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Tissue and Cellular Localization of NADH-Dependent Glutamate Synthase Protein in Leaves of Spinach

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Summary

Tissue and cellular localization of NADH-dependent glutamate synthase (NADH-GOGAT, EC1.4.1.14) in young leaves of dicotyledonous spinach (*Spinacia oleacea*) was investigated using the immunocytological method with an affinity-purified anti-NADH-GOGAT immunoglobulin G. Immunoblotting analysis showed this antibody specifically cross-reacted with NADH-GOGAT protein in crude soluble proteins from young leaf blades of spinach. When transverse sections (10 μm in thickness) prepared from the paraffin-embedded young leaf blades of spinach were stained with the anti-NADH-GOGAT antibody, strong signals for NADH-GOGAT protein were detected in companion cells of large vascular bundles. Weak signals for the NADH-GOGAT protein were also detected in vascular parenchyma cells and mesophyll cells of young leaves. Ferredoxin (Fd)-GOGAT (EC 1.4.7.1) protein was mainly located in mesophyll cells and signals for the protein were also detected in companion cells and xylem-parenchyma cells of large vascular bundles. By the way, in young leaf blades of monocotyledonous rice, NADH-GOGAT protein specifically located in vascular cells and Fd-GOGAT protein was abundant in mesophyll cells (Hayakawa et al., 1994). The differences of functions in young leaves for NADH-GOGAT proteins between monocotyledonous rice and dicotyledonous spinach are discussed.

Key words: Cellular localization, glutamate synthase, nitrogen metabolism, spinach

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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 (Ireland and Lea, 1999). 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) (Ireland and Lea, 1999).

Fd-GOGAT is the major form of GOGAT in green tissues and is located in the chloroplast stroma (Ireland and Lea, 1999). 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 and Ogren, 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^+ formed from the primary assimilation of nitrate (Sakakibara et al., 1992; Redinbaugh and Campbell, 1993; Ireland and Lea, 1999; Tobin and Yamaya, 2001).

NADH-GOGAT is a minor form of GOGAT species in green tissues and findings for its tissue and cellular localization, gene structure and expression, and function in higher plants are much limited (Ireland and Lea, 1999). Complete structures of the gene and cDNA for NADH-GOGAT have been reported in root nodules of the legume species alfalfa, in the monocotyledonous species rice, and in the dicotyledonous species *Arabidopsis* (Gregerson et al., 1993; Vance et al., 1995; Goto et al., 1998; Lancien et al., 2002). In root nodules its expression is developmentally regulated (Gregerson et al., 1993). In monocotyledonous species rice, both the activity and the protein content for NADH-GOGAT were abundant in young developing leaf blades and spikelets at the early stages of ripening (Yamaya et al., 1992; Hayakawa et al., 1993). In addition, our previous immunocytological studies reported NADH-GOGAT protein mainly located in vascular-parenchyma cells and mestome-sheath cells in young leaf blades and vascular parenchyma cells, nucellar projection, nucellar epidermis, and a few layers of aleurone cells in young grains (Hayakawa et al., 1994). This cell-type specific and age-specific expression of rice NADH-GOGAT gene was supported by analysis of a promoter activity of the gene (Kojima et al., 2000). Since these cells of young rice organs, in which NADH-GOGAT located, are important for solute transport from phloem and xylem, it was 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 senescing tissues and roots (Yamaya et al., 1992; Hayakawa et al., 1993, 1994; Tobin and

Yamaya, 2001). In specific regions of the epidermis and exodermis in roots of rice the enzyme also seemed to be important for the assimilation of exogenously supplied NH_4^+ (Yamaya et al., 1995; Hirose et al., 1997; Ishiyama et al., 1998; Hayakawa et al., 1999; Tobin and Yamaya, 2001). In addition, the characterization of a knock-out T-DNA insertion mutant (*glt1*-T) in the single NADH-GOGAT *Glt1* gene in *Arabidopsis* suggested that NADH-GOGAT has significant roles in non-photorespiratory NH_4^+ assimilation and in glutamate synthesis required for plant development (Lancien et al., 2002).

