Molecular cloning, characterization and regulation of two different NADH-glutamate synthase cDNAs in bean nodules

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ABSTRACT

NADH-dependent glutamate synthase (NADH-GOGAT) is a key enzyme in primary ammonia assimilation in Phaseolus vulgaris nodules. Two different types of cDNA clones of PvNADH-GOGAT were isolated from the nodule cDNA libraries. The full-length cDNA clones of PvNADH-GOGAT-I (7.4 kb) and PvNADH-GOGAT-II (7.0 kb), which displayed an 83% homology between them, were isolated using cDNA library screening, ‘cDNA library walking’ and RT-PCR amplification. Southern analysis employing specific 5′ cDNA probes derived from PvNADH-GOGAT-I and PvNADH-GOGAT-II indicated the existence of a single copy of each gene in the bean genome. Both these proteins contain ~100 amino acid sequences theoretically addressing each isoenzyme to different subcellular compartments. RT-PCR analysis indicated that PvNADH-GOGAT-II expression is higher than PvNADH-GOGAT-I during nodule development. Expression analysis by RT-PCR also revealed that both of these genes are differentially regulated by sucrose. On the other hand, the expression of PvNADH-GOGAT-I, but not PvNADH-GOGAT-II, was inhibited with nitrogen compounds. In situ hybridization and promoter expression analyses demonstrated that the NADH-GOGAT-I and -II genes are differentially expressed in bean root and nodule tissues. In silico analyses of the NADH-GOGAT promoters revealed the presence of potential cis elements in them that could mediate differential tissue-specific, and sugar and amino acid responsive expression of these genes.

Key-words: Phaseolus vulgaris; carbon and nitrogen compounds; gene expression; promoter analysis; PvNADH-GOGAT.

INTRODUCTION

Glutamine synthetase (GS) and glutamate synthase (GOGAT) form the main route of ammonia assimilation in higher plants (GS/GOGAT cycle) (Miflin & Lea 1976; Lancien, Gadal & Hodges 2000; Suzuki & Knaff 2005). GS catalyses the ATP-dependent amidation of glutamate resulting in the production of glutamine. GOGAT, on the other hand, catalyses the reductive transfer of the amide group of glutamine to the α-keto position of 2-oxoglutarate, forming two molecules of glutamate (Lea, Robinson & Stewart 1990). Ammonia in higher plants is derived both from the primary nitrogen sources (ammonia, nitrate and for legumes, dinitrogen) and from a number of internal nitrogen-cycling pathways such as photorespiration, amino acid metabolism and phenylpropanoid metabolism (Vance 1997). GOGAT is therefore essential not only for primary nitrogen assimilation but also to maintain the general nitrogen economy of the plant.

In higher plants, GOGAT occurs as two distinct isoforms: Fd-dependent GOGAT (Fd-GOGAT; EC 1.2.7.1) and NADH-dependent GOGAT (NADH-GOGAT; EC 1.4.1.14), and both isoforms differ from one another in molecular mass, subunit composition, enzyme kinetics, reductant specificity and metabolic function (Temple,Vance & Gantt 1998; Lea & Miflin 2003). These two molecular species of GOGAT exist in both green and non-green tissues. The Fd-GOGAT has been shown to be the most abundant isoform in green tissues and is located in the chloroplast (Walls, Lea & Millin 1979). This monomeric enzyme is an Fe-S protein with an M, about 150 kDa. In leaves, Fd-GOGAT catalyses the assimilation of NH₄⁺ derived from both the light-dependent reduction of NO₃⁻ and the NH₄⁺ generated during photorespiration, while the root isoform has been implicated in the assimilation of NH₄⁺ derived from soil NO₃⁻ (Sakakibara et al. 1991; Redinbaugh & Campbell 1993; Suzuki & Knaff 2005). Complimentary DNA of Fd-GOGAT has been isolated from a number of species. Two Fd-GOGAT genes (GLU1 and GLU2) were characterized in Arabidopsis thaliana; GLU1

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NADH-GOGAT isoforms in Phaseolus vulgaris

Poly(A)+ RNA for constructing the cDNA library was isolated from the nodules following the standard method (Sambrook, Fritsch & Maniatis 1989). Total and poly(A)+ nodule RNA for isolating full-length bean NADH-GOGATs by RT-PCR were extracted using RNasy Plant Kit and Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA), respectively. The total RNA from the plant tissues for the expression analysis by RT-PCR was isolated by employing the RNA Tripure Isolation Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA).

MATERIALS AND METHODS

Plant growth, treatments and tissue sampling

P. vulgaris cv. Negro Jamapa seeds (ProNaSe, Guanajuato, México) were surface sterilized in 20% sodium hypochlorite solution for 10 min, washed thoroughly with sterile distilled water and germinated on a moist sterile filter paper. Three-day-old seedlings were transferred to the pots containing sterile vemiculite (five plants per pot) and then inoculated with Rhizobium tropici CIAT899, Rhizobium etli CE3 or R. etli CE3 NifA−. The uninoculated plants served as the control. The plants were grown in a naturally lighted greenhouse as described by Ortega et al. (1992). For the PvNADH-GOGAT expression analysis, the nodules were harvested at various time points during 12–21 d after inoculation (DAI). In addition, roots, stems and fully expanded leaves of 13-day-old nodulated plants, and cotyledonary leaves from 3-day-old plants, and flowers and pods from mature plants were also collected for analysis. In the studies pertaining to assessing the effects of carbon and nitrogen treatments on the PvNADH-GOGAT expression, the plants were watered for 4 d, starting on the 17th DAI, with the Hoagland nutrient solution containing either sucrose (0.5, 1 and 2%), asparagine, glutamine, xanthine, allantoin (10 mM) or allopurinol (2 mM), and then the nodules were harvested for analysis. The nodules were also collected from the control plants that were treated with the Hoagland nutrient solution devoid of any carbon and nitrogen compounds. All tissues were immediately frozen in liquid nitrogen and stored at –70 °C until use.

Isolation of RNA

NADH-GOGAT plays a major role in the assimilation of ammonium derived from photorespiration, whereas the GLU2 gene may play a major role in the primary nitrogen assimilation in roots (Oliveira et al. 1997; Suzuki & Rothstein 1997; Coschigano et al. 1998).

NADH-GOGAT is found primarily in the non-photosynthetic tissues of plants, such as roots and legume root nodules, where it is located in the plastids. In Medicago sativa, it has been immunolocalized to the amyloplasts of infected cells in the nodules (Trepp et al. 1999a). Pattern of expression was found to shift during root nodule development and expression is tightly linked to effective nodules (Trepp et al. 1999b). It has been purified from legume root nodules as well as rice (Oryza sativa) cell cultures (Chen & Cullimore 1988; Anderson et al. 1989; Hayakawa et al. 1992). As Fd-GOGAT, NADH-GOGAT is an Fe-S flavoprotein (Curti et al. 1995; Suzuki & Knaff 2005). NADH-GOGAT exists as a monomer with an Mr of approximately 225–230 kDa (Yamaya et al. 1992; Gregerson et al. 1993; Vance et al. 1995). In nodules, it is the major form of GOGAT, and where it combines with cytosolic GS to assimilate NH₄⁺ produced by the nitrogen-fixing bacteroids. NADH-GOGAT activity has been found to increase markedly during nodule development (Gregerson et al. 1993; Vance et al. 1994), and is three times higher than the Fd-GOGAT activity. It has been proposed that NADH-GOGAT catalyses the rate-limiting step in ammonia assimilation in the root nodules (Trepp et al. 1999a,b).

