA in nodules from antisense transformant 7-11-10 and control plants. (A) Protein immunoblot detection of NADH-GOGAT, 50 µg of total soluble protein was separated by SDS-PAGE, electroblotted blotted and the filter probed with NADH-GOGAT antibody. (B, C) Total nodule RNA (10 µg) for control line pARC100E and antisense line 7-11-10 was fractionated by electrophoresis through a 1.5% formalydehyde-agarose gel, transferred to a nylon membrane and probed for the presence of transcripts encoding NADH-GOGAT and aldolase. Before exposure to X-ray film the signal intensity was quantified by radioanalytic scanning. The intensity of the hybridizing NADH-GOGAT signal was calculated to be 43% of the control (average of three observations).

levels (data not shown). These data correlate with the 50–60% reduction in NADH-GOGAT enzyme activity.

To assess whether the reductions in NADH-GOGAT enzyme activity and protein in antisense 7-11-10 plants were the result of changes in the steady-state levels of NADH-GOGAT transcripts, RNA blot analysis was performed. Total nodule RNA from control (pARC100E) and 7-11-10 plants was separated electrophoretically, blotted and hybridized with a ^{32}P-labelled probe generated from an *Eco*RI restriction fragment of the GOGAT cDNA internal to the antisense transgene insert. NADH-GOGAT transcript abundance in each lane was quantified by the AMBIS radioanalytic image analysis. Figure 2B shows that NADH-GOGAT transcript in genotype 7-11-10 was approximately 43% of the control (average of three observations), indicating that the reduction in NADH-GOGAT protein and activity could be directly attributed to a reduction in NADH-GOGAT mRNA. No hybridization signal corresponding to the antisense transcript of approximately 2.6 kbp was detected (data not shown). An alfalfa aldolase cDNA probe (Vance *et al.*, 1995) was used as a loading control (Fig. 2C).

Fd-GOGAT and related ammonia assimilatory enzyme activity and transcript abundance in control and antisense genotypes

Primary nitrogen assimilation in root nodules involves the concerted efforts of several enzymes including GS, AAT and PEPC, all of which exhibit nodule-enhanced patterns of expression (Vance *et al.*, 1994). The activity of these enzymes was determined in the nodules from plants of control and 7-11-10 populations. Activities of these enzymes could be linked to a common control mechanism sensitive to alterations in nodule NADH-GOGAT activity or could be effected by the consequences of such an alteration. The consistent reduction in NADH-GOGAT activity observed for 7-11-10 had no significant effect on GS, AAT and PEPC activity (Fig. 3).

Although transcripts for the ferredoxin-dependent isoform of GOGAT (Fd-GOGAT) were not detected in alfalfa root nodules (Vance *et al.*, 1995), activity has been detected in the plant fraction of legume root nodules (Suzuki *et al.*, 1984, 1988). Therefore the effects of the antisense NADH-GOGAT transgene on the expression pattern of Fd-GOGAT were assessed for any compensatory effects. Total RNA from leaves and nodules of control and the antisense plant was probed with ^{32}P-labelled Fd-GOGAT (Vance *et al.*, 1995). The steady-state amount of Fd-GOGAT transcript in leaves was similar between controls and the antisense plants (Fig. 4). A prolonged exposure of the nodule RNA blots hybridized with the Fd-GOGAT probe detected low levels of the cognate transcript. The Fd-GOGAT mRNA abundance in the antisense plant appeared slightly enhanced (Fig. 4).

