Planta (1998) 204: 288–294

Planta

Expression of NADH-dependent glutamate synthase protein in the epidermis and exodermis of rice roots in response to the supply of ammonium ions

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Received: 21 June 1997 / Accepted: 11 September 1997

Abstract. The mRNA and protein for NADH-dependent glutamate synthase (NADH-GOGAT; EC 1.4.1.14) in root tips of rice (*Oryza sativa* L. cv. Sasanishiki) plants increases dramatically within 12 h of supplying a low concentration (>0.05 mM) of ammonium ions (T. Yamaya et al., 1995, Plant Cell Physiol 36: 1197–1204). To identify the specific cells which are responsible for this rapid increase, the cellular localization of NADH-GOGAT protein was investigated immunocytologically with an affinity-purified anti-NADH-GOGAT immunoglobulin G. When root tips (>1 mm) of rice seedlings which had been grown for 26 d in water were immuno-stained, signals for the NADH-GOGAT protein were detected in the central cylinder, in the apical meristem, and in the primordia of the secondary roots. Signals for ferredoxin-dependent GOGAT (Fd-GOGAT; EC 1.4.7.1) protein were also seen in the same three areas. When the roots were supplied with 1 mM ammonium ions for 24 h, there were strong signals for the NADH-GOGAT protein in two cell layers of the root surface, i.e. epidermis and exodermis, in addition to the cells giving signals in the absence of ammonium ions. The supply of ammonium ions was less effective on the profile of signals for Fd-GOGAT. Although the supply of ammonium ions had less effect on the expression of cytosolic glutamine synthetase (GS; EC 6.3.1.2), this enzyme was also found to be located in the epidermis and exodermis, as well as in the central cylinder and cortex. The results indicate that NADH-GOGAT, coupled to the cytosolic GS reaction, is probably important for the assimilation of ammonium ions in the two cell layers of the root surface.

This paper is dedicated to Dr. Ann Oaks on the occasion of her retirement

Abbreviations: Fd-GOGAT = ferredoxin-dependent glutamate synthase; GS1 = cytosolic glutamine synthetase; IgG = immunoglobin G; NADH-GOGAT = NADH-dependent glutamate synthase

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Introduction

Glutamate synthase (GOGAT) catalyses the transfer of the amide group of glutamine formed by glutamine synthetase (GS; EC 6.3.1.2) to 2-oxoglutarate to yield two molecules of glutamate. One of the glutamate molecules can be cycled back as a substrate for the GS reaction and the other can be used for many synthetic reactions (Lea et al. 1990; Sechley et al. 1992). This GS/GOGAT cycle, as defined by Lea and Miflin (1974), represents the major pathway in the assimilation of NH_4^+ under normal metabolic conditions in plants. Two molecular species of GOGAT are found in both green and non-green tissues, one requiring NADH as reductant (NADH-GOGAT; EC 1.4.1.14) and the other requiring ferredoxin (Fd-GOGAT; EC 1.4.7.1) (Lea et al. 1990; Sechley et al. 1992). In leaves, these two GOGAT species have a distinct function: a major role of Fd-GOGAT, located in the chloroplast stroma, is the reassimilation of NH_4^+ released from photorespiration (Kendall et al. 1986; Somerville and Ogren 1980). The apparent function of NADH-GOGAT is, at least in young leaves and grains at the early stage of ripening of rice plants, in the re-utilization of glutamine transported from the phloem and xylem (Hayakawa et al. 1994). In roots, on the other hand, the functions of these two GOGAT species are not well characterized. In maize roots, Fd-GOGAT is probably involved in the assimilation of NH_4^+ formed from primary assimilation of nitrate (Redinbaugh and Campbell 1993), some of which occurs in the roots (Oaks 1992). Our preliminary studies showed that the mRNA and protein for NADH-GOGAT in whole roots or the root-tip sections of rice plants accumulate markedly within 12 h of the start of a supply of as low as 50 μM NH_4^+ (Yamaya et al. 1995). A similar response was seen in rice cells in suspension culture (Watanabe et al. 1996). Those results indicate that NADH-GOGAT in rice roots plays an important role in the generation of glutamate for the assimilation of NH_4^+ via the GS reaction. Cellular localization studies are one approach in establishing a role for NADH-GOGAT in the nitrogen metabolism of roots. However,

there has been no report on the cellular localization of either NADH-GOGAT or Fd-GOGAT protein in plant roots.