In non-legumes such as rice, NADH-GOGAT has been characterized with studies of gene organization, expression of mRNA, protein localization and protein structure (Yamaya et al. 1992, 1995; Goto et al. 1998). The expression of the NADH-GOGAT gene in rice is regulated in a cell type-specific, age-specific and nitrogen-responsive manner (Hayakawa et al. 1994, 1999). It has been located in roots, leaves, etiolated leaves, vascular bundles of unexpanded leaf tissues and the developing grains, and was shown to function in the primary assimilation of NH₄⁺ derived from soil NO₃⁻ and the re-assimilation of NH₄⁺ released during amino acid catabolism and/or during seed germination.

Full-length cDNA and genomic clones of NADH-GOGAT have been isolated from M. sativa and O. sativa, and was found that NADH-GOGAT is encoded by a single gene (Gregerson et al. 1993; Vance et al. 1995; Goto et al. 1998). The only report that described the existence of two isoforms of NADH-GOGAT in plants is in bean nodules where two isoenzymes (I and II) were identified (Chen & Cullimore 1988). The isoenzyme NADH-GOGAT-II is localized in the central tissue of the nodule, while the isoenzyme NADH-GOGAT-I is found to be present both in the cortex and central tissue of the nodules, as well as in the roots (Chen & Cullimore 1989). NADH-GOGAT-II accounted for the majority of the activity in the root nodules. Specific activity of NADH-GOGAT in roots is found to be about 27 times lower than that in the nodules, and consisted almost entirely of NADH-GOGAT-I. Both isoenzymes were partially purified, and were shown to have similar Ms of about 200 kDa with marginal differences in kinetic characteristics (Chen & Cullimore 1988).
Isolation of \textit{PvNADH-GOGAT} cDNAs

Two \textit{\lambda}ZAP cDNA libraries (constructed using \textit{\lambda}ZAP-cDNA synthesis Kit; Stratagene, La Jolla, CA, USA) of \textit{P. vulgaris} derived from poly(A)$^+$ RNA of 15-day-old nodules were screened for isolating \textit{PvNADH-GOGAT} clones. The cDNA probes were labelled with $^32$P using random primer labeling kit (Megaprime DNA labelling system; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), and employed to screen the cDNA libraries. The library screening was done on nylon membrane (Amersham Life Sciences, Little Chalfont, UK) replicas by standard procedures (Sambrook \textit{et al.} 1989). Hybridization was carried out overnight in 5X Denhardt’s solution (1X Denhardt’s solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 6X SSPE (1X SSPE is 0.15 M NaCl, 10 mM sodium phosphate and 1 mM ethylenediaminetetraacetic acid, pH 7.4), 1% sodium dodecyl sulphate (SDS) and 0.1 mg mL$^{-1}$ denatured salmon sperm DNA at 55 °C. Membrane filters were washed once at room temperature for 5 min in 2X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS, and then twice at 50 °C for 20 min each in 0.2X SSC and 0.1% SDS. Positive \textit{\lambda}ZAP clones were plaque purified and cDNA inserts were obtained in the Bluescript SK$^+$ vector by \textit{in vivo} excision according to the manufacturer's instructions (Stratagene).

Library screening was done in two steps. Initial library screening was done using a 1.6 kb cDNA probe derived from the alfalfa \textit{NADH-GOGAT} (pGOGAT7.2; Gregerson \textit{et al.} 1993). As a consequence, we isolated two clones containing cDNA inserts of 2.5 kb (\textit{PvNADH-GOGAT-I}) and 3 kb (\textit{PvNADH-GOGAT-II}). Subsequently, utilizing these partial \textit{PvNADH-GOGAT-I} and \textit{PvNADH-GOGAT-II} cDNAs as probes, longer cDNA inserts were obtained by screening a second nodule cDNA library, which was prepared using size-fractionated (3–9 kb) nodule cDNA. From the second screening, two longer clones of 5 kb each corresponding to \textit{PvNADH-GOGAT-I} and -II were isolated and sequenced. In order to expand the 5' region of the cDNA inserts, size-fractionated nodule cDNA library was used as a template for ‘cDNA walking’, and three sequential rounds of PCR (profile A; Supplemental Table S1) using Expand Long Template PCR System (Roche Diagnostics Corporation) were performed using pBluescript T3 primer in combination with gene specific 5' region primers designed for each of the \textit{PvNADH-GOGAT} cDNA insert/fragment (gene-specific reverse primers for the amplification of 5' regions; Supplemental Table S2). The gene specific 5' primers were derived from the sequence analysis of the cDNA inserts in the clones as well as the amplified fragments obtained from each round of PCR amplification.

Finally, RT-PCR was performed in order to obtain the full-length clones for each of the \textit{PvNADH-GOGAT}s. For this purpose, fresh nodule cDNA was synthesized from the total RNA isolated from 15-day-old nodules using PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA), primed with oligo (dT)$_{12-18}$ (Invitrogen, Carlsbad, CA, USA), and employed for the amplification of the full-length cDNAs using gene-specific 5' and 3' primers (for amplifying full-length clones; Supplemental Table S2) designed based on the assembled sequences of the clones obtained through both library screening and ‘cDNA library walking’ (see above). In the PCR step of the reaction, the Expand Long Template PCR System (Roche Diagnostics Corporation) was used for the amplification of the full-length 7.4 kb \textit{PvNADH-GOGAT-I} and 7.05 kb \textit{PvNADH-GOGAT-II} (profile B; Supplemental Table S1). Both fragments were cloned in pGEM-T easy vector (Promega Corp., Madison, WI, USA) and transformed into XL10 GOLD \textit{Escherichia coli} cells (Stratagene) and sequenced.

Sequence analysis

DNA sequencing of all isolated clones was done at Medigenomix (Munich, Germany), and the sequences of the full-length clones were corroborated with those sequences previously obtained from the partial clones. To manage the sequence data, the DNA strider 1.3 program (March 1988) was used. Similarity searches of databases were performed according to Altschul \textit{et al.} (1990) with the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). Transit peptide was identified and analysed with Plant-Ploc (Plant Protein Subcellular Location Prediction; http://202.120.37.186/bioinf/plant/; Chou 2005; Shen & Chou 2006; Chou & Shen 2007) and WoLF PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences; http://wolfpsort.org/; Horton \textit{et al.} 2006, 2007) programmes. The protein molecular masses and pI were estimated by using the pl/MW program at Uniprot (EMBL-EBI; http://au.expasy.org/tools/pi_tool.html).

RT-PCR analysis

The tissue samples were ground in liquid nitrogen, and the total RNA was extracted with TriPure (Roche Diagnostics Corporation) according to the instructions of the manufacturer. For RT-PCR studies, 5 µg of total RNA was converted into first-strand cDNA by using the PowerScript pre-amplification system (Clontech) and oligo (dT)$_{12-18}$ (Invitrogen). One microlitre of cDNA sample was amplified (profile C; Supplemental Table S1) in a 50 µL PCR mixture containing DNA polymerase buffer (Roche Diagnostics Corporation), 2.5 mM MgCl$_2$, 200 µM of each dNTPs, 0.1 µM of each gene-specific primers (RT-PCR primers; Supplemental Table S2) and 1 unit of Taq DNA polymerase (Roche Diagnostics Corporation).

