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Organization and Structure of Ferredoxin-Dependent Glutamate Synthase Gene and Intracellular Localization of the Enzyme Protein in Rice Plants

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Abstract

A 5822 bp long cDNA clone encoding the full length ferredoxin-dependent glutamate synthase (Fd-GOGAT; EC 1.4.7.1) protein was isolated from roots of rice (*Oryza sativa* L. cv. Sasanishiki). Its sequence was identical to those of partial cDNAs for Fd-GOGAT from green leaves and shoots of rice. The predicted open reading frame (4848 bp) encodes a 1616 amino acid protein with a molecular mass of 175034 Da that includes a 96-amino acid presequence. The combined nucleotide sequence of genomic clones for Fd-GOGAT isolated from rice was 20899 bp long and contained an entire structural gene, a 5672 bp 5'-upstream region from the first methionine and a 779 bp 3'-downstream region from the stop codon. The predicted transcribed region (15.4 kb) consisted of 33 exons separated by 32 introns. The evolution among diverse GOGAT proteins, expression of Fd-GOGAT gene and intracellular localization of this protein in rice roots are also described.

Accession numbers: AB024716, AB061357.

Key words: Fd-GOGAT gene, Glutamate synthase, immunogold electron microscopy, rice.

Abbreviations

Fd-GOGAT, ferredoxin-dependent glutamate synthase; NADH-GOGAT, NADH-dependent glutamate synthase.

Introduction

The glutamine synthetase (GS; EC 6.3.1.2) / glutamate synthase (GOGAT) cycle is a major pathway in the assimilation of NH_4^+ under normal metabolic conditions in higher plants (Lea *et al.*,

1990; Sechley *et al.*, 1992). GOGAT catalyses the transfer of the amide group of glutamine, formed by the reaction of GS, onto 2-oxoglutarate to yield two molecules of glutamate. One of the glutamate molecules can be utilized as a substrate for the synthesis of glutamine via the GS reaction and the other can be used for further metabolic reactions. At least two molecular species of GOGAT exist in higher plants; one requiring NADH as a reductant (NADH-GOGAT; EC 1.4.1.14) and the other requiring the reduced form of ferredoxin (Fd-GOGAT; EC 1.4.7.1) (Lea *et al.*, 1990; Sechley *et al.*, 1992).

Complete structures of the gene and cDNA for NADH-GOGAT have been reported in root nodules of the legume species alfalfa and in the monocotyledonous species rice (Gregerson *et al.*, 1993; Vance *et al.*, 1995; Goto *et al.*, 1998; Temple *et al.*, 1998; Tobin and Yamaya, 2001). Previous studies suggested that the apparent function of NADH-GOGAT in young leaves and in grains is reutilization of the re-mobilized glutamine, which has been exported via the phloem and xylem from senescencing tissues and roots (Yamaya *et al.*, 1992; Hayakawa *et al.*, 1993; Hayakawa *et al.*, 1994; Tobin and Yamaya, 2001). In specific regions of the epidermis and exodermis in roots the enzyme is also important for the assimilation of exogeneously supplied NH_4^+ ions (Yamaya *et al.*, 1995; Hirose *et al.*, 1997; Ishiyama *et al.*, 1998; Hayakawa *et al.*, 1999; Tobin and Yamaya, 2001). In addition, the recent characterization of a knock-out T-DNA insertion mutant (*glt1*-T) in the single NADH-GOGAT *Glt1* gene in *Arabidopsis* also suggested that NADH-GOGAT has significant roles in non-photorespiratory ammonium assimilation and in glutamate synthesis required for plant development (Lancien *et al.*, 2002).

Fd-GOGAT is the major form of GOGAT in green tissues and is located in the chloroplast stroma (Sechley *et al.*, 1992). Its major role in green leaves is the reassimilation of NH_4^+ released from the photorespiratory pathway, which is supported by analysis of conditional lethal mutants defective in Fd-GOGAT in both *Arabidopsis* and barley (Somerville *et al.*, 1980; Kendall *et al.*, 1986). In roots, Fd-GOGAT is probably localized in plastids and is thought to be involved in the assimilation of NH_4^+ ions formed from the primary assimilation of NO_3^- (Sakakibara *et al.*, 1992; Sechley *et al.*, 1992; Redinbaugh and Campbell, 1993; Tobin and Yamaya, 2001). In dicotyledonous species *Arabidopsis*, two full-length cDNAs encoding Fd-GOGATs (*Glu1* and *Glu2*) have been isolated (Coschigano *et al.*, 1998). The *Glu1* gene, which is linked to the *gls1* locus in chromosome 5 mapped by genetic analysis of photorespiratory mutants that grow normally at elevated concentrations of CO_2 but die in normal atmospheric concentrations of CO_2, is expressed at the highest levels in leaves and accumulation of its transcripts is specifically induced by light and sucrose. By contrast, the *Glu2* gene, mapped on the chromosome 2, is expressed at lower constitutive levels in leaves and preferentially in roots. These results suggested that the major role of *Glu1* gene products is reassimilation of photorespiratory NH_4^+ and also primary assimilation of nitrogen in leaves, whereas the role of *Glu2* products may be primary assimilation of nitrogen in roots. In addition, the observations of gene structures for *Glu1* and *Glu2* in *Arabidopsis* were now available in the database on the computer network. On the other hand, in monocotyledonous plants, only one full-length cDNA encoding Fd-GOGAT has been isolated from leaves of maize (Sakakibara *et al.*, 1991). In contrast to the results of *Arabidopsis*, analysis of genomic DNA in maize indicated the presence of a single-copy gene for this enzyme (Sakakibara *et al.*, 1991). In rice, Fd-GOGAT was located mainly in mesophyll cells of the leaf blades, in chloroplast-containing cross-cells of the pericarp of the grain and also in the apical meristem and the central cylinder of the roots (Hayakawa *et al.*, 1994; Ishiyama *et al.*, 1998). In order to understand more precisely the regulation of gene expression of Fd-GOGAT and physiological functions of this enzyme in the monocotyledonous species rice, we need to isolate the gene encoding this enzyme. Here we report the isolation and characterization of both the entire length cDNA from roots and the gene for Fd-GOGAT in rice plants. We also d escribe the expression of this gene in rice roots. This is the first paper describing the characterization of the gene for Fd-GOGAT and the intracellular localization of the enzyme protein in plant roots.

Materials and Methods

Plant materials

Rice (*Oryza sativa* L. cv. Sasanishiki) seedlings were grown hydroponically in a ventilated growth chamber as described previously (Hayakawa *et al.*, 1993; Yamaya *et al.*, 1995). For the isolation of total RNA and for electron microscopy, the seedlings were first cultivated in tap water adjusted to pH 5.5 with diluted HCl, in the chamber with a 13-h photoperiod giving an average photon flux density of 250 μmol quanta m^{-2} s^{-1} at 25 ℃ for 28 days post imbibition, to fully consume the materials of endosperm. The seedlings were then transferred to a

nutrient solution containing 1 mM NH_4Cl, 1mM $NaNO_3$ or no nitrogen and grown for an additional 24 h. The seedlings were then separated into roots and leaves, weighed, frozen in liquid nitrogen, and stored at -80 ℃ prior to extraction of total RNA. Crown roots were harvested from 10 randomly selected seedlings, and the region from the tips to 10 mm of the roots were cut into 1-mm-thick transverse sections. The sections were used for the electron microscopy preparation. The shoots of rice grown for 5 days in the dark after imbibition were used for the isolation of genomic DNA.