In this investigation, we examine the cellular localization of the two GOGAT proteins and of cytosolic GS by using immunocytological methods in roots of rice seedlings which were supplied or not supplied with NH_4^+.

Materials and methods

Plant material. Rice (*Oryza sativa* L. cv. Sasanishiki) seeds were soaked in distilled water at 30 °C for 1 d and 40 germinated seeds were transferred to a nylon net, floating on tap water that had been adjusted to pH 5.5, in a 12-l plastic container. Seedlings were grown in water for 26 d in a greenhouse. On the 26th day, when the endosperm had been thoroughly utilized, the seedlings were transferred to quarter-strength basal nutrient solution which contained either no nitrogen or 1 mM NH_4Cl. Seedlings were harvested 24 h later, as described previously (Yamaya et al. 1995). Approximately 10-cm-long crown roots were selected, cut into 1-cm-long segments from the tip, and weighed before being frozen in liquid nitrogen. Segments were numbered from the tip. They were stored at −80 °C for biochemical experiments. For immunocytological experiments, two cross-sections (the tip to 5 mm; 5–10 mm) of crown roots were placed in FAA solution [1.85% (v/v) formaldehyde, 5% (v/v) acetic acid, and 63% (v/v) ethanol]. At least triplicate analyses were performed with independent samples for immunoblotting and immunolocalization.

Immunocytology. The sections were fixed in FAA solution for 48 h at 4 °C. The fixed roots were then sequentially dehydrated, embedded in paraffin, and trimmed as described previously (Hayakawa et al. 1994). Segments of the trimmed roots, corresponding to the zones 0–2 mm from the tip and 8–10 mm from the tip, were sliced into 10-μm cross-sections or longitudinal sections, stretched onto a glass slide, deparafinized, rinsed, and reacted with antibodies (Hayakawa et al. 1994). For observation of the root anatomy, the 10-μm sections were also stained with 0.05% (v/v) Toluidine Blue O (Hayakawa et al. 1994). Polyclonal immunoglobulin G (IgG) raised against either NADH-GOGAT purified from rice cell cultures (Hayakawa et al. 1992), purified rice-leaf Fd-GOGAT (Yamaya et al. 1992), or a synthetic GS1-peptide-ovalbumin conjugate (Kamachi et al. 1992) was used. Each of the IgGs was purified with corresponding antigens (Yamaya et al. 1992), before use in the present study. 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., Burlingame, Calif., USA) as described previously (Hayakawa et al. 1994).

Quantification of NADH-GOGAT and Fd-GOGAT proteins. The frozen 1-cm-long root segments were ground to a fine powder and then homogenized in 50 mM potassium phosphate (pH 7.5), 0.2% (v/v) 2-mercaptoethanol, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 30 μM leupeptin, 500 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 100 mM KCl, and 0.5% (v/v) Triton X-100 [3 ml · (g FW)$^{-1}$]. The crude protein fraction was prepared from the homogenate, separated by SDS-PAGE, and immunoblotted with the corresponding affinity-purified IgG as described previously (Hayakawa et al. 1994; Yamaya et al. 1995). The immunoreacted GOGAT polypeptides were visualized and quantified densitometrically, using purified GOGAT proteins as the standard for the calibration, as described previously (Hayakawa et al. 1993). The Bradford method (1976) was used to quantify the soluble protein content. Bovine serum albumin was used as the standard.

Results

Sequential segments were taken along the 10-cm-long crown roots of rice seedlings that had been grown on water for 26 d and treated with 1 mM NH_4Cl for a further day. Contents of NADH-GOGAT and Fd-GOGAT proteins in each segment before and after the supply of NH_4Cl were estimated by immunoblotting procedures (Fig. 1). Soluble protein contents were