Plant DNA extraction and DNA gel blot analysis

Genomic DNA was isolated from the leaves of 5-day-old seedlings using the DNAzol procedure (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA). For the gel blot analyses genomic DNA was digested with PstI, XbaI, KpnI and EcoRV restriction enzymes, separated on a 0.8% agarose gel (15 µg DNA per lane) and transferred to nylon
membranes (Hybond-N’, Amersham Life Sciences). Hybridization was performed using, as labelled probes, a 611 bp and a 461 bp fragments specific for PvNADH-GOGAT-I and PvNADH-GOGAT-II, respectively. Both fragments contain the 5′ untranslated region (UTR) and transit peptide-coding regions. The probes were radio labelled with 32P by random priming with rediprime II (Amersham Life Sciences). Hybridization was carried out overnight at 65 °C in 300 mM phosphate buffer (pH 7.2) and 7% SDS. The hybrized filters were washed at 65 °C with 2X SSC (15 min), 0.2X SSC (15 min) and 0.1X SSC (10 min) plus 0.1% (w/v) SDS, and exposed to Kodak X Omat films.

Isolation of the NADH-GOGAT promoters

The 5′ flanking genomic sequences of PvNADH-GOGAT-I and PvNADH-GOGAT-II were isolated by long-distance PCR using bean DNA and GenomeWalker kit (Clontech) according to the manufacturer’s instructions. Gene specific primers employed for genome walking of the 5′ flanking regions of PvNADH-GOGAT-I and PvNADH-GOGAT-II are given in the Supplemental Table S3. Long-distance PCR was performed using Advantage 2 Polymerase Mix with the following cycling profiles. Primary PCR amplification was carried out with five initial cycles of 28 s at 94 °C and 4 min at 72 °C, followed by 20 cycles of 28 s at 94 °C and 4 min at 67 °C, and a single final cycle of 7 min at 67 °C. Secondary PCR was carried out with an initial cycle of 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 64 °C, and 2 min at 72 °C, and a single final cycle of 7 min at 72 °C. Isolated 5′ flanking regions were sequenced at Medigenomics, and the transcription start sites were determined using the Neural Network Promoter Prediction Program (Reese 2001; http://www.bgdp.org/seq_tools/promoter.html) in conjunction with the untranslated 5′ sequences of PvNADH-GOGAT-I and PvNADH-GOGAT-II cDNAs. The promoter sequences were also explored for identifying cis-acting regulatory elements in them using PLACE (Higo et al. 1999; http://www.dna.affrc.go.jp/PLACE/) as well as PlantCARE databases (Lescot et al. 2002; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Development of plant transformation vectors and agrobacterial strains carrying the binary vectors, and inoculum preparation

The binary vectors pBI-PrPvNADH-GOGAT-I-EGFPUS and pBI-PrPvNADH-GOGAT-II-EGFPUS containing the P. vulgaris NADH-GOGAT promoters transcriptionally coupled to the egfp-gusA coding fusion sequence and the 3′ nos terminator sequence were constructed as follows. Five prime flanking sequences comprising of 1501 bp PvNADH-GOGAT-I and 1366 bp PvNADH-GOGAT-II, upstream of their respective coding sequences were PCR amplified as described above, digested with SalI and XmaI (restriction sites were embedded in the forward and reverse primers used for promoter amplification; see Supplemental Table S3) and cloned upstream of the egfp-gusA coding sequence in a similarly digested pBI101-EGFPUS binary vector yielding pBI-PrPvNADH-GOGAT-I-EGFPUS and pBI-PrPvNADH-GOGAT-II-EGFPUS. The pBI101-EGFPUS vector was constructed by replacing gusA coding sequence in pBI101.1 (Clontech) with egfp-gusA chimeric reporter gene derived from pHGWFS7 (Karimi, Inze & Depicker 2002).

The binary vectors used for the bean transformation, pBI101-EGFPUS (promoterless EGFPUS, control vector), pBI-PrPvNADH-GOGAT-I-EGFPUS or pBI-PrPvNADH-GOGAT-II-EGFPUS, were transformed into Agrobacterium rhizogenes K599 by electroporation (Nagel et al. 1990). A. rhizogenes strains harbouring the transformation vectors were grown at 30 °C in Luria-Bertani (LB) plates supplemented with 50 µg mL−1 kanamycin. The bacterial cells from overnight-grown culture from a single plate were harvested and resuspended in 5 mL sterile distilled water, and used for infecting the bean seedlings for the induction of hairy root formation (see below).

Hairy root transformation and growth conditions

Bean seeds harvested from the greenhouse-grown plants were surface sterilized and germinated in pots containing sterile vermiculite in the plant growth room (maintained at a 14 h light/10 h dark cycle and a temperature of 25 °C) as described earlier (Silvente et al. 2008), and irrigated with the Summerfield nutrient solution (Summerfield, Huxley & Minchin 1977) supplemented with 5 mM KNO3. The hairy root transformation of the bean plants was performed according to Estrada-Navarrete et al. (2006) as modified by Silvente et al. (2008) using A. rhizogenes strains carrying the binary vectors pBI101-EGFPUS (control vector), pBI-PrPvNADH-GOGAT-I-EGFPUS or pBI-PrPvNADH-GOGAT-II-EGFPUS. A. rhizogenes-transformed composite bean plants were grown in pots and inoculated with 1–2 mL R. tropici CIAT899 culture (rhizobial inoculum was prepared by growing CIAT899 strain to a density of 106 cells mL−1 in peptone-yeast extract (PY) medium supplemented with nalidixic acid, 20 µg mL−1), irrigated with the Summerfield nutrient solution (Summerfield et al. 1977) having a highly reduced level (0.385 mM) of KNO3, and incubated in the plant growth chamber maintained at a 14 h light/10 h dark cycle and a temperature of 25 °C. After 4–5 d of acclimatization, the transformed plants were transferred to the naturally lit greenhouse maintained at 25 °C. The plants (five replicates per each treatment) were harvested 3–4 weeks after rhizobial inoculation, and the roots and nodules were analysed for GUS and GFP activity. GUS and GFP analysis revealed no conspicuous plant to plant variations in the pattern or level of expression of the reporter genes.
Histochemical localization of GUS activity and other microscopic methods for the visualization of GFP

GUS staining of the transgenic roots was performed at 30°C for 12–24 h essentially as described by Reddy et al. (1998). After staining, the roots were rinsed in 100 mM phosphate buffer and fixed for more than 4 h in a solution containing 3.7% formaldehyde, 5% acetic acid and 50% ethanol, examined as whole specimens and photographed under a stereomicroscope (Carl Zeiss Sfemi 2000-C; Carl Zeiss Jena GmbH, Jena, Germany).

For the GFP analysis, the roots and nodules were processed essentially according to Estrada-Navarrete et al. (2006). Briefly, the tissues were cross-linked with 100 μM m-maleimidobenzoyl-N-hydroxysuccinimide-ester, fixed with 4% paraformaldehyde, passed through dehydration ethanol-methacrylate series, and finally embedded by polymerization in butyl-methacrylate/methyl-methacrylate (4:1, vol/vol; Sigma Aldrich, St Louis, MO, USA) mixture (Electron Microscopy Sciences, Fort Washington, PA, USA). The embedded tissue was sectioned (3 μm thickness) using ultramicrotome (Leica, Bensheim, Germany), processed to remove methacrylate, mounted with citifluor (Ted Pella, Inc., Redding, CA, USA) and observed for GFP fluorescence using a Zeiss LSM 510 Meta confocal microscope equipped with Axiocord 200M. GFP excitation was obtained at 488 nm using an argon laser and an HFT UV fluorescence was detected with an LP 505-nm emission ent with Axiovert 200M. GFP excitation was obtained at 488 nm using an argon laser and an HFT UV fluorescence was detected with an LP 505-nm emission filter. The images were processed using Adobe Photoshop Elements 2.0 software (Adobe Systems Incorporated, Mountain View, CA, USA).