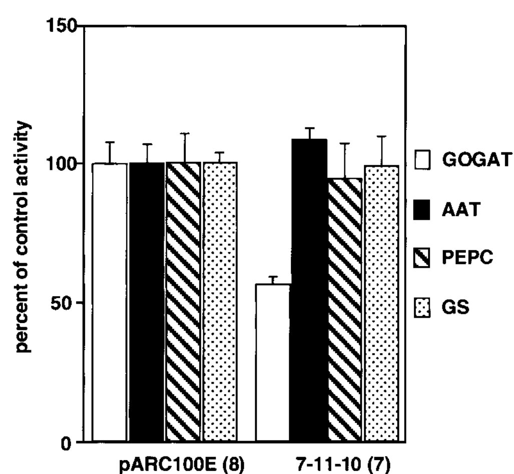

Fig. 3. Analysis of key nitrogen assimilatory enzymes in a control line and antisense genotype 7-11-10. Root nodule extracts were assayed for NADH-GOGAT, AAT, PEPC, and GS activity and are represented as a percentage of the control value. The control mean values for NADH-GOGAT, AAT, and PEPC were 50, 636 and 307 nmol NADH mg^{-1} protein min^{-1}, respectively, and 50 nmol glutamine mg^{-1} protein min^{-1} for GS. Error bars represent one standard deviation as a percentage of the control activity. The number of plants tested per genotype is reported in parentheses. The results reported are representative of two independent experiments.

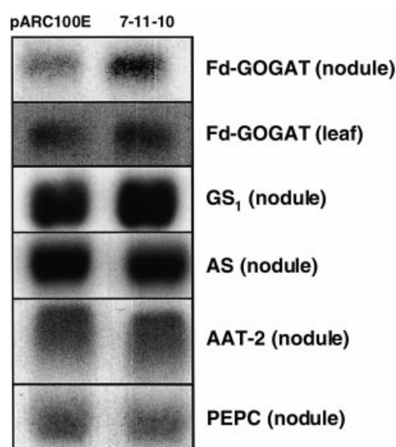

Fig. 4. Analysis mRNA abundance for several key enzymes involved in nitrogen and carbon metabolism. Total RNA from control pARC100E and antisense 7-11-10 nodules and leaves (10 μg) was separated electrophoretically, blotted, and probed for the presence of transcripts for Fd-GOGAT (leaf and nodule), GS_1, AS, AAT-2, and PEPC (nodule). The blots are representative of two observations.

Although the analysis of activity for several key ammonia assimilatory enzymes revealed no differences between the control and antisense genotype (Fig. 3), these assays cannot distinguish between the multiple isoforms that exist for some of these enzymes. Therefore, total RNA blots from control and antisense nodules were probed with the cDNAs for the nodule-enhanced forms of several enzymes. No significant differences were observed in the transcript abundance for the nodule-enhanced forms of AAT-2, GS_1, AS or PEPC between the control and antisense 7-11-10 nodule RNA (Fig. 4).

Comparison of the nitrogen content and nitrogen fixation rates between the control and antisense genotypes

Plants derived from the original transformants were inoculated with *S. meliloti* and supplied with N-free fertilizer. In a side-by-side comparison of mature plants, moderate chlorosis was seen among symbiotically dependent 7-11-10 plants, a phenotype that was not observed among plants with silent antisense or control genotypes. Both the leaves and stems of the 7-11-10 plants were pale green. To confirm that this reduction was the result of impairment in the assimilation of symbiotically derived N, and not due to a global lesion in nitrogen metabolism, uniformly trimmed nodulated plants were put on +N (KNO_3) or −N (K_2SO_4) fertilization regimes. The shoot N content of the control and silent antisense genotypes was essentially the same for the KNO_3 and K_2SO_4 treatments (mean values of 3.18% versus 3.16%, respectively). The 7-11-10 plants given KNO_3 had a significantly greater shoot percentage N ($P=0.05$) than those receiving K_2SO_4 (3.31% versus 2.78%). This amounts to a 12–16% reduction in shoot N concentration when the symbiotically sustained 7-11-10 population is compared to either control N regimes or the KNO_3 treated 7-11-10 populations. Thus, it appears that the significant reduction in shoot nitrogen content contributed to chlorosis in the symbiotically grown antisense NADH-GOGAT transformant. No consistent difference in root N content was observed (data not shown).

