Journal of Plant Biology, December 2004, 47(4) : 401-406 **SHORT COMMUNICATION**

Molecular Cloning of a cDNA Encoding Glutamine Synthetase from Root Nodules of *Elaeagnus umbellata*

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We analyzed a cDNA clone encoding cytosolic glutamine synthetase, *EuNOD-GS1*, isolated from a root nodule cDNA library of *Elaeagnus umbellata*. This clone has an insert size of 1359 bp and encodes a protein for 355 amino-acid residues, with a molecular weight of 39.2 kDa. Its expression is slightly higher in the root nodules than in the leaves or uninfected roots. Analysis of the deduced amino acid sequences and phylogeny revealed that *EuNOD-GS1* is clustered with cytosolic GS-α isoenzymes. Therefore, based on this and previous results, we propose that the main physiological role of *EuNOD-GS1* is the assimilation of ammonia from secondary and, in part, primary sources.

Keywords: cDNA, *Elaeagnus umbellata*, *Frankia*, glutamine synthetase, root nodule

Glutamine synthetase (GS; EC 6.3.1.2) is the first enzyme in the main pathway of ammonia assimilation in higher plants. This assimilation occurs primarily through the glutamate synthase cycle, in which GS acts in conjunction with glutamate synthase (Fd- or NADH-GOGAT) to convert ammonium ions and 2-oxoglutarate to glutamate, via glutamine, at the expense of ATP and reducing power (Coruzzi and Last, 2000). Plant GS isoenzymes are located in the cytosol (GS_1) or the chloroplast/plastid (GS_2). Different GS polypeptides are encoded by small multigene families whose members regulate in a tissue-specific and developmental manner (Forde and Cullimore, 1989; Morey et al., 2002).

The glutamine and glutamate produced by the GS/GOGAT cycle can be used to synthesize all other nitrogen-containing compounds, and can also perform as essential precursors for various secondary metabolites in the plant (Gallardo et al., 2003). Ammonium arises from numerous sources, both primary (such as nitrate or ammonium ions in the soil or nitrogen-fixing bacteria in root nodules) and secondary (e.g., from photorespiration in green leaves, protein catabolism, phenylpropanoid and lignin metabolisms, or the de-amination of amino acids in germinating seeds). GS expression has been extensively studied in legumes, especially in their root nodules, where GS plays a central role in the assimilation of ammonium, the product of symbiotic nitrogen fixation.

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Over the past twenty years, molecular genetic and biochemical studies on the ammonia assimilation pathway have focused on legume plants, which form symbiotic relationships with rhizobia and fix atmospheric N_2. However, little research has been performed concerning this pathway in plants that form symbiotic N_2-fixation relationships with *Frankia*, even though those species play crucial roles in forest ecosystems where nitrogen is frequently a limiting factor in tree growth. Several genes involved in ammonia assimilation have been isolated from these woody actinorhizal species (Guan et al., 1996; Mullin and Dobritsa, 1996; Franche et al., 1998; Kim et al., 1999). Their genetic transformation is also now being established (Franche et al., 1998; Smouni et al., 2002). These recent advances in the actinorhizal plant-*Frankia* symbiotic relationships shed light on the genetic modification of nitrogen metabolism as well as the elucidation of functional roles for nodulin genes in those plants.

In this report, we have isolated and characterized a cDNA clone encoding GS from the root nodule of an actinorhizal plant, *E. umbellata*. The *GS* clone, showing enhanced expression in the root nodules, encodes a cytosolic GS_1 isoform and clusters with α-type isoenzymes.

MATERIALS AND METHODS

Bacterial Strain and Plant Material

Nodulation of *E. umbellata* seedlings with *Frankia* strain EuIK1 has been described previously (Kim et al.,

1993). Seedlings that were not inoculated with *Frankia* strain were used in the isolation of total RNA from the leaves and roots. All harvested tissues were frozen in liquid nitrogen and stored at –80°C.