Construction of cDNA library and screening of Fd-GOGAT cDNA clones

Total RNA was extracted by the SDS-phenol/LiCl method, followed by the GTC-CsTFA method and poly(A)$^+$RNA was isolated from total RNA by Oligo(dT)-cellulose chromatography (Yamaya *et al.*, 1995; Hirose *et al.*, 1997). Firstly, a cDNA library was constructed in λgt11 (Stratagene, La Jolla, CA, U.S.A.) from poly(A)$^+$RNA prepared from green mature leaves of rice and the library ($1x10^4$ recombinants) was immunoscreened with affinity-purified rabbit polyclonal IgG raised against Fd-GOGAT purified from rice green leaves (Yamaya *et al.*, 1992). Next, a size-fractionated cDNA library ($8.0x10^5$ independent recombinants) was constructed in λZAPII vector (Stratagene, La Jolla, CA, U.S.A.) using a SuperScript Choice System (Life Technologies, Inc, NY, U.S.A.) and a GigapackIII Gold kit (Stratagene, La Jolla, CA, U.S.A.) from poly(A)$^+$RNA prepared from the 26-day-old roots of rice seedlings. The library ($4.0x10^5$ recombinants) was screened by the plaque hybridization method with the 5'-terminus fragment (1.1 kb) of 4.9 kb long cDNA from rice shoot (EST clone; the accession number S13501), which is homologous with the partial translational region of maize-leaf Fd-GOGAT, as a probe. The EST clone (S13501) was provided by the MAFF DNA Bank (DNA bank at National Institute of Agrobiological Sciences, Ministry of Agriculture, Forestry and Fisheries of Japan).

Construction of genomic DNA library, screening of genomic clones for Fd-GOGAT and isolation of the gene using PCR methods

The genomic DNA was prepared from 5-day-old shoots of rice grown in the dark by the CTAB method (Murray and Thompson, 1980). A genomic library was constructed in λFIXII vector (Stratagene, La Jolla, CA, U.S.A.) from partially digested rice DNA with *Sau*3AI using λFIXII/*Xho*I Partial Fill-in Vector kit (Stratagene, La Jolla, CA, U.S.A.) in accordance with the manufacturer's instructions. The unamplified genomic library ($2.8x10^5$ recombinants) was screened with the 5'-terminus fragment (1.1 kb) of the rice shoot cDNA (S13501) as a probe.

Since genomic DNA clones, isolated from the genomic DNA library, encoded only the upstream region from the exon 22 of the gene, we also used PCR methods to obtain the downstream region from the exon 22 of the gene. Eight oligonucleotide primers were synthesized, which corresponded to the sequence of intron 21 and exon 22 (f1 and f2), or the translational region at the 3'-terminus of the rice Fd-GOGAT cDNA (f3, f4, and r1 to r4). The primer sequences are as follows: f1, 5'-TGC ATT CCC TGG CAC TTA CCA AGA CA-3'; f2, 5'-TGG TGG GAG CAG ATC AAT CCA AAA GC-3'; f3, 5'-GGT GCC ACG ATT TTG AGA GAA TGG GA-3'; f4, 5'-TGC CAC TCT TCT GGC AAC TGG TAC CA-3'; r1, 5'-GGG AAT TGC AGT AGA TCA CTT CGC CG-3'; r2, 5'-TGG CAA GTA CTC GTT CAA ACT CGG CA-3'; r3, 5'-TAC TCG CAG CAA TGA TCT CCA GTG CC-3'; r4, 5'-CCT CAA CCA CTG CTT GAC CAA GGG AG-3'. The primers (f1 to f4) are forward primers and the others (r1 to r4) are reverse primers.

We obtained a 3.3 kb PCR product containing the translational region downstream from the exon 22 of the rice Fd-GOGAT gene. Using the rice genomic DNA as template, we performed PCR with the primers f1 and r1 and LA Taq DNA polymerase (TaKaRa, Tokyo, Japan), as follows: 10 min at 94 ℃ followed by 30 cycles of 30 s at 96 ℃, 2 min at 55 ℃ and 5 min at 72 ℃, and 1 cycle of 10 min at 72 ℃. Using diluted first PCR product as template, we performed the second PCR with the primers f2 and r2, under the same conditions as the first PCR. The PCR product of the second PCR reaction was digested with *Hin*dIII, purified and cloned into pBluescript II SK+ vector (Stratagene, La Jolla, CA, U.S.A.).

PCR products (1.3 kb), containing the downstream portion from the 3'-untranslational region of rice Fd-GOGAT gene were obtained by the inverse PCR method and the cassette PCR method. Rice genomic DNA was digested with *Pst*I and self-ligated. Using the self-ligated genomic DNA as template, we performed inverse PCR with the primers r3 and f4 and LA Taq DNA polymerase, as described above. Using the diluted first inverse PCR product as template, we performed the second PCR with primers r4 and f4 by the same conditions as the first inverse PCR. The cassette PCR was performed using an LA PCR in vitro Cloning kit (TaKaRa, Tokyo, Japan) in accordance with the manufac-

turer's instructions. Rice genomic DNA digested with *Xba*I was ligated with an *Xba*I cassette. Using the rice genomic DNA fragments ligated with the *Xba*I cassette as template, we performed the first cassette PCR with the gene specific forward primer (f3), cassette reverse primer and LA Taq DNA polymerase, under the same conditions as described above. Using the diluted first cassette PCR product as template, we performed second cassette PCR with the gene specific forward primer (f4) and the nested cassette reverse primer, under the same conditions as the first cassette PCR. The PCR products by the inverse PCR and the cassette PCR were purified and cloned into pGEM-T Easy vector (Promega, San Luis Obispo, CA, U.S.A.).

Sequencing and analysis of phylogenetic relationship among GOGAT proteins

The inserts of cDNA and genomic DNA clones were subcloned into pBluescript II SK+, SK-, or KS+ and nested deletion fragment were made with exonuclease III. Sequences were determined by the dideoxy chain-termination method using an automated DNA sequencer (ABI PRISM-377, Applied Biosystems Japan, Tokyo, Japan). The sequence data were analyzed with the DNASIS software system (Hitachi Software Engineering, Tokyo, Japan) and the GENETYX-MAC software system (Software Development, Tokyo, Japan) for nucleotide and deduced amino acid sequences. A BLAST (Basic Local Alignment Search Tool) search was also performed to compare sequences of nucleotides and deduced amino acids for other GOGAT species (Altschul *et al.*, 1990). For analysis of phylogenetic relationship among GOGAT proteins, the tree was calculated by the neighbor-joining method in the CLUSTAL W program (Thompson *et al.*, 1994) and PHYLIP program (Felsenstein, J., PHYLIP (Phylogeny Inference Package) ver. 3.5c., Department of Genetics, University of Washington, Seattle, U.S.A.) on the DDBJ database (DNA Data Bank of Japan, National Institute of Genetics, Mishima, Japan).