To test whether the lower shoot N content of the 7-11-10 genotype could be correlated with a lower rate of N_2 fixation, the rate of H_2 evolution from mature nodulated root systems was measured. Table 1 shows that the average apparent nitrogenase activity and total nitrogenase activity was lower in the antisense 7-11-10 plants as compared to the control pARC100E plants. However, the variability within these samples was high, contributing to a low level of statistical significance (Table 1). In a comparison of control and antisense plants maintained in growth pouches under symbiotic conditions, total dry weight from both shoot and root was significantly reduced in the antisense plant. Measurements of N_2 fixation using $^{15}N_2$ showed little or no difference in nitrogenase activity. Nodule fresh weight was also significantly reduced, which impacted nodule efficiency and apparent efficiency, resulting in reduced plant nitrogen content (Denison, J Blumenthal, Russelle, and CP Vance, unpublished data).

The antisense NADH-GOGAT plant 7-11-10 is male sterile

To verify the stability of the antisense NADH-GOGAT construct and to determine its pattern of inheritance and effect on progeny plants, flowers from 7-11-10 plants were

Table 1. *Apparent nitrogenase activity (ANA) and total nitrogenase activity (TNA) of control pARC100E and antisense 7-11-10 genotypes*

H_2 evolution of control and 7-11-10 plants was measured in a flow-through system. Units of ANA and TNA are μmol H_2 $plant^{-1}$ h^{-1}. ANA was measured in air, and TNA was measured by replacing N_2 with argon in the system.

Genotype	*n*	ANA	s.d	Difference significant at *P* =	TNA	s.d.	Difference significant at *P* =
pARC100E	12	6.34	1.55	0.12	15.32	4.04	0.33
7-11-10	12	5.48	0.98		13.87	2.93	

tripped to induce self-fertilization. No seed pods were observed on the selfed 7-11-10 plants, despite repeated attempts at self pollination. In comparison, the control pARC100E and silent antisense genotype plants successfully produced selfed seed. A separate group of 7-11-10 plants was used as a female parent in a cross with alfalfa experimental population UM3064, and these plants readily set viable seed. The inability of the antisense 7-11-10 plant to act as a male parent indicated that impaired NADH-GOGAT activity may have reduced pollen viability. Pollen from control and antisense plants was collected, suspended in water and spotted on to pollen germination medium. Extensive germination and pollen tube growth was observed within 1 h with pollen from the control plant (Fig. 5A). In contrast, pollen grains collected from 7-11-10 plants were smaller and rarely germinated even after several hours on the germination medium (Fig. 5B). The addition of glutamate to the germination medium to test whether low NADH-GOGAT activity was causing a nutritional deficiency in the pollen grain, did not restore viability. Pollen viability was also not restored by providing the 7-11-10 plants with nitrate in solution for several weeks prior to pollen collection (data not shown). The pollen from the control and antisense plants was also subjected to scanning electron microscopic analysis. Pollen from the antisense plant appeared shrunken and collapsed when compared to the control pollen (Fig. 5C, D). A reduction in the degree of pollen grain clumping was also observed with pollen from the antisense plant (data not shown).

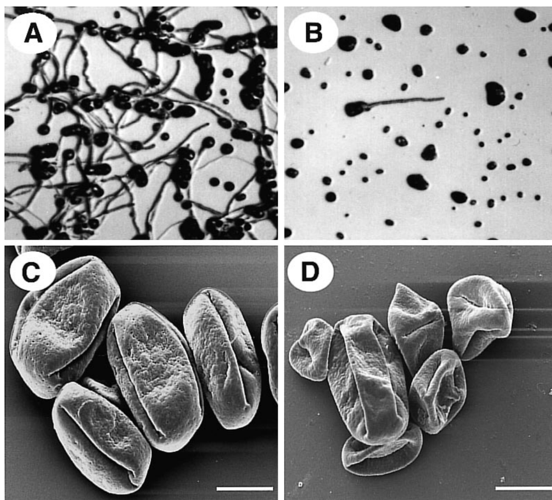

Fig. 5. Analysis of the pollen from antisense line 7-11-10. Pollen collected from newly opened flowers of control pARC100E (A) and antisense line 7-11-10 (B) was suspended in water and plated onto pollen germination medium. The germination rate of pARC100E pollen was approximately 84% (average of six observations), while the average for 7-11-10 was 6% (nine observations). Low temperature, low voltage scanning electron microscopic analysis of pollen from pARC100E (C) and 7-11-10 (D) plants. Bar = 40 μm.