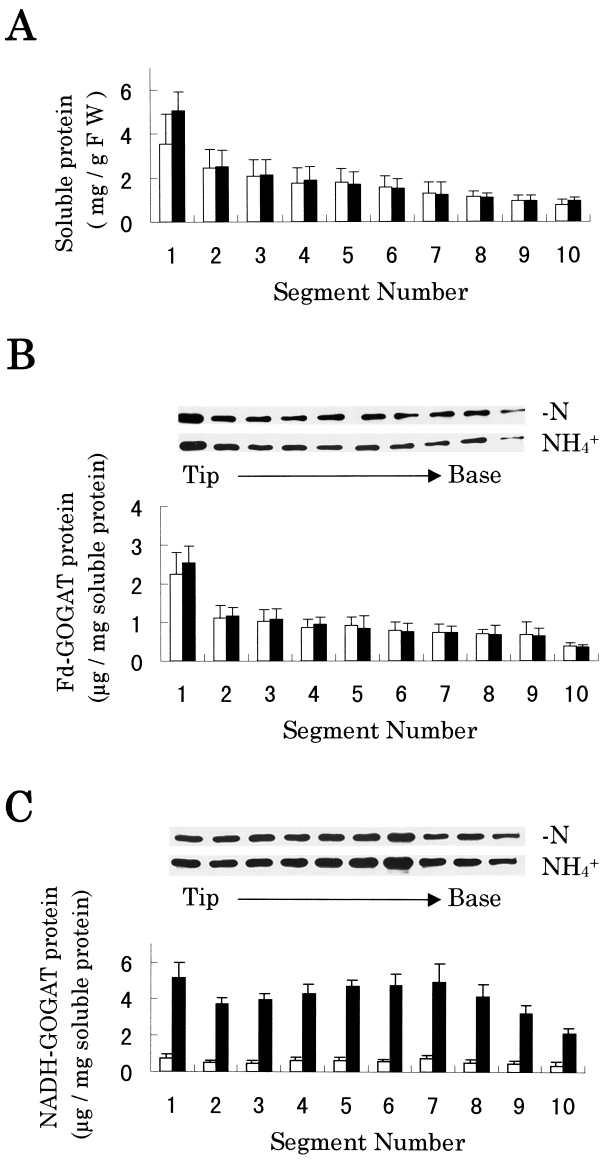


Fig. 1A–C. Determination of soluble protein content **A**, Fd-GOGAT protein content **B** and NADH-GOGAT protein content **C** in segments of rice roots. One-centimeter transverse sections were taken from the tip (segment 1) to the base of ca. 10-cm-long crown roots. No nitrogen (*open bars*) or 1 mM NH_4^+ (*closed bars*) was supplied to 26-d-old seedlings for 24 h. Data are means of independent triplicate samples and SD values ($n = 3$) are indicated. Western immunoblots are also shown. Three micrograms of protein from all segments was separated by SDS-PAGE for the immunoblotting of Fd-GOGAT, while 3 μg (segment 1) and 5 μg (segments 2–10) from the no-nitrogen roots and 0.5 μg (segment 1) and 1 μg (segments 2–10) from the NH_4^+-treated roots were applied for the detection of NADH-GOGAT protein

highest in the root-tip segments (segment number 1) in both treatments (Fig. 1A), while the fresh weight was relatively constant (results not shown). The content of Fd-GOGAT protein was also highest in the root-tip segments and gradually declined as a function of age toward the root base (Fig. 1B). There was no large difference in Fd-GOGAT content between segments prepared from the roots before or after the supply of NH_4Cl. In contrast, the content of NADH-GOGAT protein in all segments was markedly increased after the supply of NH_4Cl (Fig. 1C). The root-tip segment and segments 5–7 showed relatively higher contents of NADH-GOGAT protein. Secondary root formation was observed in the middle part of the roots (results not shown). Thus, the NH_4Cl-mediated large increase in NADH-GOGAT protein seen in the whole roots of rice (Yamaya et al. 1995) was caused by the increase in all segments, and not by an increase in any specific part of the roots. Our previous results showed that changes in the content of NADH-GOGAT protein in the whole roots was essentially the same as those in the activity of the enzyme (Yamaya et al. 1995). Although the activity of NADH-GOGAT was not measured in the current study, because of the limited amounts of samples, it is assumed that the activity also increases in all segments of the crown roots.