In situ hybridization

All RNA probes were generated from linearized pBlue-script plasmids containing either the 602 bp \( PfNADH-GOGAT-I \) fragment, the 563 bp \( PfNADH-GOGAT-II \) fragment or the 600 bp \( Pf\text{leghemoglobin} \) fragment. Both \( PfNADH-GOGAT-I \) (between 6695 and 7297 bp) and \( PfNADH-GOGAT-II \) (between 6419 and 6962 bp) fragments (probes) were derived from the non-homologous 3′ UTR and C terminal coding regions of the full-length cDNA clones. The plasmids harbouring \( PfNADH-GOGAT-I \), \( PfNADH-GOGAT-II \) or \( Pf\text{leghemoglobin} \) fragments were linearized with the appropriate restriction enzymes and transcribed in the sense and antisense directions using the T3 or T7 RNA polymerase and radio labelled with \(^3S\) UTP nucleotide. The hydrolysis of the transcribed probes and the subsequent in situ hybridization protocols have been described by Trepp et al. (1999b).

The sequence data from this paper have been deposited at the Genbank under the accession numbers \( PfNADH-GOGAT-I \) cDNA, AF314925; \( PfNADH-GOGAT-II \) cDNA, AF314924; \( PfNADH-GOGAT-I \) promoter, AB195997; and \( PfNADH-GOGAT-II \) promoter, AB195998.

RESULTS

Isolation and characterization of two bean cDNAs encoding NADH-GOGAT

In order to isolate NADH-GOGAT homologs from the bean (P. vulgaris), we first screened a 15-day-old root nodule cDNA library by hybridization with a 1.6 kb cDNA probe derived from M. sativa NADH-GOGAT. Consequently, two clones containing bean cDNA inserts of 2.5 kb \( (PfNADH-GOGAT-I) \) and 3 kb \( (PfNADH-GOGAT-II) \) were isolated. The sequencing of both cDNA clones revealed an open reading frame (ORF) encoding a partial polypeptide similar to MsNADH-GOGAT, with different 3′ UTR sequences between them. The detection of two cDNAs of \( PfNADH-GOGAT \) with different 3′ UTR sequences suggested the presence of two \( PfNADH-GOGAT \) genes in the bean. RNA gel blot analysis utilizing specific \( PfNADH-GOGAT \) clones as probes revealed 7 kb mRNAs as expected for proteins with a molecular mass higher than 200 kD. In order to obtain longer \( PfNADH-GOGAT \) cDNA clones, 2.5 and 3 kb fragments of \( PfNADH-GOGAT-I \) and \( PfNADH-GOGAT-II \), respectively, were used as hybridization probes to screen a second bean nodule cDNA library, which was prepared with size-fractionated (3–9 kb) nodule cDNA. From the second screening, two longer clones of 5 kb each corresponding to \( PfNADH-GOGAT-I \) and \( PfNADH-GOGAT-II \) were isolated. Full-length \( PfNADH-GOGAT-I \) (7406 bp) and \( PfNADH-GOGAT-II \) (7050 bp) cDNA clones were subsequently obtained by ‘cDNA library walking’ with nested amplification by PCR using the bean root nodule library as template (see material and methods). Sequence analysis of different sub-clones, as well as of RT-PCR derived full-length cDNA clones, confirmed the existence of two different \( PfNADH-GOGAT \) genes in bean.

The sequence of the \( PfNADH-GOGAT-I \) cDNA (7406 bp) showed that it contains a 435 bp 5′ UTR with several translation stop codons in all three reading frames before the first ATG, and revealed an ORF 6591 bp long, starting at residue 436. A 367 bp long 3′ UTR and a 13 bp polyA∗ were also defined. \( PfNADH-GOGAT-I \) ORF codes for a 2196 amino acid polypeptide with a predicted molecular mass of 241 kD and a theoretical pI of 6.1 (Fig. 1). \( PfNADH-GOGAT-I \) clone is 7050 bp long with a 5′ UTR of 189 bp with several translational stop codons in all three reading frames before the first ATG codon start site. The \( PfNADH-GOGAT-II \) clone showed a single ORF of 6582 bp from 190 to 6771 bp, a 3′ UTR 264 bp long and 15 bp polyA∗. Putatively, \( PfNADH-GOGAT-II \) codes for a 2192 amino acid polypeptide with a predicted molecular mass of 241 kD and a pI of 6.18 (Fig. 1). Complete coding regions of both clones showed 81% identity at the nucleotide level and 86% at the amino acid level. It is important to note that the theoretical pI predicted for the \( PfNADH-GOGAT \) deduced proteins of the present study closely correlated with the pI 6.069 and 6.159, respectively, reported for \( PfNADH-GOGAT-I \) and -II earlier (Chen & Cullimore 1988).
A BLAST search of the protein databases with the deduced amino acid sequences of \textit{PvNADH-GOGAT-I} and \textit{PvNADH-GOGAT-II} showed an identity of 85 and 83\%, respectively, with the \textit{MsNADH-GOGAT} (Fig. 1). Additionally, \textit{PvNADH-GOGAT-I} and -II polypeptides showed an identity of 78\% with \textit{A. thaliana} and 74\% with \textit{O. sativa} NADH-GOGATs. \textit{PvNADH-GOGAT-I} and -II, at amino acid level, showed an identity ranging from 39 to 46\% with Fd-GOGAT from \textit{A. thaliana} (GLU1 and GLU2), \textit{Spinacia oleracea}, \textit{Zea mays}, \textit{O. sativa} and \textit{Glycine max}.

**Functional domain analysis**

Deduced amino acid sequences indicated that each of the \textit{PvNADH-GOGAT-I} and \textit{PvNADH-GOGAT-II} contain a pre-sequence of 98 and 96 amino acids, respectively, resembling that of \textit{MsNADH-GOGAT} (Gregerson \textit{et al} 1993). The analysis using Plant-Ploc and WoLF PSORT programmes suggested a plastid targeting sequence for both \textit{PvNADH-GOGAT-I} and \textit{PvNADH-GOGAT-II}. It is extraordinary that these pre-sequences are rich in Ser and Thr residues, but reduced in Asp, Glu and Tyr content, which is characteristic of the nuclear-encoded plastid precursor protein (Karlin-Neumann & Tobin 1986; von Heijne, Steppuhn & Herrmann 1989).

In addition, the examination of the predicted polypeptide sequences of the bean NADH-GOGAT isoforms revealed three main domains that are putatively responsible for various enzymatic functions as delineated in NADH-GOGATs of alfalfa and other plants: The first domain is concerned with carrying out the glutamine amidotransferase reaction (Figs 1 & 2). Interestingly, within this domain, \textit{PvNADH-GOGAT-I} exhibited a 26-amino acid sequence, which is different from both \textit{PvNADH-GOGAT-II} and \textit{MsNADH-GOGAT} (designated in bold letters in Fig. 1 and black square in Fig. 2). The second domain (underlined in Fig. 1) is involved in the binding of FMN, and located in close proximity to three cysteine residues (indicated by diamonds in Fig. 1) putatively implicated as a binding site for [3Fe-4S] cluster. FMN and [3Fe-4S] carry out the electron transport to the intermediate 2-iminoglutарате to form glutamate from 2-oxoglutarate. The third domain is an NADH-binding domain (double underlined in Fig. 1) and contains five conserved residues (identified by arrowheads in Fig. 1) that are important for the binding of the cofactor (Gregerson \textit{et al} 1993).