In order to understand more precise physiological functions of NADH-GOGAT in higher plants, we examined tissue and cellular localization of NADH-GOGAT protein in young leaves of the dicotyledonous spinach and the result was compared to that of the monocotyledonous rice (Hayakawa et al., 1994).

Materials and Methods

Plant materials

Spinach (*Spinacia oleracea* L.) seedlings were grown on a synthetic culture soil (Kureha, Tokyo, Japan) in small container in a greenhouse. When unexpanded leaf blades at the 2nd nodal position were emerging from the 1st sheaths, the lower young fully expanded leaf blades at the 1st nodal position were harvested. Rice (*Oryza sativa* L. cv. Sasanishiki) plants were grown in hydroponic culture in a greenhouse and unexpanded leaf blades at the 11th nodal position before emerging from the 10th sheaths were harvested according to Sugiyama et al. (2004). For the protein extraction, the samples were weighed, frozen in liquid nitrogen, and stored at −80°C until they were extracted. For the immunocytological experiment, approximately 5-mm thick cross-sections of fresh leaf blades of spinach were fixed in FAA solution [1.85% (v/v) formaldehyde, 5% (v/v) acetic acid and 63% (v/v) ethanol] for 24 h at 4°C.

Antibody preparations

Polyclonal immunoglobulin G (IgG) raised against either NADH-GOGAT purified from rice cell cultures (Hayakawa et al., 1992) or purified rice-leaf Fd-GOGAT (Yamaya et al., 1992) was used. Each of the IgGs was affinity-purified with corresponding antigens (Yamaya et al., 1992) before use in the present study. Anti-rice NADH-GOGAT IgG and anti-rice Fd-GOGAT IgG preabsorbed with excess amounts of the corresponding antigens were used as controls, respectively.

Preparation of protein extracts and immunoblotting analysis

The frozen samples were first ground to a fine powder in a mortar with a pestle in the presence of washed quartz sand with liquid nitrogen. The powder was

homogenized in 50 mM KH_2PO_4-KOH buffer (pH 7.5) containing 0.2% (v/v) 2-mercaptoethanol, 1 mM EDTA, 20 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 100 mM KCl and 0.05% (v/v) Triton X-100 [2 ml (g fresh weight)$^{-1}$]. The homogenate was centrifuged at 18,500$\times$g for 20 min at 4°C and the supernatant was used as a soluble protein fraction for the immunoblotting analysis. The protein content was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Immunoblotting after SDS-PAGE was performed as described previously (Yamaya et al., 1992). The dilutions of primary antibodies used in immunoblotting analyses were at 1 : 100 for the affinity-purified anti-NADH-GOGAT IgG and at 1 : 200 for the affinity-purified anti-Fd-GOGAT IgG, respectively. The same dilution of anti-GOGAT antibodies that had been preabsorbed with antigens were used as controls. After primary immunostaining, the reacted peptides were detected with a goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad, CA, USA), followed by visualization with the ECL plus western blotting detection reagents (Amersham Bioscience, NJ, USA) as substrates on a X-ray film (X-OMAT, Kodak, CA, USA).

Immunocytological experiment

Immunocytological experiment using the light microscope was performed with the purified ant-rice GOGAT antibodies, as described previously (Hayakawa et al., 1994). Cross-sections of the young leaf blades fixed with FAA solution were sequentially dehydrated, embedded in paraffin, sliced into 10-μm transverse sections, stretched onto a glass slide, deparafinized, and rinsed. The sections were incubated with the purified anti-rice NADH-GOGAT IgG (the dilution at 1 : 10) or the purified anti-Fd-GOGAT IgG (at 1 : 20) as primary antibodies. Control sections were incubated with the same dilution of anti-rice GOGAT antibodies that had been preabsorbed with antigens. Antigens on the surface of the tissue sections were visualized with ABC reagent containing AvidinDH and biotinylated horseradish peroxidase (Vectastain ABC Elite kit; Vector Lab. Inc., CA, USA) and observed using a Leica DMRB light microscope (Leica Microsystems, Wetzlar, Germany).