Genomic Southern blot and northern blot analyses

For genomic Southern blot analysis, genomic DNA (1 μg) prepared from 5-day-old shoots of rice seedlings was digested with *Eco*RI, *Hin*dIII, *Eco*RV, *Xba*I and *Bgl*II as recommended by the manufacturer (TaKaRa, Tokyo, Japan) and the digested DNA was electrophoresed through a 0.8% agarose gel, denatured, transferred to a nylon membrane and probed with 1316 bp partial cDNA (F2) for rice Fd-GOGAT.

Extraction of total RNA from roots, fully expanded leaf blades and nongreen developing leaf blades of the 28-day-old rice seedlings by the SDS-phenol/LiCl method and northern blot analysis with 430 bp cDNA fragment encoding 3'-terminus translational region for rice Fd-GOGAT as a probe were performed as described previously (Yamaya *et al.*, 1995; Hirose *et al.*, 1997).

Immunogold electron microscopy analysis

The immunogold electron microscopy analysis of Fd-GOGAT protein in crown-root tips (<10 mm) of rice seedlings grown with 1 mM NH_4^+ for 24 h were performed as described previously (Hayakawa *et al.*, 1999). The ultrathin sections on the nickel grids were incubated with affinity-purified anti-Fd-GOGAT IgG (Yamaya *et al.*, 1992), which was diluted 1 : 2, as the primary antibody. Control sections were incubated with the same dilution of anti-Fd-GOGAT IgG that had been preabsorbed with excess amounts of purified Fd-GOGAT protein as described previously (Hayakawa *et al.*, 1994). After the immunolabeling and staining with uranyl acetate and lead citrate, the gold particles were observed using a Hitachi H-8100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

Results and Discussion

Cloning and Characterization of Fd-GOGAT cDNA from Rice

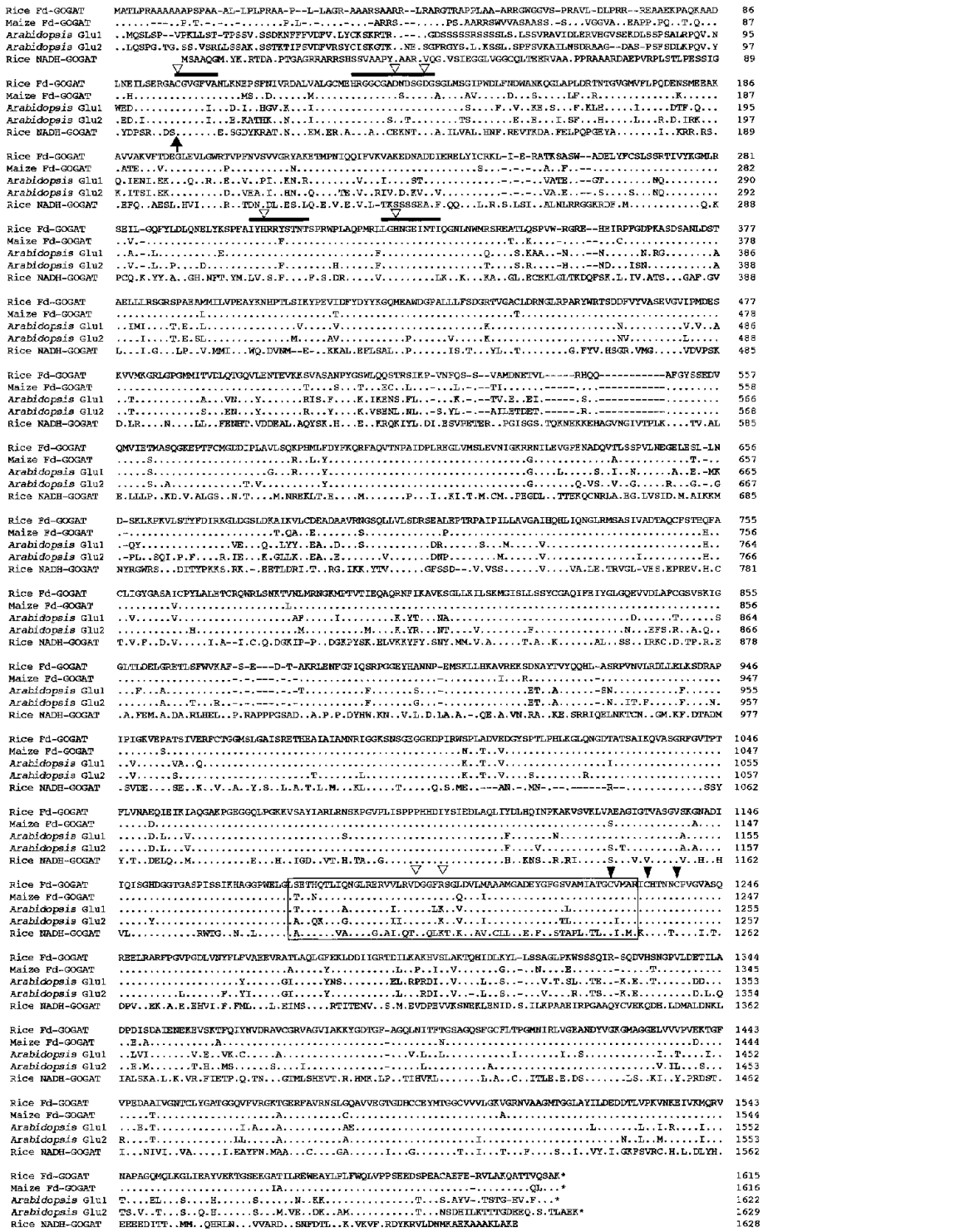
Two immunopositive clones containing inserts of 572 and 1316 bp (named F1 and F2, respectively) were obtained by immunoscreening of rice leaf cDNA library constructed in λgt11 using affinity-purified rabbit polyclonal IgG raised against Fd-GOGAT purified from rice green leaves (Yamaya *et al.*, 1992). Sequence analyses of F1 and F2 showed that they belong to the same class and encode polypeptides similar to the C-terminus region of maize leaf Fd-GOGAT (95% identity for F2) (Sakakibara *et al.*, 1991). Since the apparent molecular mass of Fd-GOGAT in rice leaves and roots was approximately 160 kDa, the transcripts were 5 kb longer than the corresponding these proteins (Yamaya *et al.*, 1992). The nucleotide sequence of the 3'-terminus region of an EST clone (S13501) found by the database search in rice ESTs for the longest cDNA encoding proteins with sequence similarity to the N-terminus region of maize Fd-GOGAT was completely identical to those of cDNAs (F1 and F2) for rice leaf Fd-GOGAT. This cDNA, however, covers only a 4.9 kb of cDNA for rice shoot Fd-GOGAT.