**Genomic organization of NADH-GOGATs in the bean**

In order to confirm if the two \textit{PvNADH-GOGAT} clones represent two different genes and to assess whether there may be other homologs of these two NADH-GOGATs in the bean, genomic DNA Southern blot analysis was performed. As is shown in Fig. 3, a single hybridizing fragment was detected in each of the genomic DNA digestions tested. When a 611 bp probe containing 435 bp of the 5' UTR and 176 bp of the N-terminal coding region was used for the specific detection of \textit{PvNADH-GOGAT-I}, a single hybridization fragment was detected in each of the DNA digestions tested (Fig. 3a). On the other hand, when a specific probe for the detection of \textit{PvNADH-GOGAT-II} (461 bp long fragment containing 189 bp of the 5' UTR and 272 bp of the N-terminal coding region) was used, KpnI and EcoRV digestions generated each a single hybridizing fragment of almost the same size (Fig. 3b). PstI and XbaI digestions also generated each a unique hybridizing fragment of almost the same size but larger than those fragments generated by KpnI and EcoRV digestions (Fig. 3b). These results indicated that \textit{PvNADH-GOGAT-I} and \textit{PvNADH-GOGAT-II} represent two different \textit{NADH-GOGAT} genes and that there is only a single copy of each gene in the \textit{P. vulgaris} genome.

**Expression of NADH-GOGATs in bean tissues**

To determine whether the two \textit{P. vulgaris} NADH-GOGATs are differentially expressed in various plant tissues, RT-PCR analysis was performed using RNA isolated from roots, stem, young cotyledonary leaves, mature leaves, flowers, pods and nodules. As is shown in Fig. 4, transcripts of both \textit{PvNADH-GOGATs} were detected in all tissues analysed. In most tissues, excepting mature leaves, \textit{PvNADH-GOGAT-II} expression was found to be higher than that of \textit{PvNADH-GOGAT-I} (Fig. 4a,b). RT-PCR analysis utilizing mRNA isolated from the nodules harvested from the plants 12–21 d after rhizobial inoculation showed high and nearly constant levels of expression of \textit{PvNADH-GOGAT-II} during all stages of nodule development as compared to \textit{PvNADH-GOGAT-I} (Fig. 4a,b). In contrast, \textit{PvNADH-GOGAT-I} showed a peak in expression only at day 18 following inoculation and a decline thereafter. These results indicated that both \textit{NADH-GOGAT} genes are differentially regulated in various bean tissues including the nodules.

**Relationship between \textit{PvNADH-GOGAT} expression and nitrogen fixation**

As already mentioned, NADH-GOGAT plays an important role in the ammonia assimilation pathway. In order to delineate the extent of the participation of individual \textit{PvNADH-GOGATs} in the assimilation of the ammonia derived from the symbiotically nitrogen-fixing rhizobial cells, we analysed the expression of \textit{PvNADH-GOGAT-I} and -II in \textit{fix+} and \textit{fix−} nodules induced respectively by the CE3 wild type and the NifA− mutant strains of \textit{R. etli}. RT-PCR analysis demonstrated that \textit{PvNADH-GOGAT-I} expression was significantly lowered in non-fixing nodules (Fig. 4a,b). In contrast, the level of \textit{PvNADH-GOGAT-II} expression remained unaffected in \textit{fix−} nodules, until at least 18 dpi, as compared to \textit{fix+} nodules. As these \textit{fix−} nodules...
have a normal morphology and contain bacteroides in the infected cells, this result suggests that the expression of \( \text{PvNADH-GOGAT-I} \), but not of \( \text{PvNADH-GOGAT-II} \), is affected by the nitrogen status of the nodule and not is due by a developmental deficiency.

In an analogous study in \( \text{P. vulgaris} \), working with fix\(^+\) and fix\(^-\) \text{Rhizobium} \) strains, Chen, Bennett & Cullimore (1990) observed changes in the activity of \( \text{NADH-GOGAT-I} \) isoenzyme in the nodules in response to nitrogen fixation, while the induction of \( \text{NADH-GOGAT-II} \) isoenzyme remained unaltered. Based on these findings, it was concluded that unlike \( \text{NADH-GOGAT-I} \), the induction of the \( \text{NADH-GOGAT-II} \) isoenzyme occurs independently of nitrogen fixation, but that nitrogen fixation was clearly required to increase and maintain the magnitude of the induction in \( \text{P. vulgaris} \).

**Regulation of \( \text{PvNADH-GOGAT} \) expression by carbon and nitrogen compounds**

As the nitrogen status of the nodule affected the \( \text{PvNADH-GOGAT-I} \) but not \( \text{PvNADH-GOGAT-II} \) expression, we decided to study the effect of different nitrogen compounds as well as sucrose on the expression of these two \( \text{NADH-GOGATs} \) in nodules. RT-PCR analysis revealed that the transcript levels of \( \text{PvNADH-GOGAT-I} \) increased in the nodules derived from the plants treated with 0.5% sucrose, but no further enhancement was noticed when supplemented with higher sucrose concentrations (Fig. 5a,b). In contrast, the \( \text{PvNADH-GOGAT-II} \) expression increased concomitantly with the increase in sucrose concentration. The quantitative analysis of the sugars revealed that indeed the levels of metabolizable sugars are enhanced in the nodules of the plants supplemented with exogenous sucrose, fructose or glucose (Silvente et al. 2008). These findings, together with the above results, indicate that both \( \text{PvNADH-GOGATs} \) are positively regulated by carbon reserves, thus, implicating carbon availability as a limiting factor in the GS/GOGAT ammonia assimilation pathway in nodules. The presence of different nitrogenous compounds in the nutrient solution did not affect the expression of \( \text{PvNADH-GOGAT-II} \) in the nodules (Fig. 5a,b). However, \( \text{PvNADH-GOGAT-I} \) expression declined in the presence of amides (asparagine and glutamine), and most noticeably with ureides (xanthine and allantoine) and with allopurinol,
which is an inhibitor of ureide synthesis. It is intriguing that both the ureides and the ureide inhibitor, like allopurinol, down regulate the expression of PvNADH-GOGAT-I. The inhibition of PvNADH-GOGAT-I by allopurinol probably suggests that the metabolites upstream of xanthine production (such as purine pathway intermediates) also negatively affect the PvNADH-GOGAT-I expression.

The above results indicate in one hand that both nodule PvNADH-GOGATs are positively regulated by carbon availability and in the other, that nodule PvNADH-GOGAT-I is negatively regulated by metabolic intermediates of the ureide biosynthetic pathway.