Results

Antibody specificity and detection of GOGAT proteins in young leaves of spinach

In immunoblotting analysis after SDS-PAGE, our affinity-purified anti-NADH-GOGAT IgG and affinity-purified anti-Fd-GOGAT IgG cross-reacted mono-specifically with NADH-GOGAT polypeptide with a molecular mass of approximately 200 kDa and with Fd-GOGAT polypeptide with that of approximately 160 kDa in crude soluble protein extracts prepared from young rice leaves,

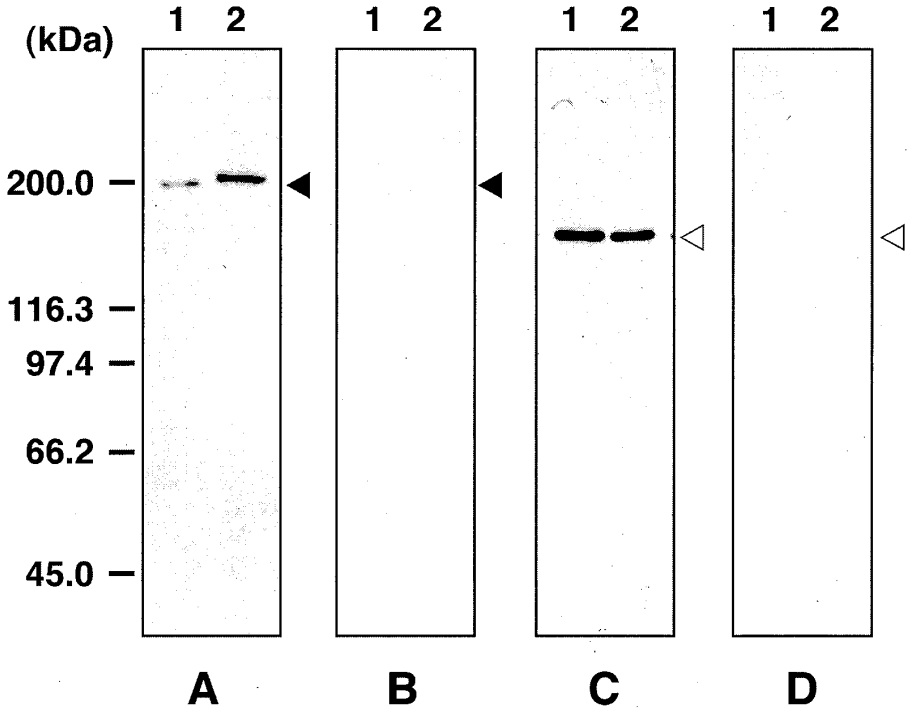

FIG. 1. Immuno-detection of NADH-GOGAT and Fd-GOGAT proteins in crude protein extracts from young leaf blades of rice and spinach plants with the affinity-purified anti-rice NADH-GOGAT IgG and the affinity-purified anti-rice Fd-GOGAT IgG. (A) Immunoblot of crude protein extracts (10 μg) from rice leaf blades (lane 1) and spinach leaf blades (lane 2) with the affinity-purified anti-rice NADH-GOGAT IgG. (B) Same as (A) except that the anti-NADH-GOGAT IgG was preabsorbed with excess amounts of the NADH-GOGAT protein purified from rice cultured cells. (C) Same as (A) except that the affinity-purified anti-rice Fd-GOGAT IgG was used. (D) Same as (C) except that the anti-Fd-GOGAT IgG was preabsorbed with excess amounts of the Fd-GOGAT protein purified from rice leaves. The numbers at the left refer to the positions of molecular weight markers for protein in kilodaltons. The blackened and whitened arrowheads indicate polypeptides of NADH-GOGAT and Fd-GOGAT, respectively.