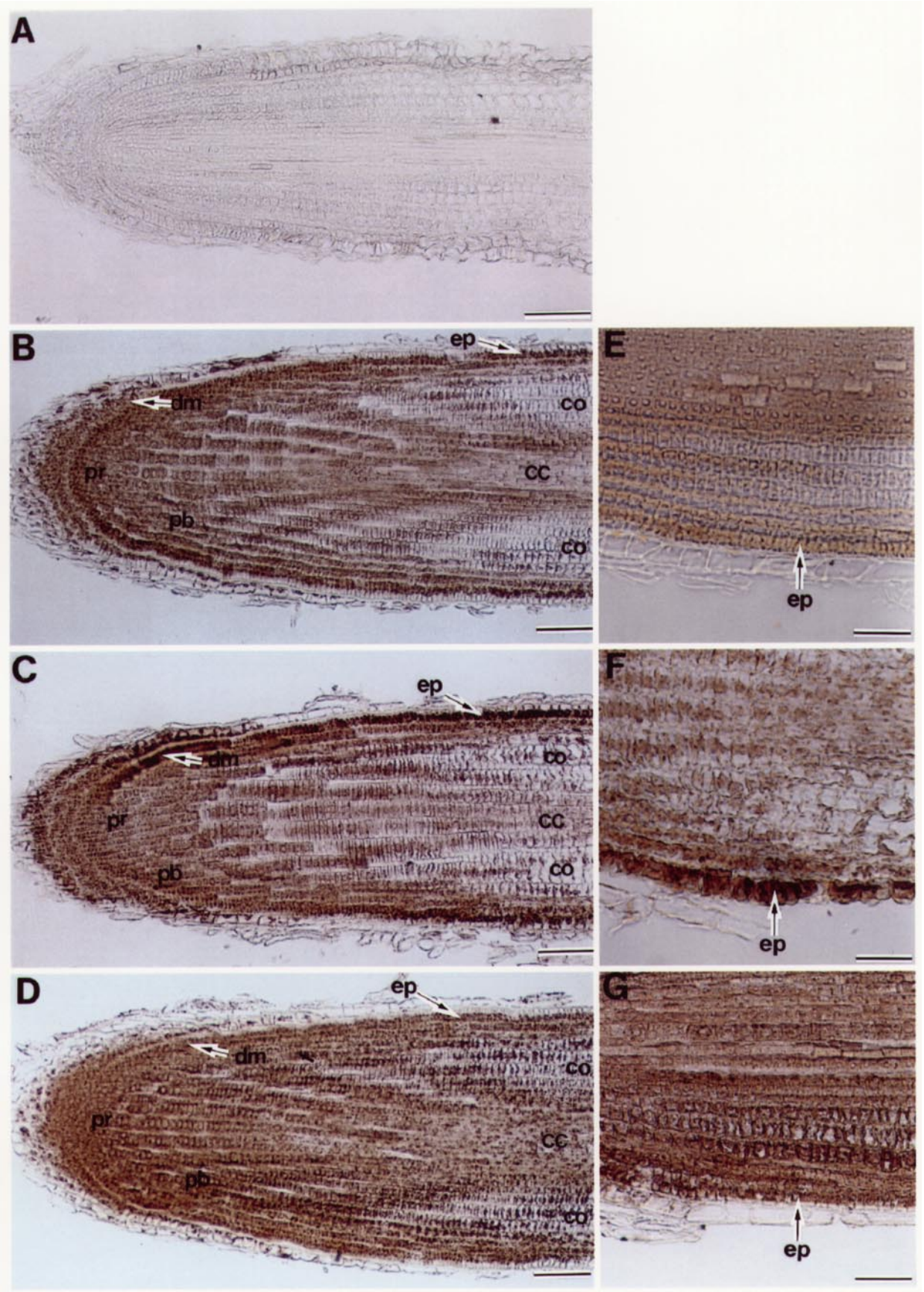
Our affinity-purified anti-NADH-GOGAT IgG (Hayakawa et al. 1994), anti-Fd-GOGAT IgG (Hayakawa et al. 1994), and anti-cytosolic-GS (Sakurai et al. 1996) cross-reacted monospecifically with the corresponding antigens in rice plants. The root-tip segments were chosen to locate both NADH- and Fd-GOGAT, because of the high contents of these proteins. Ten-micrometer longitudinal sections of the root tip (0–2 mm from the tip) were prepared from paraffin-embedded root segments, and GOGAT proteins were stained with either the NADH-GOGAT IgG or Fd-GOGAT IgG (Fig. 2). Signals for NADH-GOGAT were detected in the region of the central cylinder and apical meristem, i.e. dermatogen cells, plerome cells, and periblem cells (Fig. 2B). The signal detected in root cap cells, epidermal cells, exodermal cells, and sclerenchyma cells was relatively minor. Cortical cells from the tip to ca. 250 μM were stained by the NADH-GOGAT IgG, but those beyond the 250-μm region were not. The supply of NH_4Cl resulted in a more intense staining for the NADH-GOGAT protein in the epidermal region (Fig. 2C,F), than was found in the roots in the absence of NH_4Cl (Fig. 2B,E). However, the supply of NH_4Cl had basically no effect on the distribution of Fd-GOGAT protein which was detected in the area of the central cylinder, apical meristem, and cortical cells below ca. 250 μm from the tip in both treated (Fig. 2D,G) and untreated (data not shown) roots.