Isolation and characterization of PvNADH-GOGAT-I and PvNADH-GOGAT-II promoters

To study how PvNADH-GOGAT-I and PvNADH-GOGAT-II genes are expressed in the roots and nodules, we isolated the promoter regions of these genes from the bean genomic DNA, employing the genome walking strategy, and utilized them to study their activities during root and nodule development in bean hairy root system (see materials and methods). The sequencing revealed that the cloned PvNADH-GOGAT-I and PvNADH-GOGAT-II promoters were 1501 and 1366 bp long, respectively. The determination of the transcriptional start sites in the promoters, employing the Neural Network Promoter Prediction Program (see materials and methods), enabled the precise delineation of a 436 bp long 5’ untranslated region (5’ UTR) in PvNADH-GOGAT-I and a 192 bp long 5’ UTR in PvNADH-GOGAT-II cDNA transcripts (Supplementary Fig. S1). A TATA box (TATATAT) was recognized in the promoter of PvNADH-GOGAT-I at positions −460 to −466 bp upstream of the translational initiation site (+1), whereas in the PvNADH-GOGAT-II promoter, the TATA box (TATAAA) was found to be at positions −215 to −220 bp. Putative CAAT box motifs were located at −930 to −933 bp and −254 to −257 bp, respectively, in the PvNADH-GOGAT-I and PvNADH-GOGAT-II promoters. Thus, the proximal regions of PvNADH-GOGAT-I and PvNADH-GOGAT-II promoters consist of the elements shown to be essential for the initiation of transcription in the promoters of other plants.

Tissue-specific expression patterns of PvNADH-GOGAT-I and PvNADH-GOGAT-II genes during root and nodule development

To elucidate the patterns of expression of PvNADH-GOGAT-I and PvNADH-GOGAT-II genes during root and nodule development, we have utilized PvNADH-GOGAT promoters fused to the egfp-gusA chimeric reporter gene (Fig. 6 top panel) and produced compound bean plants with transgenic hairy roots through A. rhizogenes-mediated transformation (see materials and methods). The hairy roots of the plants transformed with the control vector (promoterless egfp-gusA vector) served as the control. The transgenic hairy roots from the symbiotically grown control plants did not exhibit any GUS activity or GFP fluorescence...
On the other hand, the histochemical staining of the roots transformed with both GOGAT promoters revealed relatively a strong GUS activity in the nodule primordia (Fig. 6c,d,m) as well as in the mature nodules (Fig. 6e,f,n,o). A detailed examination with confocal microscopy, as revealed by EGFP expression, established that the activity of both promoters was mainly restricted to the infected cortical cells in the central zone of the nodules, although more intensely with the \textit{PvNADH-GOGAT-II} promoter than the \textit{PvNADH-GOGAT-I} promoter (Fig. 6h,i,q,r). In contrast to the infected cortical cells, the uninfected cells virtually showed no EGFP expression. The outer cortical cells of the nodules, on the other hand, exhibited a low EGFP induction with both the promoters; here again in the outer cortical cells the expression with \textit{PrPvNADH-GOGAT-I} being less intense than \textit{PrPvNADH-GOGAT-II}.

In order to further verify the sites of expression of both \textit{PvNADH-GOGAT-I} and \textit{PvNADH-GOGAT-II} promoters, we performed \textit{in situ} hybridization studies. For this, longitudinal sections of paraffin-embedded \textit{P. vulgaris} mature root nodule tissues were hybridized with sense or antisense $^{35}$S UTP-labelled RNA probes (Fig. 7) for either \textit{PvNADH-GOGAT-I} or -II. Results showed that both \textit{PvNADH-GOGAT-I} and -II are expressed within the central tissue of the bean root nodule (Fig. 7a,d). A higher magnification of this region demonstrated that \textit{PvNADH-GOGAT-I} and -II mRNAs are mostly localized within the infected cells (Fig. 7j,k). As expected, the nodule sections probed with the \textit{Pv-leghemoglobin} transcript, used as a positive control, showed a high expression in the central tissue of the nodule (Fig. 7g), specifically, the infected cells. Thus, the \textit{in situ} hybridization results have provided additional confirmation showing the infected cells as the major sites of expression of both \textit{NADH-GOGAT} genes, as evidenced with the expression of respective promoters in the bean nodules (see above; Fig. 6).

### In silico identification of the potential \textit{cis}-acting elements that regulate tissue-specific, and sugar and amino acid responsive expression of the \textit{NADH-GOGAT} promoters

Since \textit{PvNADH-GOGAT-I} and \textit{PvNADH-GOGAT-II} exhibited differential expression in root tissues (Fig. 6), we performed in silico analysis to assess whether any differences could be deciphered in these promoters with regard to the putative \textit{cis}-elements that were shown to mediate tissue-specific expression in other plants. PLACE and PlantCARE database searches revealed the presence of a large array of putative \textit{cis}-acting elements in both \textit{PvNADH-GOGAT-I} (~95 motifs) and \textit{PvNADH-GOGAT-II} (~70 motifs) promoters that potentially participate in the regulation of gene expression in response to various cellular and environmental cues. In both \textit{PvNADH-GOGAT} promoters, we found \textit{cis}-acting elements that were shown to mediate tissue-specific expression in various other plants (Fig. 8a–c). Even though

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**Figure 5.** (a) RT-PCR analyses of bean \textit{PvNADH-dependent glutamate synthase (NADH-GOGAT)}-I and -II mRNA levels in the nodules of the bean plants treated with different carbon and nitrogen compounds. Bean aquaporin expression is used as an internal reference. Nodules nursed with (left panel) 0.5, 1.0 or 2.0% sucrose; and (right panel) 10 mM asparagine (Asn), glutamine (Gln), xanthine (Xan), allantoin (Alla) or 2 mM allopurinol (Allop). C, untreated nodules served as control. (b) Histogram depicting the ratios of Xan, allantoin (Alla) or 2 mM allopurinol (Allop). C, untreated panel) 10 mM asparagine (Asn), glutamine (Gln), xanthine (Xan), allantoin (Alla) or 2 mM allopurinol (Allop). C, untreated nodules served as control. Nodules treated with different carbon and nitrogen compounds. Bean root (Fig. 6a–f) as compared to the roots carrying the \textit{PvNADH-GOGAT-I} promoter (Fig. 6h,i,q,r). In contrast to the infected cortical cells, the uninfected cells virtually showed no EGFP expression. The outer cortical cells of the nodules, on the other hand, exhibited a low EGFP induction with both the promoters; here again in the outer cortical cells the expression with \textit{PrPvNADH-GOGAT-I} being less intense than \textit{PrPvNADH-GOGAT-II}.
both promoters harbour ed meristic and vascular tissue-specific regulatory motifs, the \( \text{PvNADH-GOGAT-I} \) promoter contained more of them, both in terms of number and diversity, than \( \text{PrPvNADH-GOGAT-II} \) (Fig. 8a,b). In contrast, \( \text{PvNADH-GOGAT-II} \) contained a higher concentration of nodule-specific cis-elements in its promoter (Fig. 6c). In both promoters, we have also located several sugar responsive plant cis-acting elements including those responsible for sucrose-inducible expression (Fig. 8d). cis-Elements that regulate sugar repression were also detected in both promoters, though more of such motifs were found in \( \text{PrPvNADH-GOGAT-I} \) than in \( \text{PrPvNADH-GOGAT-II} \) (Fig. 8e). In addition, both promoters harboured consensus sequences of cis-elements that resembled amino acid active motifs encountered in mammalian asparagine synthetase and \( \text{CHOP} \) (homolog of CAAAT/enhancer-binding protein) gene promoters (Guerrini et al. 1993; Bruhat et al. 2000, 2002; Barbosa-Tessmann et al. 2000; Fig. 8f). Consensus sequences of the conserved cis-elements located in both \( \text{NADH-GOGAT} \) promoters that potentially mediate tissue-specific, and sugar and amino acid responsive expression, together with relevant references, are included in Fig. 8 and its legend. In addition to the above mentioned cis-elements, both the promoters also exhibited stretches of AT-rich regions and numerous perfect direct and inverted repeats of varying lengths up to 14 bp (not shown). Future work can only decipher the functional significance of these repeat motifs.