respectively (Fig. 1A, C, lane 1; Yamaya et al., 1992). NADH-GOGAT and Fd-GOGAT polypeptides were also specifically detected in crude soluble protein extracts from young leaves of spinach (Fig. 1A, C, lane 2). These polypeptide bands were no longer detected when anti-rice GOGAT IgGs, which had been preabsorbed with excess amounts of corresponding antigens, were used as primary antibodies, respectively (Fig. 1B, D). This observation is an important control for assessing the meaning of the results obtained in the following immunocytological experiment.

Tissue and cellular localization of GOGAT proteins in young leaves of spinach

Ten-micrometer transverse sections of young leaf blades of spinach were

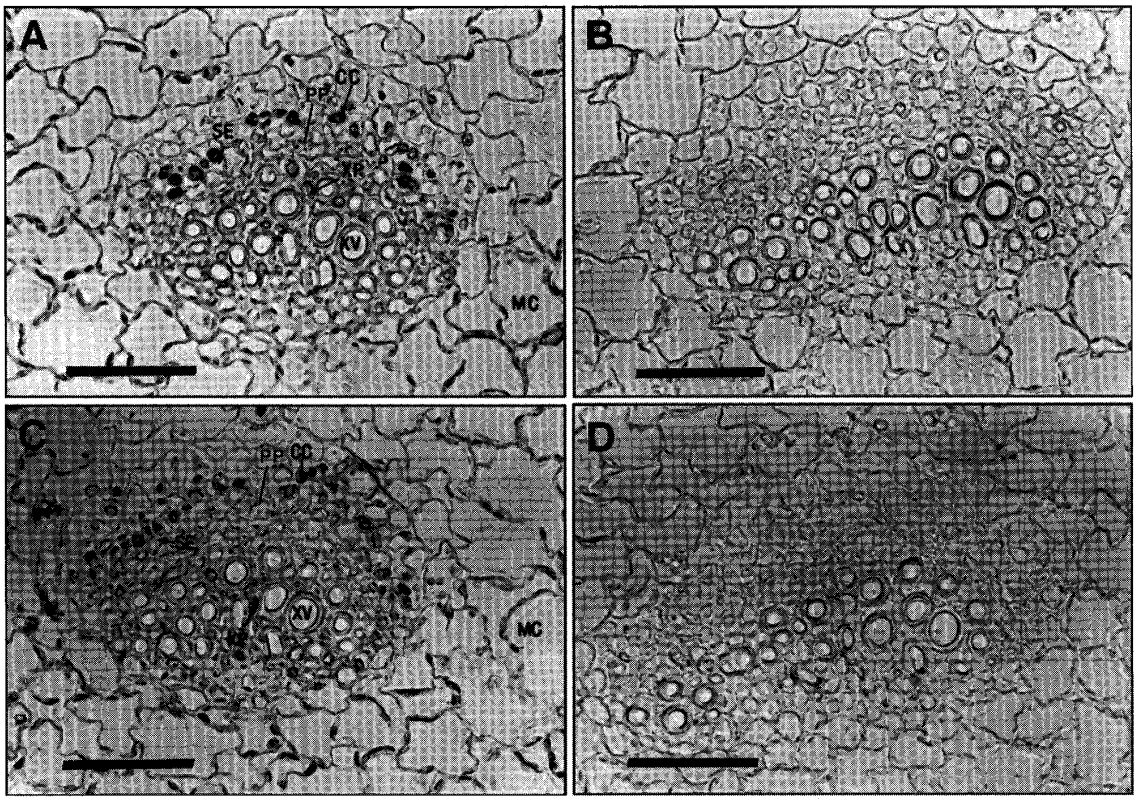

FIG. 2. Cellular localization of NADH-GOGAT and Fd-GOGAT proteins in young leaf blades of spinach. (A) The transverse section of a young leaf blade immunostained with the affinity-purified anti-rice NADH-GOGAT IgG. The close-up view of the large vascular bundle is shown. (B) Same as (A) except that the anti-NADH-GOGAT IgG was preabsorbed with excess amounts of the NADH-GOGAT protein purified from rice cultured cells. (C) Same as (A) except that the affinity-purified anti-rice Fd-GOGAT IgG was used. (D) Same as (C) except that the anti-Fd-GOGAT IgG was preabsorbed with excess amounts of the Fd-GOGAT protein purified from rice leaves. CC, companion cell; MC, mesophyll cell; PP, phloem parenchyma cell; SE, sieve element; XP, xylem parenchyma cell; XV, vessel element. Scale bars indicate 50 μm.

prepared from paraffin-embedded leaf tissues and NADH-GOGAT protein was detected immunocytologically (Fig. 2). Strong signals for NADH-GOGAT protein were detected in companion cells of large vascular bundles (Fig. 2A). Weak signals for the NADH-GOGAT protein were also detected in vascular parenchyma cells and mesophyll cells of young leaves. In contrast, Fd-GOGAT protein was mainly located in mesophyll cells and signals for the protein were also detected in companion cells and xylem-parenchyma cells of large vascular bundles (Fig. 2C). There were no clear signals when anti-rice GOGAT IgGs preabsorbed with excess amount of corresponding antigens were used as primary antibodies (Fig. 2B, D).

Discussion