Isolation of Nucleic Acids

Total RNA and genomic DNA were isolated according to the method of Kim and An (1999). RNase-free DNase (Promega, USA) was used to remove genomic DNA contamination in the RNA samples during purification.

cDNA Library Screening, Cloning, and Sequence Analysis

Construction and screening of a root nodule cDNA library was performed according to the technique described by Kim and An (1999). A full-length *GS* cDNA clone was isolated from 1×10^5 recombinant

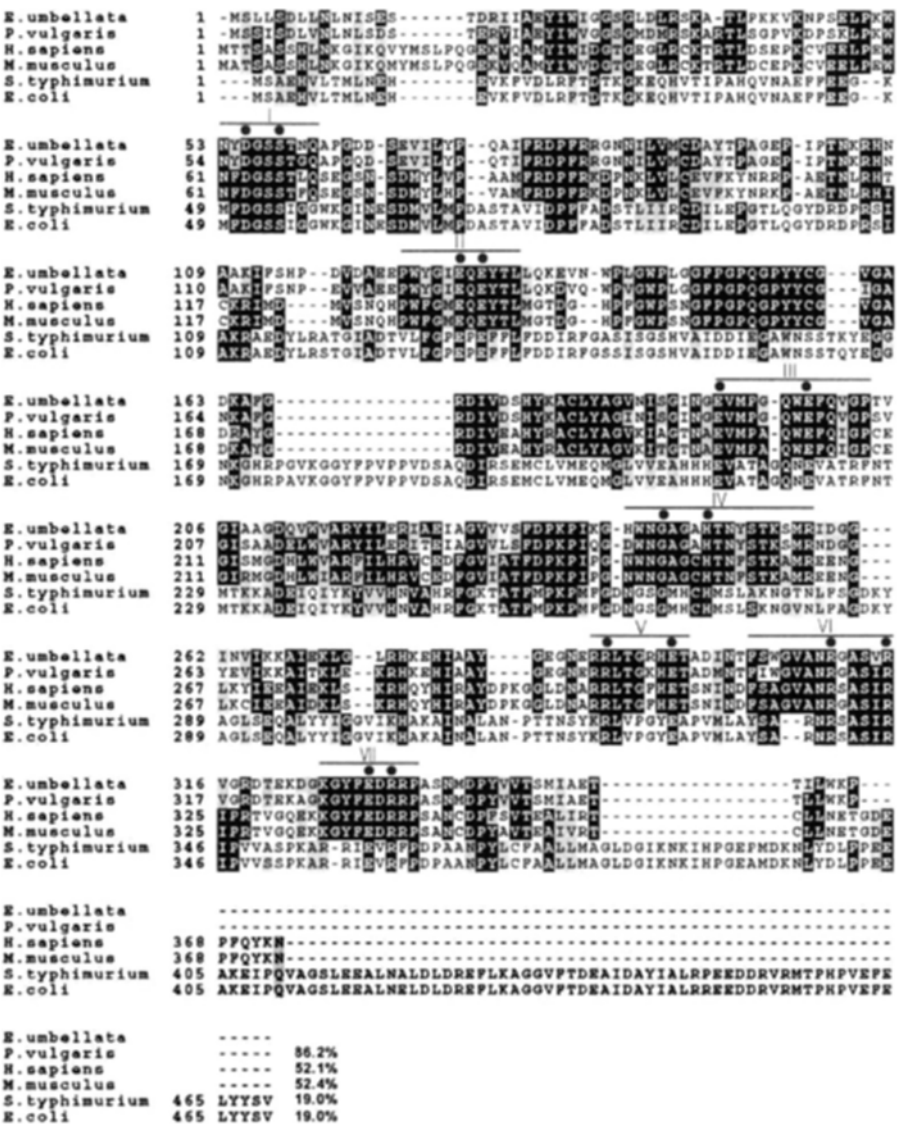

Figure 1. Multiple sequence alignments of glutamine synthetases (GSs) from bacteria, mammals, and plants. Active-site residues (black circles) are completely conserved among different GS species. Sources and GenBank accession numbers used in this alignment are *Escherichia coli* (NP_418306), *Salmonella typhimurium* (AAA27134), *Homo sapiens* (AAH18992), *Mus musculus* (AAH15086), *Phaseolus vulgaris* GSγ1 (CAA32759), and *E. umbellata* GS1 (in this study, AY620818). Percentage values at ends of sequences indicate identities among *E. umbellata* GS_1 and GSs from other organisms.