**DISCUSSION**

Legume plants can obtain their nitrogen from atmospheric \( \text{N}_2 \) through symbiotic association with rhizobia. Ammonia, which is fixed by rhizobia, is assimilated in the plant via the joint action of GS and GOGAT, which convert glutamine and 2-oxoglutarate into glutamate. These two reactions are now generally agreed to be the primary route of nitrogen assimilation in plants, and the products glutamine and glutamate, donate their amido and amino groups for the synthesis of most of the nitrogen-containing compounds of the cell. One interesting point is that, in non-nodulated bean plants, the assimilated ammonia is mobilized in the form of amides, but when bean plants are symbiotically fixing nitrogen, ureides are the main nitrogen-containing compounds involved in the transport of the nitrogenase-derived ammonia to the rest of the plant (Atkins 1982). To complement our understanding of the ammonia assimilation process in bean nodules, we isolated and characterized two \( \text{NADH-GOGAT} \) cDNAs, studied their expression in different plant tissues and assessed their regulation by carbon and nitrogen compounds in the root nodules. This research extends our understanding of legume root nodule nitrogen metabolism and \( \text{NADH-GOGAT} \) by: (1) demonstrating that \( \text{NADH-GOGAT} \) in bean is encoded by two separate genes having distinct patterns of expression; (2) the expression of the \( \text{PvNADH-GOGAT-I} \) is reduced by products of nitrogen fixation; (3) in the 5′-upstream region of both \( \text{PvNADH-GOGAT} \) genes selected cis-elements are conserved between the two; (4) the 5′-upstream region of both GOGAT genes drives reporter gene expression to infected cells of root nodules; and (5) effective nitrogen fixation is not required for the expression of either \( \text{PvNADH-GOGAT} \) genes, suggesting that the regulation of the \( \text{NADH-GOGAT} \) genes in bean nodules may be under developmental control.

Two distinct clones of 7406 bp (\( \text{PvNADH-GOGAT-I} \)) and 7050 bp (\( \text{PvNADH-GOGAT-II} \)) cDNAs capable of encoding a 241 kD protein each, were isolated and characterized. Both clones presented, at the amino acid level, an identity ranging from 74 to 85% with other \( \text{NADH-GOGAT} \) enzymes and a similarity lower than 46% with Fd-GOGAT enzymes already characterized from different plants. The analysis of the deduced amino acid sequence of each of the \( \text{PvNADH-GOGATs} \) showed an amido transferase domain between amino acids 96 and 454 (Figs 1 & 2). In addition, three regions involved in cofactor binding are also detected; namely, the sequences similar to those implicated in FMN binding (between the residues 1180 and 1253) (Fig. 1, underlined), which are adjacent to three conserved cysteine residues (1251, 1257 and 1262) (Fig. 1, diamonds) that are involved in binding the [3Fe-4S] cluster (Kauff et al. 1991; Sakakibara et al. 1991; Vanoni & Curti 2005), and a third domain containing five highly conserved amino acids (Fig. 1, arrowheads), which are critical for \( \text{NADH-GOGAT} \) binding (Scrutton, Berry & Perham 1990; Suzuki & Kauff 2005). These regions in \( \text{PvNADH-GOGATs} \) are very similar and almost in the same positions as that described in MsNADH-GOGAT (Gregerson et al. 1993). Further, the amino acid sequence also exhibited a region (residues 1–98) encoding a transit peptide that predicts a plastid localization for both \( \text{PvNADH-GOGATs} \). These data are in
Figure 7. Localization of \textit{Pv}NADH-dependent glutamate synthase (NADH-GOGAT)-I and II transcripts in \textit{Phaseolus vulgaris} root nodules by \textit{in situ} hybridization. Longitudinal sections of paraaffin embedded \textit{P. vulgaris} mature root nodules probed with sense and antisense $^{35}$S UTP-labelled RNA probes. The signal specific to the radio-labelled probe is seen as white silver grains when tissue is viewed under dark field optics and as dark particles when viewed under bright field. (a and b) Nodule sections probed with \textit{Pv}NADH-GOGAT-I radio-labelled antisense (a) and sense (b) riboprobe. (d and e) Nodule sections probed with \textit{Pv}NADH-GOGAT-II radio-labelled antisense (d) and sense (e) riboprobe. (g and h) Nodule sections probed with \textit{Pv}-leghemoglobin radio-labelled antisense (g) and sense (h) riboprobe. Panels (c, f and i) are bright field views of (a, d and g) to show nodule anatomy. (j and k) High magnification of portions of the panels (a) and (d), respectively, that show signal (dark grains) located predominately in the infected cells of the central tissue. Magnification for (a–i) = 40$\times$, (j and k) = 400$\times$. Bar length equals 0.5 mm (a–i), 0.05 mm (j and k). ct, central tissue; oc, outer cortex; in, infected cell; un, uninfected cell.
concordance with the previous results, which indicated that in bean nodules, the two NADH-GOGAT isoenzymes are localized in the plastids (Chen & Cullimore 1989). In contrast to the above, however, the amino acid analysis also revealed a region that distinguishes \( P. v. GOGATs \) from each other. A sequence comprising of 26 amino acids in the amido transferase domain of \( P. v. GOGAT-I \) is found to be completely different from the corresponding sequence in \( P. v. GOGAT-II \) (Fig. 2, designated in bold letters). This dissimilarity in the amino acid composition at the amido transferase domain between both \( P. v. GOGATs \) could explain the different affinities for L-glutamine between the two NADH-GOGAT isoenzymes reported by Chen & Cullimore (1988).

The data presented here demonstrate that contrary to what has been reported for other plants, in \( P. v. GOGATs \) is encoded by two different genes, and strongly suggest that these genes encode the two NADH-GOGAT isoenzymes reported by Chen & Cullimore (1988). The fact that the \( P. v. GOGAT-II \), as compared to \( P. v. GOGAT-I \), is strongly expressed in nodules (present study; Figs 4a,b & 6i, a,r) and the NADH-GOGAT-II isoenzyme, in contrast to NADH-GOGAT-I isoform, accounted for most of the nodule GOGAT activity (Chen & Cullimore 1988), will allow us to suggest that the \( P. v. GOGAT-II \) gene most likely encodes the NADH-GOGAT-II isoenzyme, while the \( P. v. GOGAT-I \) gene code for the NADH-GOGAT-I isoenzyme. This conclusion gains further support from the finding that the expression of \( NADH-GOGAT-I \), but not \( NADH-GOGAT-II \), is affected by nitrogen fixation in the nodules (Fig. 4a,b), as was similarly reported for NADH-GOGAT-I and II isoenzyme activities in response to nitrogen fixation (Chen et al. 1990).