A previous study suggested that Fd-GOGAT in

rice roots was an immunologically distinct protein from the enzyme in rice leaves (Suzuki *et al.*, 1982). To obtain full-length cDNAs encoding rice root Fd-GOGAT, we constructed a size-fractionated cDNA library (8.0×10^5 independent recombinants) in λZAPII vector from poly(A)$^+$RNA prepared from 26-day-old roots of rice seedlings. The library (4.0×10^5 recombinants) was screened by the plaque hybridization method with the 5′-terminus fragment (1.1 kb) of rice shoot cDNA (S13501) as a probe and ten positive clones were isolated. Because they showed the same pattern on the restriction map, these clones appeared to have derived from the same mRNA. The longest clone designated as pOSFGGT52 was chosen for sequence analysis. The complete nucleotide sequence of this insert (5822 bp) was determined and deposited in the DDBJ DNA databank under the accession number AB024716. The nucleotide sequences of green leaf cDNAs (F1 and F2) and a shoot cDNA (S13501) were identical to that of the 3′-terminus region of the root cDNA (pOSFGGT52). These results suggest that rice shoots and roots contain only one expressed gene for Fd-GOGAT.

The pOSFGGT52 contained 411 bp and 563 bp of 5′-untranslated sequence and 3′-untranslated sequence, respectively. The cDNA showed a large open reading frame of 4848 bp which encodes a 1616 amino acid protein with a molecular mass of 175034 Da following the first ATG codon found in the cDNA. Although no in-frame stop codon was found upstream from the predicted start codon, this start codon occurs at an almost similar position to that of maize Fd-GOGAT cDNA (Sakakibara *et al.*, 1991). A comparison of the deduced amino acid sequence encoded by pOSFGGT52 with other plant GOGAT sequences (Sakakibara *et al.*, 1991; Coschigano *et al.*, 1998; Goto *et al.*, 1998) is shown in **Fig. 1**. The rice Fd-GOGAT protein is more similar to the enzyme sequence of the monocotyledonous maize (90% identity) than to two enzyme sequences of the dicotyledonous *Arabidopsis* (79% identity for Glu1 and 76% identity for Glu2, respectively). Furthermore, the amino acid sequence of rice Fd-GOGAT is similar to those of N-terminal region (from 1 to 1628) of rice NADH-GOGAT (Goto *et al.*, 1998) and *E. coli* NADPH-GOGAT large subunit (Oliver *et al.*, 1987) (40% and 39% identity, respectively) and lacks the additional 538 amino acids at the C-terminus of rice NADH-GOGAT that encodes the NADH-binding domain. The amino acid sequence from Cys^{98} to Asn^{106} of maize Fd-GOGAT, which corresponds to N-terminal region of the mature protein, is completely conserved in the sequence from Cys^{97} to Asn^{107} of rice Fd-GOGAT. This result suggests rice Fd-GOGAT has a 96-amino acid presequence at the N-terminal region. The predicted mature protein consisting of 1520 amino acids with a molecular mass of 165010 Da was in good agreement with the value of Fd-GOGAT polypeptide purified from rice leaves (Yamaya *et al.*, 1992).

As shown in **Fig. 1**, the putative regions required for binding of substrates and prosthetic groups, i.e., *purF*-type glutamine amide transfer, binding of FMN and insertion of the $[3Fe\text{-}4S]^{1+,0}$ cluster, among the deduced amino acids sequences of plant GOGATs are highly conserved (Sakakibara *et al.*, 1991; Coschigano *et al.*, 1998; Goto *et al.*, 1998). The primary structures for domains in Fd-GOGATs from rice, maize (Sakakibara *et al.*, 1991) and *Arabidopsis* (Coschigano *et al.*, 1998) and the N-terminus region of NADH-GOGAT from rice (Goto *et al.*, 1998) were compared to the *purF*-type glutamine amide transferase domain of glutamine phosphoribosylpyrophosphate amide transferase from *E. coli* (Mei and Zalkin, 1989), the FMN binding domain of yeast flavocytochrome b_2 (Lederer *et al.*, 1985) and the $[3Fe\text{-}4S]^{1+,0}$ cluster binding region of the iron-sulfur protein subunit (FrdB) of fumarate reductase from *E. coli* (Johnson *et al.*, 1988). The Cys^1-Asp^{29}-His^{101} catalytic triad of glutamine phosphoribosylpyrophosphate amide transferase from *E. coli*, which is implicated in the glutamine amide transfer function of *purF*-type glutamine amide transferases (Mei and Zalkin, 1989), are conserved as Cys^{97}-, Asp^{133} or Asp^{138}-, His^{305} or His^{327} in the deduced amino acid residues of rice Fd-GOGAT and this catalytic triad are also conserved in Fd-GOGATs from maize (Sakakibara *et al.*, 1991) and *Arabidopsis* (Coschigano *et al.*, 1998). In the putative FMN binding region, Asp^{409} and Arg^{413} of yeast flavocytochrome b_2, which interact with the ribityl side chain of FMN (Lederer *et al.*, 1985), are conserved as Asp^{1196} and Arg^{1200} of rice Fd-GOGAT, and Asp^{1197} and Arg^{1201} of maize Fd-GOGAT (Sakakibara *et al.*, 1991). These two amino acid residues also conserved to Asp^{1205} and Lys^{1209} of *Arabidopsis* Glu1, Asp^{1207} and Lys^{1211} of *Arabidopsis* Glu2 (Coschigano *et al.*, 1998) and Asp^{1212} and Lys^{1216} of rice NADH-GOGAT (Goto *et al.*, 1998), such as Asp^{1070} and Lys^{1074} of *A. brasilense* GltS α-subunit (Pelanda *et al.*, 1993). In the putative $[3Fe\text{-}4S]^{1+,0}$ cluster binding regions of plant GOGAT, Cys-$(X)_5$-Cys-$(X)_3$ or $_4$-Cys-Pro sequences are conserved (Sakakibara *et al.*, 1991; Coschigano *et al.*, 1998; Goto *et al.*, 1998) and these three cysteine residues are important for ligation to the $[3Fe\text{-}4S]^{1+,0}$ cluster in FrdB from *E. coli*. (Johnson *et al.*, 1988).