phages using, as a probe, the partial *GS* cDNA clone that had been obtained via screening by the hybridization-competition method (Kim, 1998). Positive phage clones were changed into phagemid clones according to the manufacturer's *in vivo* excision protocol (Stratagene, USA), and the nucleotide sequences were determined with a BigDye Terminator Cycle Ready Reaction Kit (Applied Biosystems, USA) and an ABI 3730 DNA Analyzer (Applied Biosystems). The sequences were analyzed with the ExPASy Molecular Biology Server (URL http://kr.expasy.org) and the BLAST program (Altschul et al., 1990). A phylogenetic tree was then generated using the PHYLIP program.

RNA and DNA Gel Blot Analyses

RNA and DNA gel blot analyses, using the partial *GS* cDNA clone as a probe, have been described previously (Kim et al., 2003).

RESULTS AND DISCUSSION

We have isolated a partial cDNA clone (insert size of approximately 600 bp) that shares high sequence homology with the previously reported glutamine synthetases (GSs) from a root nodule cDNA library of *E. umbellata* (Kim, 1998). To isolate a full-length cDNA clone corresponding to the partial *GS* clone, we again screened the library using the partial clone as a probe. A full-length cDNA clone, *EuNOD-GS1* (*E. umbellata* Nodule Glutamine Synthetase 1), was isolated from this screening and analyzed to further characterize its molecular nature.

The *GS* cDNA clone is 1359 bp long and contains an open reading frame of 1068 b, with an ATG initiation codon at position 36 and a termination codon at position 1065 (TGA). The 3'-untranslated region is 256 b long and possesses a putative poly(A) signal at position 1187 (AATAA). The deduced peptide contains 355 amino acids, with a calculated molecular mass of 39.2 kDa. No organellar targeting or signal peptide sequences were found, suggesting that EuNOD-GS1 might be a cytosolic GS1 isoenzyme.

EuNOD-GS1 shows high sequence identities (>80%) with cytosolic GS_1 isoforms from various plant species (Fig. 1). The plant cytosolic GS isoenzymes (355 to 357 residues) are significantly smaller than those of either mammals (372 to 373 residues) or bacteria (443 to 478 residues). However, comparisons among these plant, mammalian, and bacterial GS sequences revealed a number of strongly conserved regions