The antisense alfalfa transformant has reduced levels of NADH-GOGAT enzyme activity and transcript in developing flower buds

In the antisense construct pA2TAGA, the NADH-GOGAT cDNA fragment was under the control of the nodule-enhanced AAT-2 promoter. A construct containing the AAT-2 promoter fragment directed GUS expression in pollen (DA Samac, RG Gregerson and CP Vance, unpublished data). To determine if the non-viability of 7-11-10 pollen was due to a direct biochemical effect of the antisense transgene during flower development and pollen formation, flower buds of 7-11-10 and a control genotype were assayed for NADH-GOGAT activity. As shown in Fig. 6A, the NADH-GOGAT activity measured in pARC100E plants was approximately 8.5 nmol NADH min^{-1} mg^{-1} protein, about one-eighth the activity of nodule extracts on a protein basis. In contrast, the NADH-GOGAT activity of antisense 7-11-10 flower buds was not above background (Fig. 6A).

A blot of total RNA extracted from flower buds was hybridized with an NADH-GOGAT probe. NADH-GOGAT message was readily detectable in the flowers of control plants, while the corresponding message in the antisense 7-11-10 lane was barely detected (Fig. 6B). The same blot was stripped and probed with the Fd-GOGAT probe. As shown in Fig. 6B, Fd-GOGAT transcript was present in alfalfa flower buds, but its level was unaffected in the antisense genotype.

Time-course of NADH-GOGAT enzyme activity and RNA in developing flower buds

The presence of significant amounts of NADH-GOGAT activity and the corresponding transcript in the control

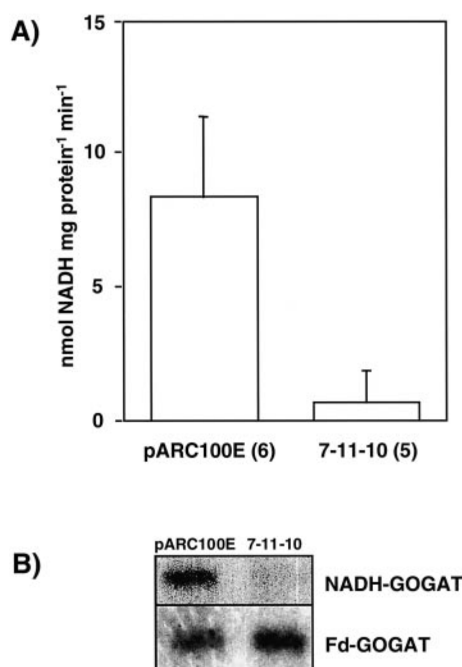

Fig. 6. NADH-GOGAT activity and mRNA level in flower buds of control pARC100E and antisense line 7-11-10. (A) Unopened flower buds of control pARC100E and antisense line 7-11-10 were assayed for NADH-GOGAT activity. The number of plants tested for each genotype is reported in parentheses. The error bars represent one standard deviation. (B) Total RNA (10 μg) isolated from control and antisense unopened flower buds was separated electrophoretically, blotted, and probed for the presence of NADH-GOGAT and Fd-GOGAT transcripts.