This is the first paper describing the tissue and cellular distribution of NADH-GOGAT protein in leaves of dicotyledonous plants. In immunoblotting analysis with a polyclonal affinity-purified anti-rice cell NADH-GOGAT IgG, a single polypeptide band of approximately 200 kDa was specifically detected in a crude protein fraction from young leaves of spinach (Fig. 1A, lane 2). Although NADH-GOGAT protein has not been yet highly purified from leaves of spinach, the enzyme proteins highly purified from rice cell cultures and alfalfa root nodules have been shown to be a monomer with a molecular mass of 196–200 kDa (Anderson et al., 1989 ; Hayakawa et al., 1992). Thus, our anti-rice NADH-GOGAT IgG can specifically recognize NADH-GOGAT protein from spinach leaves. Our immunocytological study with this antibody showed that NADH-GOGAT protein located in companion cells and parenchyma cells of large vascular bundles, and in mesophyll cells in young leaves of dicotyledonous spinach (Fig. 2A). In contrast, NADH-GOGAT protein in young leaves of monocotyledonous rice specifically located in vascular parenchyma cells (metaxylem- and metaphloem-parenchyma cells) and mestome-sheath cells of vascular bundles (Hayakawa et al., 1994). At this stage of development, more than half of the nitrogen in young leaves of rice plants is remobilized from older, senescing leaves through the phloem and the remainder is transported from roots through the xylem (Mae and Ohira, 1981). It is known that the major form of nitrogen in both the xylem (Fukumorita and Chino, 1982) and phloem (Hayashi and Chino, 1990) of rice plants is glutamine, while the major free amino acid in young rice leaves is glutamate (T. Yamaya, unpublished data). The glutamine could be generated by the reaction catalized by cytosolic GS in vascular bundles of senescing leaves (Sakurai et al., 1996 ; Tabuchi et al., 2005). The metaxylem-parenchyma cells are considered to be active in the absorption of solutes from the vessel element, since they contain abundant mitochondria and endoplasmic reticulum (Chonan et al., 1981). In addition, the parenchyma cells of metaxylem and metaphloem and mestome-sheath cells are interconnected by numerous plasmodesmata (Chonan et al., 1981). Therefore, NADH-GOGAT located in those rice cells was suggested to be active in the synthesis of glutamate from glutamine that is transported through xylem and phloem to the young leaf tissues of rice plants. The location of NADH-GOGAT protein in vascular parenchyma cells of young leaves of dicotyledonous spinach was the similar situation to that of the enzyme protein in young rice leaves (Fig. 2A). The content of NADH-GOGAT protein, however, seemed to be abundant in companion cells of young spinach leaves. Companion cells are important in the regulation of phloem loading (Van Bel, 1993). Thus, NADH-GOGAT in vascular cells of young spinach leaves seemed to be important for synthesis of glutamate like the enzyme in vascular cells in young rice leaves.