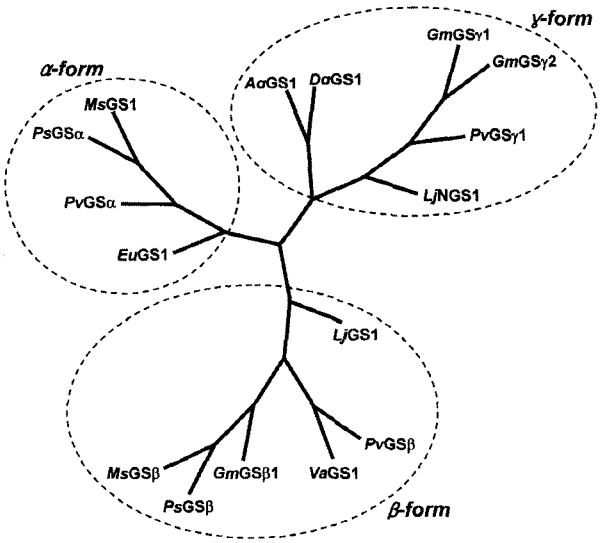

Figure 2. Phylogenetic tree of GS1 genes in legumes and actinorhizal plants. Deduced amino acid sequences were aligned with CLUSTAL W program and a neighbor-joining tree was generated via PHYLIP program. Sources and GenBank accession numbers of GS1 sequences used here are *E. umbellata* (in this study, *Eu*GS1), *Alnus glutinosa* (*Ag*GS1, Y08681), *Datisca glomerata* (*Dg*GS1, AAR29057), *Glycine max* (*Gm*GSβ1, AF301590; *Gm*GSγ1 and γ2, AAC97935 and CAA57346, respectively), *Lotus japonicus* (*Lj*GS1, X94299), *Lotus luteus* (*Ll*NGS1, X71399), *Medicago sativa* (*Ms*GS1, AAB41554; *Ms*GSβ, CAA27570), *Pisum sativum* (*Ps*GSα, P07694; *Ps*GSβ, P08282), *Phaseolus vulgaris* (*Pv*GSα, X04002; *Pv*GSβ, P04770; *Pv*GSγ1, CAA32759), and *Vigna aconitifolia* (*Va*GS1, 2106409A).

(Regions I to VII). These conserved residues play critical roles as active sites, suggesting a similar catalytic mechanism among the different GS species (Eisenberg et al., 2000). In EuNOD-GS1, all these active-site residues are completely conserved (Fig. 1), indicating that *EuNOD-GS1* encodes a functional GS isoenzyme.

Plant GS isoenzymes are classified, by their cellular localization, into two major isoforms (Forde and Cullimore, 1989) -- GS_1, cytosolic form, and GS_2, plastid form. Three distinct genes (*gln*α, *gln*β, and *gln*γ) encode the cytosolic GS_1 isoenzymes, in an organ-specific manner and a pattern of developmental expression (Forde and Cullimore, 1989; Morey et al., 2002). The plastid GS isoforms are encoded by *gln*δ genes (Forde and Cullimore, 1989). To determine which type of GS isoform is clustered with EuNOD-GS1, we generated a phylogenetic tree for GS_1 genes from leguminous and actinorhizal species, i.e., plants that form symbiotic N_2-fixations with rhizobia and *Frankia*, respectively. Our analysis demonstrated that EuNOD-GS1 is grouped with GS-α isoforms (Fig. 2).

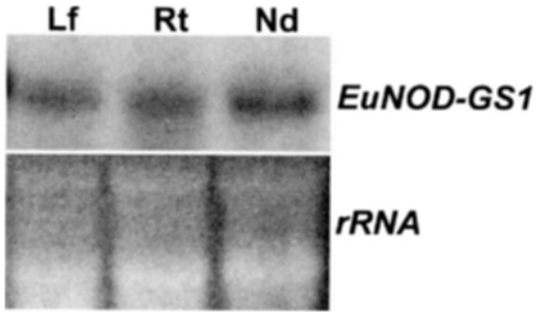

Figure 3. Expression pattern of *EuNOD-GS1* gene from different organs. Lf, leaves; Rt, uninoculated roots; Nd, root nodules.

Genes that encode GS_1 isoenzymes are highly or specifically expressed in the developing root nodules, where ammonium is assimilated as the product of symbiotic nitrogen fixation (Vance, 1990). Here, the clone encoding EuNOD-GS1 was isolated from a root nodule cDNA library using the hybridization-competition method. We hypothesized that this gene would have a nodule-specific or -enhanced expression pattern. Therefore, we purified total RNA from leaves, uninoculated roots, and root nodules, and subjected these to northern hybridization using the partial *GS* clone as a probe. Transcript levels for *EuNOD-GS1* were slightly higher in the nodules than in the leaves and uninoculated roots (Fig. 3).

To determine the number of *EuNOD-GS1* genes in the *E. umbellata* genome, we performed genomic Southern hybridization under a low-stringency condition, using the partial *GS* cDNA clone as a probe (Fig. 4). Total genomic DNA was digested with three restriction enzymes, *Eco*RI, *Hin*dIII, and *Xba*I, none of which cuts the full length of the cDNA clone. More than five signals were detected in each lane (Fig. 3), a hybridization pattern that suggests EuNOD-GS1 is encoded by a small family of genes, as has been reported in other plant species (Tingey et al., 1987; Peterman and Goodman, 1991; Morey et al., 2002).