To locate the two GOGAT proteins in the elongation zone of rice roots, 10-μm longitudinal sections and cross-sections were taken at 8–10 mm from the tip and prepared and stained with the corresponding IgGs (Fig. 3). In the roots without the supply of NH_4Cl, the signals for NADH-GOGAT were mainly detected in the area of the central cylinder, including the primordia of the secondary roots, and the signals were faint in epidermis and exodermis (Fig. 3A,B). When NH_4Cl was supplied, however, both epidermis and exodermis were strongly stained by the anti-NADH-GOGAT IgG, in addition to the area found in the roots in the absence of NH_4Cl (Fig. 3C,D). The Fd-GOGAT protein was detected mainly in the area of the central cylinder, including the primordia of the secondary roots (Fig. 3E,F). The signals for Fd-GOGAT were also observed in other areas, but were very weak. In contrast to NADH-GOGAT, however, the staining profiles were basically the same with or without the supply of NH_4Cl (results not shown). It is worth noting that both GOGAT proteins were clearly present in the primordia of the secondary roots (Fig. 3A,C,E), whether NH_4Cl was supplied or not. Cytosolic GS protein was detected in most cell types, i.e. epidermis, exodermis, cortex, and in the area of the central cylinder in the roots with (Fig. 3G) or without the supply of NH_4Cl (results not shown). Because plastidic GS can hardly be detected in rice roots (Kamachi et al. 1991), detection with anti-GS2 IgG was not attempted in the present study.

Fig. 2A–G. Cellular localization of NADH-GOGAT and Fd-GOGAT proteins in root tips (0–2 mm from the tip) of rice. **A** Longitudinal section of a root treated with 1 mM NH_4^+ for 24 h. The section was stained with affinity-purified NADH-GOGAT IgG pretreated with an excess amount of the NADH-GOGAT protein as the primary antibody. **B, E** Longitudinal section of a root grown in the absence of a nitrogen supply, and stained with affinity-purified NADH-GOGAT IgG as the primary antibody. **C, F** Longitudinal section of a root treated with 1 mM NH_4^+ for 24 h, and stained with affinity-purified NADH-GOGAT IgG as the primary antibody. **D, G** Longitudinal section of a root treated with 1 mM NH_4^+ for 24 h, and stained with affinity-purified Fd-GOGAT IgG as the primary antibody. *cc*, central cylinder; *co*, cortex; *dm*, dermatogen; *ep*, epidermis; *pb*, periblem; *pr*, plerome. Bars = 50 μm **A–D** and 12.5 μm **E, G**