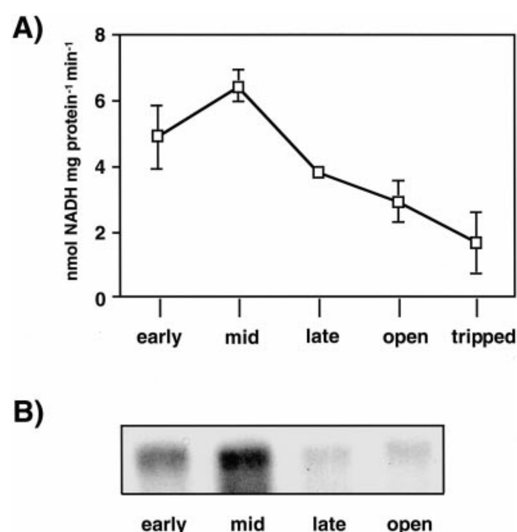

Fig. 7. NADH-GOGAT activity and mRNA level during alfalfa flower maturation. (A) The level of NADH-GOGAT activity in control alfalfa flowers was measured at five stages of development: early bud, clusters of buds less than 3 mm in length; mid buds greater than 3 mm but with no petal exposed; late buds with petals exposed but unopened; open flower, a newly opened but untripped flower and tripped flower, flowers deliberately tripped 2 d prior to collection. The error bars represent one standard deviation. (B) Total RNA (10 μg) from alfalfa flower buds at the early through open stages was isolated separated electrophoretically, blotted, and probed for the presence of transcripts for NADH-GOGAT.

flowers and the reduced amounts in flowers of the male sterile 7-11-10 genotype, indicates that NADH-GOGAT activity is essential for normal flower development. The level of NADH-GOGAT enzyme activity and transcript abundance at different stages of flower development of control plants was determined. Figure 7A shows the NADH-GOGAT activity at five stages of flower development. The flowers were categorized by gross morphological features: early bud, clusters of buds less than 3 mm in length; mid-buds, greater than 3 mm but with no petal exposed; late buds, petals exposed but unopened; open flower, a newly opened but untripped flower; and tripped flower, flowers deliberately tripped 2 d prior to collection. NADH-GOGAT activity increased from the early bud stage to a maximum (*c.* 6–8 nmol NADH mg^{-1} protein min^{-1}) at the midbud stage and then decreased gradually during the late bud, open flower and tripped flower stages (Fig. 7A).

To determine whether this course of NADH-GOGAT activity was related to the amount of NADH-GOGAT mRNA in the flower, total RNA was isolated from flower buds and flowers at the early bud, mid-bud, late bud, and open flower stages. Figure 7B shows that there is a strong correlation between the amount of NADH-GOGAT activity at these stages and the amount of mRNA, suggesting transcriptional control of NADH-GOGAT activity during flower development.

Inheritance of the antisense transgene and NADH-GOGAT activity in the out-cross progeny

When provided with a viable source of pollen obtained from alfalfa experimental population UM3064, NADH-GOGAT antisense 7-11-10 plants were able to produce seed. Of the 100 seeds collected, 58 germinated and were grown to maturity in the glasshouse. The presence of the antisense NADH-GOGAT transgene in progeny plants was determined by PCR and DNA blot analysis (data not shown). Of the 58 progeny plants, 38 were found to contain the transgene. All the progeny were assayed for root nodule NADH-GOGAT activity. Figure 8A shows the frequency distribution of plants according to nodule NADH-GOGAT activity. The activity of progeny containing the transgene ranged from 8 to 84 nmol NADH mg^{-1} protein min^{-1} with a mean activity of 36. Progeny without the transgene had an activity range of 36–86 nmol NADH mg^{-1} protein min^{-1} and mean of 64. A pARC100E population grown under the same conditions had an NADH-GOGAT activity of 76 nmol NADH mg^{-1} protein min^{-1}. The difference between the mean activities is significant ($P=0.01$).