The evolution of GOGAT proteins

The Fd-GOGAT proteins have remarkable similarities to the large subunits of NADPH-GOGAT proteins in bacteria and eukaryote, which are encoded by *GltB* genes. Fd-GOGAT proteins are also similar to the N-terminus regions of NADH-GOGAT proteins in higher plants, which are like the NADPH-GOGAT large subunits in bacteria (Sakakibara *et al.*, 1991; Gregerson *et al.*, 1993; Coschigano *et al.*, 1998; Goto *et al.*, 1998). To examine the evolutional relationships among the GOGAT proteins from archaea, bacteria and eukaryote, we constructed a phylogenetic tree using the amino acid sequences of regions common to Fd-GOGAT, *GltB* gene products of NADPH-GOGAT and N-terminus regions of NADH-GOGAT, which are like *GltB* gene products, from an archaebacteria, eubacteria, cyanobacteria, red algae, a fungus and higher plants, for which entire amino acid sequences are available from the GenBank database with an exception of the spinach Fd-GOGAT, whose amino acid sequence lacks the N-terminal presequence (**Fig. 2**). With the exclusion of the *Synechocystis* sp. *gltB* gene product, which was

Fig. 1 Deduced amino acid sequence alignment of rice Fd-GOGAT with maize Fd-GOGAT, *Arabidopsis* Fd-GOGATs and rice NADH-GOGAT. Numberings of amino acid residue for each polypeptide are indicated at the right side. Dots indicate residues identical to the first sequence. Dashes indicate gaps introduced to maximize sequence similarity. The first amino acid residues of the predicted mature polypeptides are indicated by an arrow. Asterisks denote the carboxy termini of the polypeptides. The putative *purF*-type glutamine amide transfer regions are indicated by overlines and the putative FMN binding regions are boxed. The predicted residues for Cys-Asp-His catalytic triads of *purF*-type glutamine amide transferase and those interacted with the ribityl side chain of FMN are indicated by whitened arrowheads. Three cysteines putatively involved in binding a [3Fe-4S]$^{1+,0}$ cluster are indicated by blackened arrowheads. Rice Fd-GOGAT is the predicted translational product of the rice root cDNA (pOSFGGT52, this study). Maize Fd-GOGAT is the Fd-GOGAT precursor in maize leaves (Sakakibara *et al.*, 1991). *Arabidopsis* Glu1 is the *Arabidopsis* Fd-GOGAT1 precursor (*Glu1*) and *Arabidopsis* Glu2 is the *Arabidopsis* Fd-GOGAT2 precursor (*Glu2*) (Coschigano *et al.*, 1998). Rice NADH-GOGAT is only the N-terminal region of the NADH-GOGAT precursor in rice roots, that shows identity to the maize Fd-GOGAT and to the large subunit of *E. coli* NADPH-GOGAT (Goto *et al.*, 1998). The amino acid sequence alignment was done by using the MAlign program in the GENETYX-MAC software system (Software Development, Tokyo, Japan).

originally reported as a Fd-GOGAT but now is suggested to be the large subunit of NADH-GOGAT (Dincturk and Knaff, 2000), every Fd-GOGAT proteins clustered together. In addition, monocotyledonous Fd-GOGATs, dicotyledonous Fd-GOGATs, red algae Fd-GOGATs and cyanobacteria Fd-GOGATs were separately clustered. This result shows that eukaryotic Fd-GOGATs from both higher plants and red algae are very closely related to the prokaryotic Fd-GOGATs from cyanobacteria. Temple *et al.* (1998) hypothesized, based on the location analysis of Fd-GOGAT genes in the plastid genomes of the red algae, that the origin of plastids is endosymbiotic bacteria and that higher plant Fd-GOGAT genes in the nuclear genomes may be transferred from the genome in the bacterial precursors of plastids. Our results also well supported their hypothesis. On the other hand, eukaryotic NADH-GOGATs from higher plants and a yeast were clustered together and branched from eubacterial NADPH-GOGAT (**Fig. 2**). Although the evolution of NADH-GOGAT was separated from that of Fd-GOGAT, both GOGAT genes seem to share common ancestors of the bacterial-type *gltB* from eubacteria.

Cloning and structural characterization of Fd-GOGAT gene from rice

A genomic library was constructed in λFIXII vector from partially digested genomic DNA prepared from 5-day-old shoots of rice grown in the dark. The unamplified genomic library (2.8×10^5 recombinants) was screened with the 5'-terminus fragment (1.1 kb) of the rice shoot cDNA (S13501), which encoded the partial translational region at the N-terminus of rice shoot Fd-GOGAT, as a probe, and nine positive clones were isolated. The restriction mapping of these clones, followed by Southern blotting analysis using the 5'-terminus fragment (1.1 kb) of rice shoot cDNA (S13501) and rice leaf cDNA (F2, 1.3 kb) as probes showed that these clones apparently correspond the upstream portions for the rice Fd-GOGAT gene. Since two clones, containing inserts of 13 and 14 kb (named λFD13 and λFD41, respectively), appeared to cover the longest region (17 kb) for the rice Fd-GOGAT gene, these two clones were chosen for sequence analysis. The alignment analysis of the combined nucleotide sequence (17179 bp) of λFD13 and λFD41 with that of full-length Fd-GOGAT cDNA (pOSFGGT52) from rice roots showed that this genomic DNA sequence contained the 5672 bp 5'-upstream region from the first methionine and 22 exons encoded the 3398 bp of 5'-terminus portion of entire open reading frame for Fd-GOGAT. We used PCR to obtain the downstream region from the exon 22 of the rice Fd-GOGAT gene. A 3.3 kb PCR product containing the translational region downstream from the exon 22 of the rice Fd-GOGAT gene and 1.3 kb PCR product containing the downstream portion from the 3'-untranslational region of the gene were obtained and sequenced.

The entire nucleotide sequence of rice Fd-GOGAT gene, which was a combination of the nucleotide sequences of λFD13, λFD41 and two PCR clones, was 20899 bp long and has been deposited in the DDBJ DNA databank under the accession number AB061357. The result of se-