In the leaves, the major physiological roles of plastid GS_2 are the assimilation of ammonia reduced from nitrate and the re-assimilation of photorespiratory ammonia. In the roots, cytosolic GS_1 assimilates ammonia, which is generated by the concerted reactions of nitrate and nitrite reductases that use NO_3 derived from the soil (Lam et al., 1996; Lea and Ireland, 1999). In root nodules, the primary function of GS1 is the rapid assimilation of ammonia excreted into the plant cytosol of infected cells by N_2-fixing bacteroids (Atkins, 1987). EuNOD-GS1 is classified as a cytosolic and α-form GS isoenzyme in terms of its

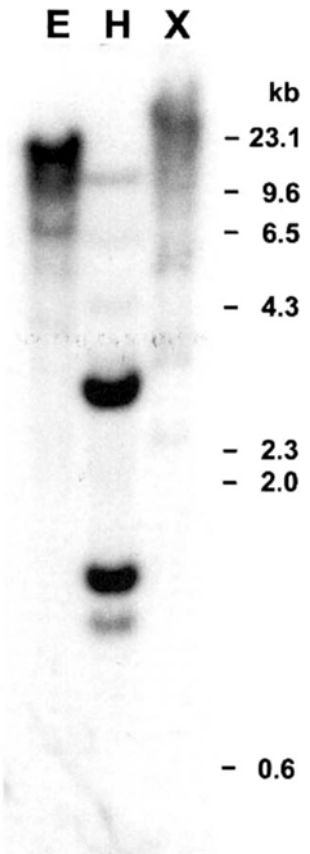

Figure 4. Genomic Southern analysis of *EuNOD-GS1* gene. DNA blot was probed with ^{32}P-labeled partial *GS* cDNA clone and washed under a low-stringency condition. E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

molecular characters, phylogenic analysis, and expression pattern. Previous studies of legume species have shown that transcripts of *gln*α genes are either highly detected in the nodules (Stanford et al., 1993; Temple et al., 1995) or are not (Gebhardt et al., 1986; Morey et al., 2002). The β-forms are found in all plant tissue types, and have an especially high level of expression in N_2-fixing root nodules (Tingey et al., 1987; Roche et al., 1993; Morey et al., 2002). In contrast, the *gln*γ group of genes is highly or specifically expressed only in those nodules (Bennett et al., 1989; Morey et al., 2002).

Based on previous results and these current data, we propose that the primary physiological role of EuNOD-GS1 in the tissues investigated here is the assimilation of ammonia from secondary sources (e.g., protein degradation, de-amination of amino acids, phenylpropanoid pathway). In addition, EuNOD-GS1 may partially function to assimilate ammonia from the primary source, i.e., the product of nitrogen-fixation

by symbiotic *Frankia* in the root nodules of actinorhizal plants, because its expression level was increased during root nodule development (data not shown).

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LITERATURE CITED

Altschul SF, Gish W, Miller W, Meyers EW, Lipman D (1990) Basic local alignment search tool. J Mol Biol 215: 403-410

Atkins CA (1987) Metabolism and translocation of fixed nitrogen in the nodulated legume. Plant Sci 100: 157-169

Bennett MJ, Lightfoot DA, Cullimore JV (1989) cDNA sequence and differential expression of the gene encoding the glutamine synthetase γ polypeptide of *Phaseolus vulgaris* L. Plant Mol Biol 12: 553-565

Coruzzi G, Last R (2000) Amino acids. *In* BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, pp 358-410

Eisenberg D, Gill HS, Pfluegl, GMU, Rotstein SH (2000) Structure-function relationships of glutamine synthetases. Biochim Biophys Acta 1477: 122-145

Forde BG, Cullimore JV (1989) The molecular biology of glutamine synthetase in higher plants. *In* BJ Miflin, HF Miflin, eds, Oxford Surveys of Plant Molecular and Cell-Biology. Oxford University Press, pp 247-296

Franche C, Laplaze L, Duhoux E, Bogusz D (1998) Actinorhizal symbiosis: Recent advances in plant molecular and genetic transformation studies. Crit Rev Plant Sci 17: 1-28

Gallardo F, Fu JM, Jing ZP, Kirby EG, Canovas FM (2003) Genetic modification of amino acid metabolism in woody plants. Plant Physiol Biochem 41: 587-594