Total RNA was isolated from the root nodules of several out-cross progeny plants exhibiting low levels of NADH-GOGAT activity (OX 27, 30, 33, and 35) and

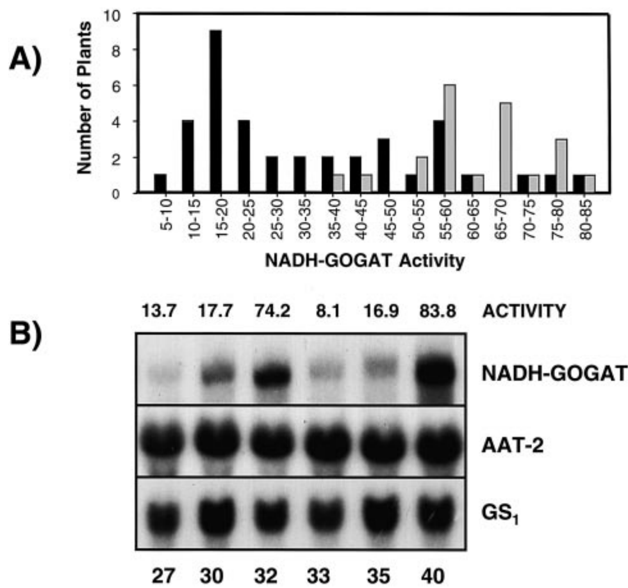

Fig. 8. Analysis of nodule NADH-GOGAT activity and transcript level among the out-crossed progeny of the antisense 7-11-10 genotype. (A) Progeny plants resulting from the crossing of the 7-11-10 genotype with experimental population UM3064 were assayed for root nodule NADH-GOGAT activity. The frequency distribution of activities for plants containing the transgene (filled columns) and without the transgene (hatched columns) were ranked by NADH-GOGAT activity in 5 nmol NADH mg^{-1} protein min^{-1} increments. (B) Total nodule RNA (20 μg) isolated from out-cross lines (27, 30, 32, 33, 35, and 40) was fractionated by electrophoresis through a formalydehyde-agarose gel, transferred to a nylon membrane and probed for the presence of transcripts encoding NADH-GOGAT, AAT-2 and GS_1. Root nodule NADH-GOGAT activity (nmol NADH mg^{-1} protein min^{-1}).

compared to plants found to contain no transgene (OX 32 and 40). RNA blot analysis revealed that the plants with low NADH-GOGAT activity also contained reduced levels of NADH-GOGAT transcript. The transcript abundance for AAT-2 and GS_1 were again unaffected (Fig. 8B).

Discussion

Our understanding of plant nitrogen metabolism has been greatly aided by the isolation and study of mutants defective in their ability to catalyse defined biochemical reactions (Lam *et al.*, 1995; Radwanski and Last, 1995; Oliveira *et al.*, 1997). Investigations into the expression, regulation and biochemical roles of key ammonia assimilatory enzymes in the root nodules of legumes would be greatly aided by the characterization of mutants deficient in particular enzymes, but otherwise effective in symbiosis. The tetraploid nature of alfalfa precludes the generation of such mutants using established mutagenesis techniques. The activity of the nodule-enhanced NADH-dependent form of glutamate synthase has been specifically reduced using an antisense transgene constructed from a portion of the NADH-GOGAT cDNA (Gregerson *et al.*, 1993) in antisense orientation behind the nodule-enhanced aspartate amino transferase-2 promoter (Gregerson *et al.*, 1994). By specifically targeting the expression of the antisense transgene in the root nodule, it was hoped that it would be possible to determine whether NADH-GOGAT might represent a major controlling step in the assimilation of symbiotically fixed ammonia. Previous studies have demonstrated that in both plant and bacterially conditioned ineffective alfalfa nodules, the expression of NADH-GOGAT is more severely impacted than GS. This indicates that NADH-GOGAT is differentially regulated and may represent the primary regulatory point in nodule primary N assimilatory metabolism (Egli *et al.*, 1989; Vance *et al.*, 1995; Temple *et al.*, 1998*b*).