Discussion

This is the first paper describing the cellular localization of two GOGAT proteins in plant roots and especially determining the specific cells that are responsible for the marked and rapid increase in NADH-GOGAT protein caused by the supply of NH_4Cl to rice roots (Yamaya et al. 1995). These GOGAT species in roots have received much less attention than those in leaves (Sechley et al. 1992), even though they must have crucial roles in maintaining appropriate levels of glutamate in roots. Nitrogen is available in the soil as NH_4^+ or NO_3^-. If the source of nitrogen is NO_3^-, as in the case of most crop plants, some of the NO_3^- could be reduced in the roots, but the majority is transferred first to the xylem and then to the shoots where it can be processed. On the other hand, NH_4^+ taken up by roots or produced by the reduction of NO_3^- is first assimilated within the roots to yield the amide group of glutamine (Lewis et al. 1983; Oaks 1992). The major form of nitrogen that is available for growth of rice plants in paddy fields is NH_4^+, but the site, as well as the mechanism, for its assimilation is largely unknown.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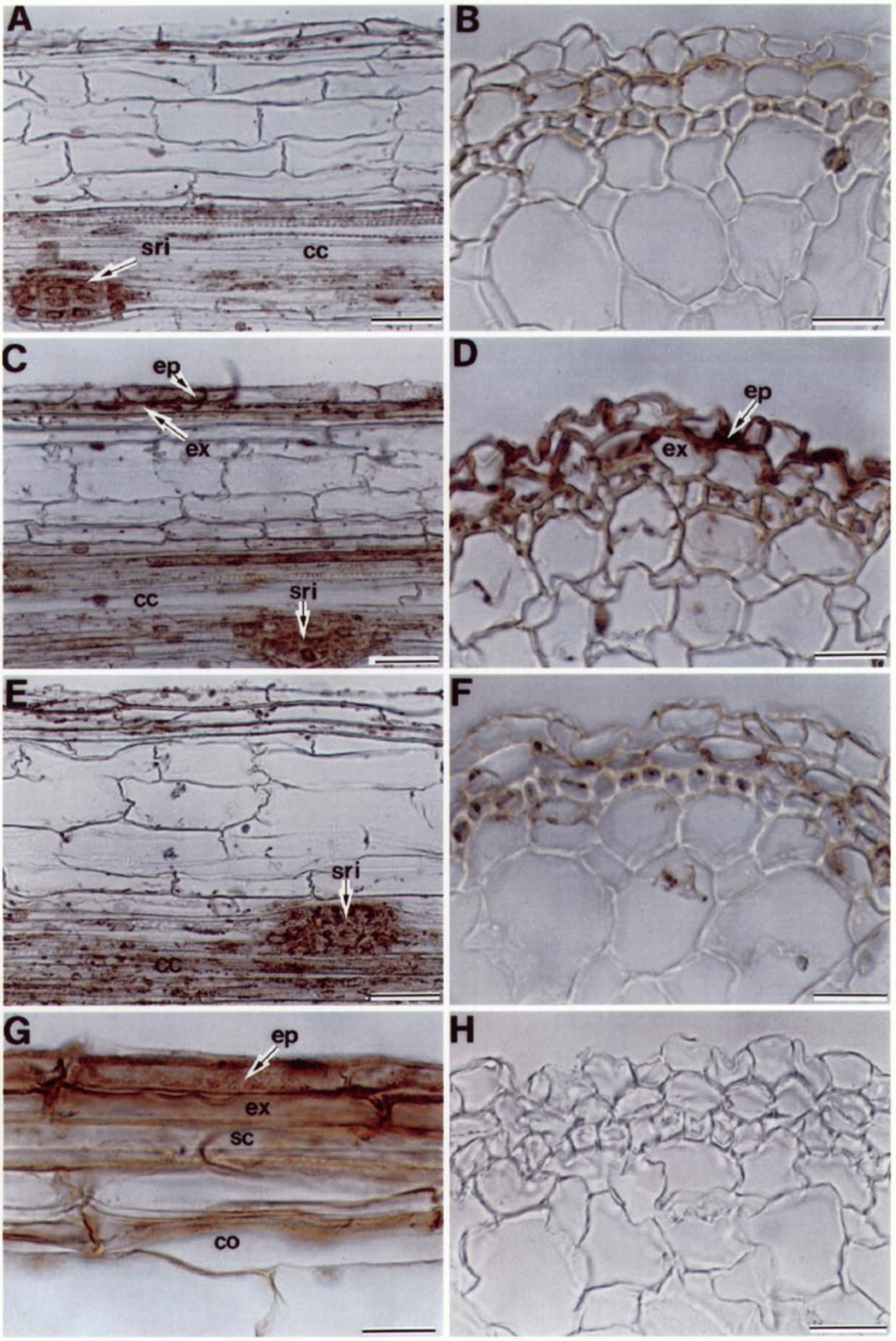
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Fig. 3A–H. Cellular localization of NADH-GOGAT, Fd-GOGAT and cytosolic GS proteins in rice roots at 8–10 mm from the tip. **A, B** Longitudinal **A** and transverse-sections **B** of untreated roots were stained with affinity-purified NADH-GOGAT IgG as the primary antibody. **C, D** Longitudinal **C** and transverse sections **D** of roots treated with 1 mM NH_4^+ for 24 h were stained with affinity-purified NADH-GOGAT IgG as the primary antibody. **E, F** Longitudinal **E** and transverse sections **F** of roots treated with 1 mM NH_4^+ for 24 h were stained with affinity-purified Fd-GOGAT IgG as the primary antibody. **G** Longitudinal section of roots treated with 1 mM NH_4^+ for 24 h was stained with affinity-purified GS1 IgG as the primary antibody. **H** Transverse section of roots treated with 1 mM NH_4^+ for 24 h was stained with affinity-purified NADH-GOGAT pretreated with an excess amount of the NADH-GOGAT protein as the primary antibody. *cc*, central cylinder; *co*, cortex; *ep*, epidermis; *ex*, exodermis; *sc*, sclerenchyma; *sri*, secondary root initial. Bars = 25 μm **A, C, E** and12.5 μm **B, D, F–H**