The interaction between cis-regulatory elements present in a promoter region and transacting factors is an indispensable requirement for regulating gene expression. Specific cis-regulatory elements in the promoter are probably shared by the genes that are responsive to similar cellular cues/factors. In silico analysis against the plant regulatory element databases revealed the presence of cis sequences potentially responsible for organ-/tissue-specific, and sugar responsive expression in the promoters of both \( P. v. GOGAT-I \) and -II (Fig. 8). The analysis showed that even though both promoters contained meristematic and vascular tissue-specific regulatory motifs, \( P. v. GOGAT-I \) promoter harboured more of such cis-elements, both in terms of number and diversity, than \( P. v. GOGAT-II \) (Fig. 8a,b). In contrast, \( P. v. GOGAT-II \) contained higher concentration of nodule-specific cis-elements in its promoter (Fig. 8c). In consonance with these observations, we found a relatively higher activity of \( P. v. GOGAT-I \) promoter in the meristematic tissues (particularly lateral root primordia), as well as in the vascular traces (Fig. 6a–d), while \( P. v. GOGAT-II \) promoter exhibited greater activity in the nodules (Fig. 6q,r). In both promoters, we have also located several sugar responsive plant cis-acting elements (Fig. 8d,e) which perhaps participate in the sucrose-mediated regulation of the expression of both these NADH-GOGAT genes as well (Fig. 5a). RT-PCR analyses showed that the \( P. v. GOGAT-I \) expression, but not that of \( P. v. GOGAT-II \), is regulated by glutamine and asparagine (Fig. 5b). Searches of plant regulatory element databases did not present any records of cis-elements that mediate amino acid responsive expression of plant promoters. On the other hand, the examination of the promoter sequences of both \( P. v. GOGAT \) genes against animal genome sequences revealed the presence of consensus sequences resembling amino acid responsive cis-elements encountered in mammalian asparagine synthetase and \( CHOP \) gene promoters (Fig. 8f). In the mammalian asparagine synthetase gene, the palindromic sequence CATGATG localized in the proximal portion of the promoter region was found to be necessary for the regulation by amino acid availability (Guerrini et al. 1993). In our studies, the \( P. v. GOGAT-I \) promoter was also found to carry the palindromic sequence CATGATG in its proximal region. Future work dealing with deletion and mutational analyses of the \( NADH-GOGAT \) promoters can only clarify the precise significance and function of the above described potential cis-acting elements in the regulation of tissue-specific, and sugar and amino acid responsive expression of these genes in bean roots.

As compared to \( P. v. GOGAT-II \), relatively low expression of \( P. v. GOGAT-I \) in nodules (Figs 4 & 6i) and its down regulation in the presence of amides and ureides (Fig. 5b) strongly suggest that this NADH-GOGAT isofom may not be playing a main role in the ammonia assimilation process in bean plant, which is a ureide transporter (Atkins 1982). However, a moderate enhancement in the expression of \( P. v. GOGAT-I \) in nodules at day 18 (Fig. 4) probably implies that this isofom may be performing a supportive role in ammonia assimilation during the active phase of nitrogen fixation. In contrast, high levels of expression of \( P. v. GOGAT-II \) in nodules (Fig. 4), particularly in the nitrogen-fixing infected cells (Figs 6r & 7d), and the maintenance of elevated levels of expression even in the presence of excessive concentrations of amides and ureides (Fig. 5b) point to the possibility that \( P. v. GOGAT-II \) may be the main isofom that mediates ammonia assimilation. The differential susceptibility for expression of these two NADH-GOGATs to various nitrogen compounds could be an adaptative mechanism allowing the \( N_2 \)-fixing bean plants to mobilize ammonia, derived from nitrogenase activity, efficiently in the form of ureides (Atkins 1982) instead of amides.

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Figure 8. Diagram depicting the occurrence and location of various putative motifs within \textit{PvNADH-dependent glutamate synthase (NADH-GOGAT)}-I and -II promoters that mediate organ-/tissue-specific, and sugar and amino acid responsive expression. Consensus sequences for (a) meristem-specific expression: CCAGGTGG (Kosugi, Suzuka & Ohashi 1995), GCCACT, CGCTCC (Mehsi, Taoka & Iwabuchi 2000), TTAATGG (Kamiya et al. 2003), ACGTCA (Terada et al. 1995) and KGTC CCCAT (Kosugi et al. 1995; Kosugi & Ohashi 1997; Klinedinst et al. 2000); (b) vascular expression: GTTAGTTC (Patzlaff et al. 2003), AGCGGG (Lacombe et al. 2000), CAGAA GATA (Yin et al. 1997) and TAGTTGG (Elliott & Shirsat 1998; Nontachaiyapoom et al. 2007); (c) nodule-specific expression: AAT AA, AAAAGAT, CTCTT and CACCC (Sandal, Bojsen & Marcker 1987; Stougaard et al. 2004; Fehlberg et al. 2005); (d) sugar-inducible expression: CTAAAC (Zourelidou et al. 2002), TGACT (Sun et al. 2003), TATCCA (Lu, Lim & Yu 1998; Lu et al. 2002), CAT AAT (Li et al. 2006), GATGGAGT, CATGACC, CATGC CCTC, SAKGCRKG (Geisler, Kleczkowski & Karpinski (2006), AATAGAAA (Sun et al. 2003), AATACTAAT (Grierson et al. 1994), TGGACGG and TGGACCA (Ma eo et al. 2001; Morikami et al. 2005); (e) sugar repression:CACG TG (Menkens, Schindler & Cashmore 1995; Urwin & Jenkins 1997), TACGTA (Toyofuku, Umemura & Yamaguchi 1998), CACCG (Niu, Helentjaris & Bate 2002), TAACAAA, CCTTTT (Morita et al. 2003), AATAA, AAAGAT, CTCTT and CACCC (Sandal, Bojsen & Marcker 1987; Stougaard et al. 2004; Fehlberg et al. 2005); (f) amino acid responsive expression: CATGATG (Guerrini L., Gong S.S., Mangasarian K. & Basilico C. 1993), ATKGCA TCA (B r uh a t et al. 2000) and TGATGAA AC (Barbosa-Tessmann et al. 2000; Bruhat et al. 2002). Numbers following the motif sequences depict the degree of conservation of the cis-elements (1.00 depicts the perfect match). Actual cis-element sequences, together with the highest degree of conservation of a given motif, are represented on the right side of the panels.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:
Figure S1. Sequences of the *Phaseolus vulgaris* NADH-dependent glutamate synthase (*NADH-GOGAT*-I and *NADH-GOGAT*-II) 5′ flanking regions. The upstream 1501 bp and 1366 bp flanking regions of the *NADH-GOGAT*-I and *NADH-GOGAT*-II genes, respectively, from the putative translation start codons are shown. TATA box is highlighted in red, and CAAT boxes in green, and predicted transcription start site is shaded in blue. Uppercase letters denoted in black correspond to the 1065 bp and 1174 bp regions of the putative *NADH-GOGAT*-I and *NADH-GOGAT*-II promoters, whereas the letters indicated in blue correspond to the 436 bp and 192 bp 5′ untranslated regions of the respective cDNAs. The first nucleotide upstream of the *NADH-GOGAT*-I and *NADH-GOGAT*-II ORFs is labelled as −1.

Table S1. Profiles of PCR cycles employed for isolating full-length *PvNADH*-dependent glutamate synthase (*NADH-GOGAT*)-I and -II cDNAs, and RT-PCR.

Table S2. Primers used for obtaining the 5′ regions of *PvNADH*-dependent glutamate synthase (*NADH-GOGAT*)-I and -II full-length cDNAs, and RT-PCR analysis.

Table S3. PCR primers used for genome walking of the 5′ flanking region and amplification of the full-length promoters of *PvNADH*-dependent glutamate synthase (*NADH-GOGAT*)-I and -II genes.

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