On the other hand, our affinity-purified anti-rice leaf Fd-GOGAT IgG specifically recognized spinach leaf Fd-GOGAT protein with a molecular mass of 160 kDa (Fig. 1C, lane 2; Hirasawa and Tamura, 1984). Fd-GOGAT protein mainly located in mesophyll cells and the protein was also detected in companion cells and xylem-parenchyma cells of large vascular bundles in young spinach leaves (Fig. 2C). In young rice leaves, Fd-GOGAT mainly localized in mesophyll cells (Hayakawa et al., 1994). In leaves of higher plants, Fd-GOGAT mainly locates in the chloroplast stroma (Ireland and Lea, 1999). Because *Arabidopsis* and barley mutants deficient in Fd-GOGAT were conditional lethals that only survived under non-photorespiratory conditions, it is concluded that Fd-GOGAT plays a crucial role in the re-assimilation of NH_4^+ released from glycine decarboxylation during photorespiration (Somerville and Ogren, 1980; Kendall et al., 1986). Localization of Fd-GOGAT in mesophyll cells is consistent with results that show that Fd-GOGAT is active in photorespiratory nitrogen metabolism.

By the way, the barley mutants deficient in Fd-GOGAT contained only around 1% of the wild-type Fd-GOGAT activity in leaves but normal levels of NADH-GOGAT activity in leaves and roots (Blackwell et al., 1988). This suggested that NADH-GOGAT is important in the primary assimilation of NH_4^+ that is produced by nitrate reduction or from direct uptake (Blackwell et al., 1988). NADH-GOGAT also located in mesophyll cells of young spinach leaves (Fig. 2A). The nitrate reduction mainly occurs in mesophyll cells, in which cytosolic NADH-nitrate reductase (NADH-NR, EC1.6.6.1) locates (Ireland and Lea, 1999). Nitrite, which is produced from nitrate by the NR enzyme reaction, is subsequently reduced to NH_4^+ in chloroplasts by Fd-nitrite reductase (EC1.7.7.1) enzyme reaction (Ireland and Lea, 1999). In addition, our immunogold-localization study showed that NADH-GOGAT protein localized in plastids in rice (Hayakawa et al., 1999). Furthermore, the translational product from the rice NADH-GOGAT gene has a 99 amino acid presequence at the N-terminal region (Goto et al., 1998). The computer analysis for the presequence of rice NADH-GOGAT with the PSORT program (http://psort.ims.u-tokyo.ac.jp/) predicted this presequence contains the transit peptide for targeting to the plastid/chloroplast (Hayakawa et al., 1999). NADH-GOGAT together with Fd-GOGAT in mesophyll cells of young spinach leaves may be concerned with assimilation of NH_4^+ that is produced by nitrite reduction.

Recently, Masclaux-Daubresse et al. (2006) reported that Fd-GOGAT protein located in the phloem companion cells-sieve element complex in the leaf veins of dicotyledonous tobacco, and the glutamate level was constant in the phloem sap of the plant. They suggested a role of Fd-GOGAT in supplying glutamate for the synthesis and transport of amino acids. Fd-GOGAT in companion cells and xylem-parenchyma cells of large vascular bundles in young spinach leaves (Fig.

2C) may be important for synthesis of glutamate, and play a overlapping role in glutamate synthesis with NADH-GOGAT in vascular cells.

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