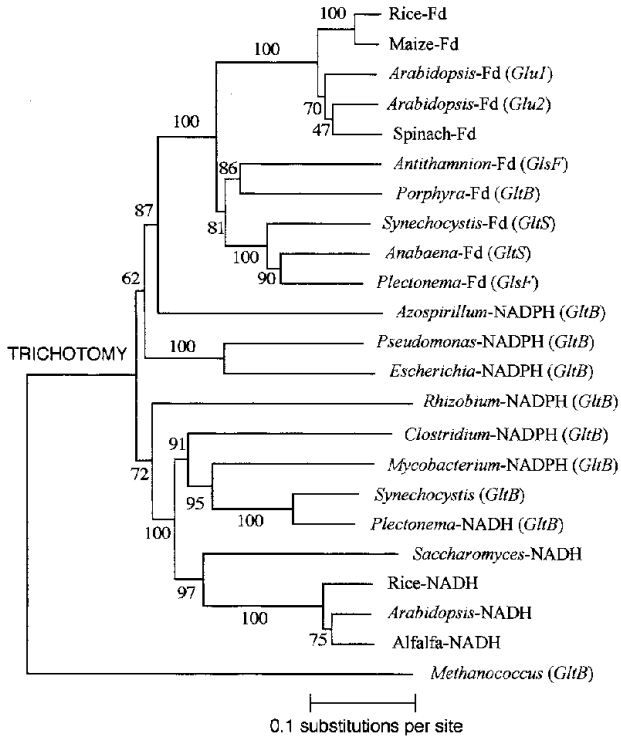


Fig. 2 The unrooted phylogenetic tree of the deduced amino acid sequences of the Fd-GOGATs, the large subunits of NADPH-GOGATs and the large subunit like regions of NADH-GOGATs. The tree was calculated by the neighbor-joining method found in the CLUSTAL W program (Thompson *et al.*, 1994) and PHYLIP program (Felsenstein, J., PHYLIP (Phylogeny Inference Package) ver. 3.5c., Department of Genetics, University of Washington, Seattle, U.S.A.). Numbers at the branch points represent the bootstrap values for percentage of 1,000 replicate trees. The length of the bar represents 0.1 substitutions per site. The informations of the deduced amino acid sequences of GOGATs were obtained from the GenBank database. The accession numbers and the deduced amino acid sequence regions used were: Rice-Fd (*Oryza sativa*), AB024716, 97 to 1615 ; Maize-Fd (*Zea mays*), M59190, 98 to 1616 ; Spinach-Fd (*Spinacia oleracea*), AF061515, 1 to 1517; *Arabidopsis*-Fd (*Glu1*) (*Arabidopsis thaliana*), U39287, 106 to 1622; *Arabidopsis*-Fd (*Glu2*) (*Arabidopsis thaliana*), U39288, 108 to 1629; *Antithamnion*-Fd (*GlsF*) (*Antithamnion* sp.), Z21705, 27 to 1536; *Porphyra*-Fd (*GltB*) (*Porphyra purpurea*), U38804, 34 to 1538; *Anabaena*-Fd (*GltS*) (*Anabaena* sp.), AJ249913, 35 to 1548; *Plectonema*-Fd (*GlsF*) (*Plectonema boryanum*), D85735, 39 to 1551; *Synechocystis*-Fd (*GltS*) (*Synechocystis* sp.), D78371, 37 to 1556; *Methanococcus* (*GltB*) (*Methanococcus jannaschii*), U67575, 1 to 510; *Azospirillum*-NADPH (*GltB*) (*Azospirillum brasilense*), AF192408, 37 to 1515; *Pseudomonas*-NADPH (*GltB*) (*Pseudomonas aeruginosa*), U81261, 15 to 1482; *Escherichia*-NADPH (*GltB*) (*Escherichia coli*), M18747, 43 to 1514; *Rhizobium*-NADPH (*GltB*) (*Rhizobium etli*), AF107264, 46 to 1581; *Clostridium*-NADPH (*GltB*) (*Clostridium acetobutylicum*), AF082880, 21 to 1518; *Mycobacterium*-NADPH (*GltB*) (*Mycobacterium tuberculosis*), Z83864, 18 to 1527; *Synechocystis* (*GltB*) (*Synechocystis* sp.), X80485, 43 to 1550; *Plectonema*-NADH (*GltB*) (*Plectonema boryanum*), D85230, 24 to 1530; *Saccharomyces*-NADH (*Saccharomyces cerevisiae*), X89221, 54 to 1569; Rice-NADH (*Oryza sativa*), AB008845, 100 to 1628; *Arabidopsis*-NADH (*Arabidopsis thaliana*), AB020754, 117 to 1664; Alfalfa-NADH (*Medicago sativa*), L01660, 102 to 1638.

quence alignment of the rice Fd-GOGAT gene with rice root Fd-GOGAT cDNA showed that the 15.4 kb structural gene of rice Fd-GOGAT consisted of 33 exons, that ranged in size from 59 bp (exon 15) to 713 bp (exon 33), separated by 32 introns (**Fig. 3A**). All introns confirm the splice site GT....AG consensus boundary sequences. The structural genes of *Arabidopsis* Fd-GOGATs (8.6 kb for *Glu1* and