As described previously (Yamaya et al. 1995), the mRNA, protein, and activity of NADH-GOGAT in whole roots of rice seedlings accumulated within 12 h of the start of supplying a low concentration of NH_4Cl. The current immunolocalization studies clearly showed that the accumulation of NADH-GOGAT protein specifically occurred in two cell layers of the root surface, i.e. epidermis and exodermis, in response to the supply of NH_4Cl. This accumulation was strongly correlated with the marked increase in NADH-GOGAT protein in the roots as determined by Western blotting. However, it cannot be concluded that whether the latter increase is totally caused by the accumulation of the enzyme protein in those two cell layers, because of the presence of the enzyme protein in other cell types, such as the apical meristem and central cylinder, both in the presence and absence of NH_4Cl, and because of the lack of quantitative estimation of the NADH-GOGAT protein in each cell type under the current localization studies. However, because cytosolic GS protein was also located in the epidermis and exodermis, most of the NH_4^+ taken up by the roots could be immediately assimilated by the cytosolic GS/NADH-GOGAT system in these two cell layers. Recent anatomical studies indicate that there is a Casparian strip between the exodermis and cortex in rice roots (Morita et al. 1996), indicating that solute transport between these cell types should be a symplastic process. The NADH-GOGAT in the epidermis and exodermis could have the function of providing the glutamate required for the cytosolic GS reaction to assimilate most of the NH_4^+ in those compartments. Also, the formation of glutamate by GOGAT may be related to the cell-to-cell movement of nitrogen in root tissues. Absorption of NH_4^+ by rice roots occurs in both the root-tip area and the area where the secondary roots are actively developing (Tatsumi 1982). The distribution of NADH-GOGAT protein along the roots as shown in Fig. 1C also supports a function for NADH-GOGAT here. Because there was little change in the expression of Fd-GOGAT protein in the epidermal and exodermal cells before and after the supply of NH_4Cl, the Fd-GOGAT is probably not directly related to the primary assimilation of the ions in rice roots under the current experimental conditions.

When rice plants were grown without a supply of NH_4Cl, both NADH-GOGAT and Fd-GOGAT proteins were detected in the central cylinder, the apical meristem area (plerome cells, periblem cells, dermatogen cells) and the primordia of the secondary roots. Functions for NADH-GOGAT and Fd-GOGAT located in these cell types are not yet easily understood. The two GOGATs located in the apical meristem and the primordia of the secondary roots, which are actively developing tissues, would be involved in the synthesis of glutamate from the glutamine that is transported through the phloem (Hayashi and Chino 1990), as is the function of NADH-GOGAT in young grains and leaf blades of rice (Hayakawa et al. 1994). Although the specific cell types in the central cylinder could not be identified at the magnification used in the present study, this area could also be important for the transport of solutes from the phloem to the actively developing roots, as well as from root surface to xylem vessel elements. Because the major forms of nitrogen in both xylem sap and phloem sap of rice are glutamine and asparagine (Fukumorita and Chino 1982; Hayashi and Chino 1990), cytosolic GS and both GOGAT species detected in the central cylinder may function in the long-distance transport of nitrogen, and the two GOGATs may function in the re-utilization of glutamine transported from the shoots. Further work is required to resolve the function of the two GOGAT species in roots. In contrasts to rice grains and leaves, spatial separation of these GOGAT proteins was not observed in the developing meristem and central cylinder, indicating that they could have identical or overlapping roles for some aspects of nitrogen metabolism in rice roots in the absence of a nitrogen supply.

We thank Dr. Ann Oaks, University of Guelph, Canada, and Dr. Alyson K. Tobin, University of St. Andrews, UK, for their helpful comments and critical reading of the manuscript. This work was supported in part by a program of Research for the Future from the Japan Society for the Promotion of Science (JSPS-RFTF96L00604), in part by Grant-in-Aid for Scientific Research on Priority Area (Nos. 09274101 and 09274102), and in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 08044187).

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