Gebhardt C, Oliver JE, Forde BG, Saarelainen R, Miflin BJ (1986) Primary structure and differential expression of glutamine synthetase genes in nodules, roots and leaves of *Phaseolus vulgaris*. EMBO J 5: 1429-1435

Guan C, Ribeiro A, Akkermans ADL, Jing Y, van Kammen A, Bisseling T, Pawloski K (1996) Nitrogen metabolism in actinorhizal nodules of *Alnus glutinosa*: Expression of glutamine synthetase and acetylornithine transaminase. Plant Mol Biol 32: 1177-1184

Kim HB (1998) Structures and expression patterns of cDNA clones encoding asparagine synthetase, chitinase, and polyubiquitin from the root nodule of *Elaeagnus umbellata*. Ph.D. thesis. Seoul National University, Seoul

Kim HB, An CS (1999) Isolation and characterization of a cDNA clone encoding polyubiquitin from the root nodule of *Elaeagnus umbellata*. Can J Bot 77: 1270-1278

Kim HB, Lee SH, An CS (1999) Isolation and characterization of a cDNA clone encoding asparagine synthetase from root nodules of *Elaeagnus umbellata*. Plant Sci 149: 85-94

Kim HB, Oh CJ, Lee H, An CS (2003) A type-I chalcone isomerase mRNA is highly expressed in the root nodules of *Elaeagnus umbellata*. J Plant Biol 46: 263-270

Kim SC, Ku CD, Park MC, Kim CH, Song SD, An CS (1993) Isolation of symbiotic *Frankia* EuIK1 strain from root nodule of *Elaeagnus umbellata*. Kor J Bot 36:177-182

Lam HM, Coschigano KT, Oliveria IC, Melo-Oliveria R, Coruzzi GM (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47: 569-593

Lea PJ, Ireland RJ (1999) Nitrogen metabolism in higher plants, *In* BK Singh, ed, Plant Amino Acids, Biochemistry and Biotechnology. Marcel Dekker, New York, pp 1-47

Morey KJ, Ortega JL, Sengupta-Gopalan C (2002) Cytosolic glutamine synthetase in soybean is encoded by a multigene family, and the members are regulated in an organ-specific and developmental manner. Plant Physiol 128: 182-193

Mullin BC, Dobritsa SV (1996) Molecular analysis of actinorhizal symbiotic systems: Progress to date. Plant Soil 186: 9-20

Peterman TK, Goodman HM (1991) The glutamine synthetase gene family of *Arabidopsis thaliana*. Light-regulation and differential expression in leaves, roots and seeds. Mol Gen Genet 230: 145-154

Roche D, Temple SJ, Sengupta-Gopalan C (1993) Two classes of differentially regulated glutamine synthetase genes are expressed in the soybean nodule: A nodule-specific class and a constitutively expressed class. Plant Mol Biol 22: 971-983

Smouni A, Laplaze L, Bogusz D, Guermache F, Auguy F, Duhoux E, Franche C (2002) The 35S promoter is not constitutively expressed in the transgenic tropical actinorhizal tree *Casuarina glauca*. Funct Plant Biol 29: 649-656

Stanford AC, Larsen K, Barker DG, Cullimore JV (1993) Differential expression within the glutamine synthetase gene family of the model legume *Medicago truncatula*. Plant Physiol 103: 73-81

Temple SJ, Heard J, Ganter G, Dunn K, Sengupta-Gopalan C (1995) Characterization of a nodule-enhanced glutamine-synthetase from alfalfa: Nucleotide-sequence, *in situ* localization, and transcript analysis. Mol Plant-Microbe Interact 8: 218-227

Tingey SV, Walker EL, Coruzzi GM (1987) Glutamine syn-

thetase genes of pea encode distinct polypeptides which are differentially expressed in leaves, roots and nodules. EMBO J 6: 1-9

Vance CP (1990) Symbiotic nitrogen fixation: Recent genetic advances. *In* BJ Miflin, PJ Lea, eds, The Biochemistry of Plants: Intermediary Nitrogen Metablism. Academic Press, New York, pp 43-88