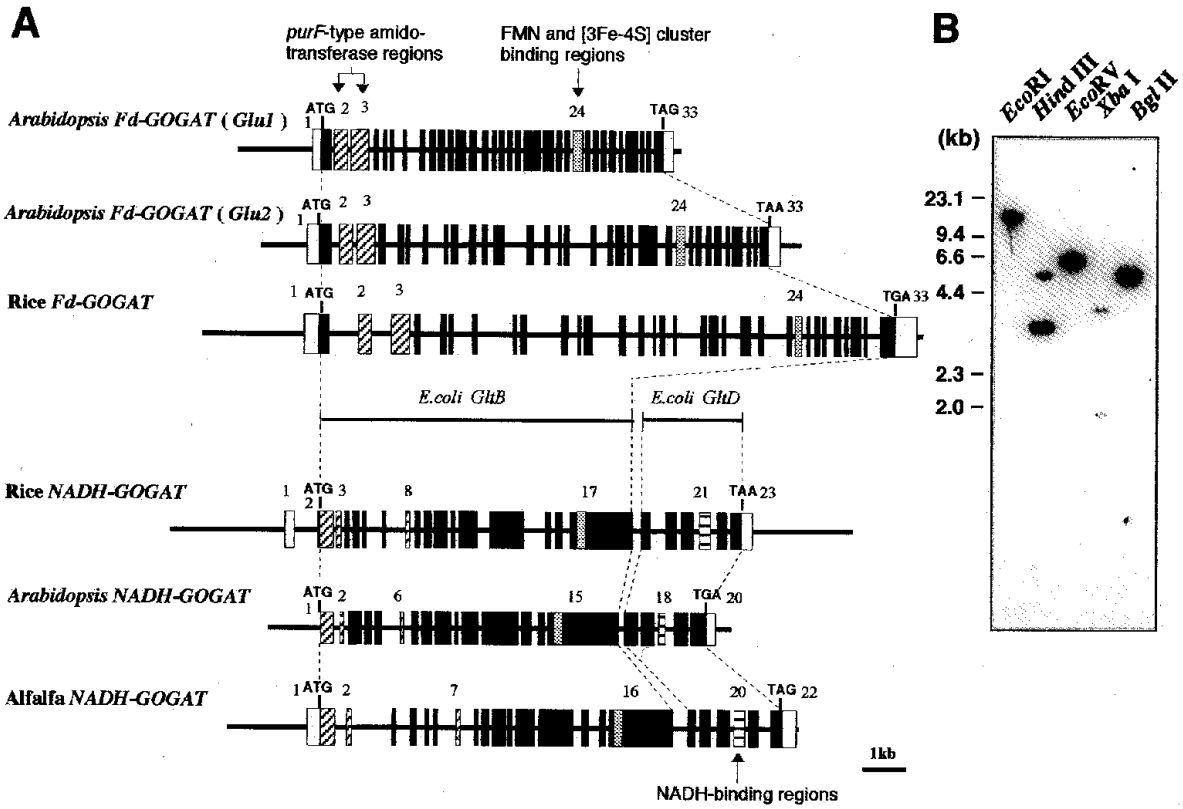


Fig. 3 Diagrammatic comparison of the structures of Fd-GOGAT genes from rice and *Arabidopsis* (accession number AL391716 for *Glu1*; accession number AC005662 for *Glu2*; Coschigano *et al.*, 1998) and NADH-GOGAT genes from rice (Goto *et al.*, 1998), alfalfa (Vance *et al.*, 1995) and *Arabidopsis* (accession number AB020754 for the gene; accession number AY099795 for cDNA) (A) and Southern blot analysis of Fd-GOGAT gene sequences in the rice genome (B). (A) Exons are indicated by boxed regions whereas introns, the 5'- and 3'-non-transcribed regions are represented by lines. The whitened boxes of exons correspond to the untranslated sequences. The exons encoding the regions for *purF*-type glutamine amide transfer, the binding of FMN and insertion of the [3Fe-4S]$^{1+,0}$ cluster and NADH-binding are indicated by the slashed boxes with slanting lines, the dotted boxes and the slashed boxes with horizontal lines, respectively. The alignment of the *E.coli* NADPH-GOGAT large and small subunit genes (*GltB* and *GltD*, Oliver *et al.*, 1987) with the encoded homologous regions in the GOGAT genes from higher plants is also shown. (B) Genomic DNA (1 μg) prepared from 5-day-old shoots of rice seedlings was digested with *Eco*RI, *Hin*dIII, *Eco*RV, *Xba*I and *Bgl*II. The digested DNA was electrophoresed through a 0.8% agarose gel, denatured, transferred to a nylon membrane and probed with 1316 bp partial cDNA (F2) for rice Fd-GOGAT. The membrane was finally washed in 0.1 x SSC, 0.1% SDS at 68 ℃ for 20 min and hybridization signals were detected. The numbers at the left refer to the positions of DNA molecular length markers in kilobases.

11.0 kb for *Glu2*) were also composed of 33 exons and 32 introns, which were deduced from sequence alignments of the putative *Arabidopsis* Fd-GOGAT genes, available from the sequence database of *Arabidopsis* DNA chromosome 5 (BAC clone F21E1, accession number AL391716 for *Glu1*) and chromosome 2 (BAC clone F13H10, accession number AC005662 for *Glu2*), with respective cDNAs (Coschigano *et al.*, 1998) (**Fig. 3A**). The positions of the insertion of each introns in rice Fd-GOGAT gene, *Arabidopsis Glu1* and *Arabidopsis Glu2* were highly conserved. The putative *purF*-type glutamine amide transfer region was encoded in the exon 2 and exon 3, and the putative region for the binding of FMN and insertion of the [3Fe-4S]$^{1+,0}$ cluster were encoded in the exon 24. On the other hand, the exon-intron organizations of NADH-GOGAT genes from *Arabidopsis*, alfalfa and rice were very similar (**Fig. 3A**). Although Fd-GOGAT proteins and N-terminus regions of

NADH-GOGAT proteins from higher plants have marked similarities to the large subunit of NADPH-GOGAT protein from *E.coli*, which is encoded by the *GltB* gene (Oliver *et al.*, 1987), the exon-intron organizations of the regions encoding homologous regions for the *E.coli GltB* product in plant Fd-GOGAT genes clearly distinct from those in plant NADH-GOGAT genes. This result also suggests the evolutional differences between Fd-GOGAT and NADH-GOGAT genes as discussed above.

To estimate the copy number of Fd-GOGAT genes in the rice genome, we performed genomic Southern blot analysis. Rice genomic DNA digested with *Eco*RI, *Hin*dIII, *Eco*RV, *Xba*I and *Bgl*II, probed with 1316 bp partial cDNA (F2) for rice Fd-GOGAT, and finally washed in the highly stringent condition, i.e., 0.1 x SSC, 0.1% SDS at 68℃ for 20 min, gave a pattern of hybridization signals consistent with the pattern presumed from the restriction-enzyme cleavage sites in the region of our isolated genomic DNA for rice Fd-GOGAT (**Fig. 3B**). This suggests that the Fd-GOGAT gene exists as a single-copy gene in the rice genome. When we used the low stringent washing condition, i.e., 2.0 x SSC, 0.1% SDS at 42℃ for 20 min, an additional one hybridization band was observed in the lanes of genomic DNA digested with *Hin*dIII, *Eco*RV, *Xba*I and *Bgl*II (data not shown). Since these additional hybridization bands could not be caused by the same restriction of genomic DNA for rice NADH-GOGAT (Goto *et al.*, 1998), this result suggests the presence of the additional Fd-GOGAT gene or pseudo-gene in the rice genome as in *Arabidopsis* genome (Coschigano *et al.*, 1998). Because Fd-GOGAT polypeptides from both roots and green leaves of rice showed the same mobilities on our two-dimensional immunoblot analysis after isoelectric focusing followed by SDS-PAGE (data not shown) and the nucleotide sequences of all cDNA clones for Fd-GOGAT were identical to those of exons of the Fd-GOGAT gene isolated here, only one Fd-GOGAT gene is mainly expressed in roots and green leaves of rice. Another gene would be silent or expressed at very low levels in the tissue tested, or be specifically expressed on a particular stage or tissues of development of rice plants.

Expression of Fd-GOGAT gene and intracellular localization of the enzyme protein in rice roots

The accumulation on steady-state levels of transcript for Fd-GOGAT was compared following the separation of total RNA prepared from roots, fully expanded leaf blades and nongreen developing leaf blades of rice seedlings (**Fig. 4A**). A single major band of the transcript (5.8 kb) which corresponded to the size of Fd-GOGAT cDNA was detected in all extracts, but the intensities of the signals from roots and nongreen developing leaf blades were much lower than those from fully expanded leaf blades. This accumulation pattern of the transcript for Fd-GOGAT was identical to that of protein for the enzyme, i.e., approx 1.5 μg (g fresh weight)$^{-1}$ for roots of rice seedlings, approx 111 μg (g fresh weight)$^{-1}$ for fully expanded blades of the 7th leaves and approximately 7.8 μg (g fresh weight)$^{-1}$ for nongreen developing blades of the 10th leaves in rice plants (Yamaya *et al.*, 1992, 1995; Ishiyama *et al.*, 1998). The accumulation level of transcript for Fd-GOGAT in roots of rice seedlings did not largely change by the treatment with 1 mM NH_4^+, 1 mM No_3^- or without a source of nitrogen (data not shown) as in the activity and protein contents in the roots (Yamaya *et al.*, 1995).