Among the clonal populations derived from six surviving antisense transgenic lines, it was found that NADH-GOGAT activity in nodules of the 7-11-10 genotype was consistently reduced to about 50% that of the control activity. The original number of transformants was low and among these only the 7-11-10 line had significantly lower nodule NADH-GOGAT activity. This may indicate lethal activity during embryo induction or plantlet development, resulting from significantly altered nitrogen metabolism. Subsequent attempts to generate more transgenic alfalfa lines containing the same construct have resulted in a large numbers of 'silent antisense' genotypes (SJ Temple and CP Vance, unpublished results). Lethal activity associated with nitrogen metabolism altering transgenes during plant regeneration has been observed previously in attempts to reduce the expression of cytosolic GS in alfalfa (Temple *et al.*, 1994) and during the regeneration of antisense Fd-GOGAT tobacco plants (Hirel *et al.* 1997). Because of its significantly reduced NADH-GOGAT activity, the 7-11-10 genotype was maintained and propagated for further physiological and molecular characterization.

In the nodules of the plant-conditioned ineffective alfalfa genotype in_1Sa, the activity of not only NADH-GOGAT, but also GS, AAT, and PEPC are significantly reduced, demonstrating the pleiotropic effect of the genetic lesion on ammonia assimilatory enzymes (Egli *et al.*, 1989). However, the enzyme activities of AAT, PEPC and GS and the transcript levels for GS_1, AS, AAT-2, and PEPC were unaffected in the antisense 7-11-10 genotype. This suggests that the reduced NADH-GOGAT activity is not the result of a non-specific mutation, and that the down-regulation of NADH-GOGAT does not induce altered ammonia assimilatory enzyme activity at other points in the pathway. The lower NADH-GOGAT activity was directly attributable to both lower NADH-GOGAT protein and mRNA in the nodule, each reduced in proportion to the measured activity.

Although Fd-GOGAT is the predominant form of the enzyme in green tissues (Lea *et al.*, 1990; Vance *et al.*, 1995), activity has been detected in the plant fraction of legume root nodules (Suzuki *et al.*, 1984, 1988). Findings

from the study of Fd-GOGAT null mutants indicates that no compensatory mechanisms exist between the two GOGAT forms and points to distinct biochemical roles for the two forms of the enzyme (Blackwell *et al.*, 1987; Lea and Forde, 1994). The results of the RNA blot analysis performed on the leaves and nodules of the 7-11-10 antisense line using an Fd-GOGAT probe, suggests that Fd-GOGAT may compensate slightly for reduced NADH-GOGAT nitrogen assimilatory activity. However, since Fd-GOGAT activity was not evaluated and Fd-GOGAT mRNA amounts are very low in alfalfa nodules the role for this enzyme remains questionable. The impaired ability of the low NADH-GOGAT genotype to assimilate symbiotically fixed N_2 indicates that the contribution Fd-GOGAT makes to nodule ammonia assimilation, is minor in comparison to NADH-GOGAT.

In glasshouse and growth chamber experiments, mature 7-11-10 plants were moderately chlorotic when grown under symbiotic N_2 fixation conditions. This chlorosis could be attributed to a lower shoot percentage N, with a reduction of N content by 10–20% when compared to control plants under the same conditions. Both the chlorosis and the lower N status of 7-11-10 plants were alleviated by the addition of N fertilizer, indicating that this genotype was not impaired with respect to nitrate uptake, reduction, and assimilation. Together these results demonstrate that the lower N content of the 7-11-10 genotype results from an impairment in its ability to assimilate ammonia derived from symbiosis. This resulted in a reduction in both shoot and root dry weight and N content. The lack of significant differences in whole plant nitrogenase activity between the antisense NADH-GOGAT plant and the controls support the interpretation that the effect of the antisense gene is specifically on nodule N assimilation rather than N_2 fixation. Nitrogenase specific activity on a nodule fresh weight basis was significantly higher in the 7-11-10 antisense plants ($3.17\ mg\ g^{-1}$ fw), than the control plants ($1.65\ mg\ g^{-1}$ fw). These findings further support the feedback control hypothesis for nitrogenase activity (Hartwig, 1998) and suggest that the N signal for feedback control is downstream from NH.

The association of the antisense transgene with male sterility in the 7-11-10 genotype came as a surprise and did not appear to be a somatic mutation resulting from tissue culture and regeneration. The shrivelled, non-viable pollen points to a metabolic lesion in the metabolism of flower tissues critical for pollen development. An investigation into the activity and expression of NADH-GOGAT through the course of flower development demonstrated that the gene is most highly expressed in the early stages of development, perhaps at a critical time in pollen formation. In rice, NADH-GOGAT activity and protein increased 6- and 4-fold, respectively, in the apical spiklets during the first 15 d after flowering, subsequently the levels declined rapidly at the grain filling stage (Hayakawa *et al.*, 1993). The authors suggested that in rice, NADH-GOGAT is responsible for the synthesis of glutamate from the glutamine that is transported from senescing tissues to the spikelets. Although changes in Fd-GOGAT paralleled those for NADH-GOGAT, the relative abundance of NADH-GOGAT protein was about three times higher from 5–15 d after flowering (Hayakawa *et al.*, 1993). Preliminary attempts to use immunocytochemistry to locate NADH-GOGAT in the flower show that NADH-GOGAT occurs in the tapetum (Trepp and CP Vance, unpublished results). The transcripts for the nodule-enhanced forms of malate dehydrogenase, PEPC, GS_1 in developing flower buds have also been detected (Temple *et al.*, 1998*a*; Schoenbeck and Vance, unpublished results). The effectiveness of the nodule-enhanced AAT-2 promoter in reducing NADH-GOGAT in the flower is itself suggestive that this gene is also expressed in flowers. A promoter GUS fusion using the AAT-2 promoter is expressed in alfalfa pollen (DA Samac, RG Gregerson and CP Vance, unpublished data).

The inheritance pattern of the antisense transgene among the F_1 generation demonstrates that the low nodule NADH-GOGAT activity phenotype segregates exclusively with the transgene although not every plant inheriting the transgene had reduced NADH-GOGAT activity. The distribution of NADH-GOGAT activity shows a single mode of distribution for plants without the transgene, centred at about the wild-type activity. For plants containing the transgene, a bimodal distribution was observed. The majority of the plants had low levels of NADH-GOGAT activity, while a smaller group had wild-type levels. The NADH-GOGAT transcript abundance in a sample of these plants correlated with enzyme activity. Based on DNA hybridization data and the frequency of the transgene among the progeny, it appears that more than one copy of the transgene is present in the 7-11-10 parent. A single locus of transgene insertion in the 7-11-10 parent would be inherited by 50% of the F_1 progeny, whereas a second unlinked insertion would lead to at least one copy of the transgene in 75% of the progeny (Stanford, 1951). A chi-squared value for the two loci insertion of 2.78 is not rejected at $P=0.05$. The slightly lower than predicted value of 64% of the progeny plants carrying the transgene may be explained by the limited sample size and the possibility that some progeny carrying the transgene were non-viable resulting from NADH-GOGAT levels insufficient to support even minimal N assimilation. The wild-type levels of enzyme activity in some of the transgene positive plants suggests that silencing occurred in some progeny. This is not unexpected based on reports of the silencing of homologous sequences in transgenic plants (Meyer and Saedler, 1996).

In conclusion, NADH-GOGAT activity in alfalfa root nodules has been down-regulated to less than half that of the wild type. The activity of other key root nodule

nitrogen and carbon assimilatory enzymes was not affected. When these plants were grown under symbiotic conditions, N assimilation was significantly reduced. A critical role was also demonstrated for NADH-GOGAT in the reproductive development of alfalfa. The reduced level of NADH-GOGAT activity was stable among clonally propagated plants derived from the original transformant and was inherited with the transgene among outcross progeny. This new generation of low-GOGAT plants will serve as a valuable tool to investigate N and C metabolism in symbiotic systems further.

Acknowledgements

We thank Drs Michael Russelle and Joanne Lamb for their technical assistance. This work was supported by 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. 981-13-0105 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 vendor.

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