Ishiyama *et al.* (1998) reported that the content of Fd-GOGAT protein was the highest in root-tip sections of rice seedlings and gradually decreased as a function of age toward the root base. This enzyme protein was found mainly in the apical meristem and central cylinder in rice roots. Subcellular fractionation studies have shown that most Fd-GOGAT protein is located in the plastids in roots of maize plants (Sakakibara *et al.*, 1992). Fd can be reduced through the oxidative pentose phosphate pathway in plastids of pea roots (Bowsher *et al.*, 1992). Our immunogold electron microscopy analysis showed that immunolabeling with the affinity-purified anti-Fd-GOGAT IgG was detected specifically in plastids in the companion cells of the central cylinder (**Fig. 4B, C**) and the sclerenchyma cells (**Fig. 4D**) in rice roots. Background labeling of other cellular compartments, i.e., nucleus, cytosol, vacuole, mitochondria and cell wall, was extremely low or negligible. The control sections, which were labeled with anti-Fd-GOGAT IgG that had been preabsorbed with excess amounts of purified Fd-GOGAT, showed very little immunogold labeling (data not shown). This result strongly suggests that Fd-GOGAT is specifically localized in the plastids of rice roots and a 96-amino acid presequence at the N-terminal region of rice root Fd-GOGAT may contain the transit peptide for targeting to plastids. In rice, the area of the central cylinder could be essential for the transport of solutes from the phloem to the actively developing cells. Companion cells are important in the regulation of phloem loading (Van Bel, 1993). Solute transport also occurs from the root surface to the xylem vessel elements. The major forms of nitrogen in both xylem sap and phloem sap of rice are glutamine and asparagine (Fukumorita and Chino, 1982; Hayashi

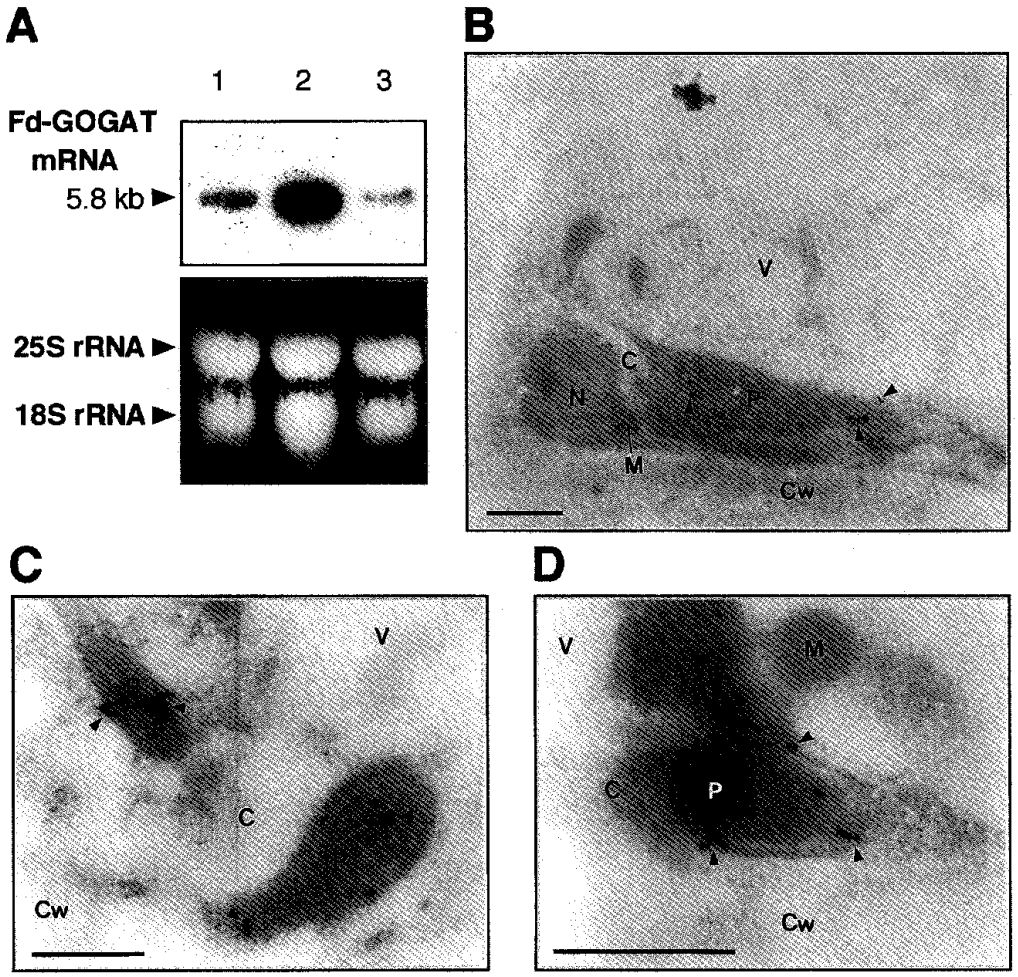


Fig. 4 Northern blot analyses of the expression of Fd-GOGAT gene in rice seedlings (A) and intracellular localization of Fd-GOGAT protein in rice roots (B, C, D). (A) Ten μg of total RNA, prepared from roots (lane 1), fully expanded leaf blades (lane 2) and nongreen developing leaf blades (lane 3) of the 28-day-old rice seedlings, were separated by agarose gel electrophoresis, transferred to a nylon membrane and then hybridized with 430 bp cDNA fragment encoding 3'-terminus translational region for rice Fd-GOGAT as a probe (the upper panel). 25S and 18S ribosomal RNAs stained by ethidium bromide before blotting were also shown as control (the lower panel). (B, C, D) The immunogold electron microscopy analysis of Fd-GOGAT protein in rice roots. Root tissue was from a seedling grown with 1 mM NH_4^+ for 24 h and sampled from the tip (<10 mm). The sections were incubated with affinity-purified anti-Fd-GOGAT IgG as the primary antibody. (B) and (C), companion cells of the central cylinder; (D), sclerenchyma cell. Note the high immunolabeling of the plastid. In the control section incubated with anti-Fd-GOGAT IgG pretreated with an excess amount of Fd-GOGAT protein, the very low amount of gold label was observed (data not shown). P, Plastid; C, cytosol; Cw, cell wall; M, mitochondrion; N, nucleus; V, vacuole. Arrowheads indicate gold label. Bar = 0.5 μm.

and Chino, 1990). Our previous immunolocalization studies showed that NADH-GOGAT protein is localized in the plastids of vascular parenchyma cells in the central cylinder of the rice-root tips (Hayakawa *et al.*, 1999) and cytosolic GS protein was detected in the central cylinder in rice (Ishiyama *et al.*, 1998). Although more work is required to argue precise functions for Fd-GOGAT and NADH-GOGAT in these tissues, these GOGATs probably have different roles in the utilization of glutamine transported from the shoots. In rice, the area of the central cylinder is essential for adaptation to growth under anaerobic conditions. Reggiani *et al.* (2000) suggested that Fd-GOGAT plays an important role in the amino acid accumulation in rice roots subjected to anaerobic conditions. Existence of Fd-GOGAT in the companion cells, which are adjacent to the sieve elements, of

the central cylinder in rice roots is advantageous for access and efficient utilization of glutamine and carbohydrate transported through the sieve elements under anaerobic conditions.

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