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Tissue Distribution of Glutamate Synthase and Glutamine Synthetase in Rice Leaves[1]

Occurrence of NADH-Dependent Glutamate Synthase Protein and Activity in the Unexpanded, Nongreen Leaf Blades

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ABSTRACT

To further explore the function of NADH-dependent glutamate synthase (GOGAT), the tissue distribution of NADH-GOGAT protein and activity was investigated in rice (*Oryza sativa* L.) leaves. The distributions of ferredoxin (Fd)-dependent GOGAT, plastidic glutamine synthetase, and cytosolic glutamine synthetase proteins were also determined in the same tissues. High levels of NADH-GOGAT protein (33.1 μg protein/g fresh weight) and activity were detected in the 10th leaf blade before emergence. The unexpanded, nongreen portion of the 9th leaf blade contained more than 50% of the NADH-GOGAT protein and activity per gram fresh weight when compared with the 10th leaf. The expanding, green portion of the 9th leaf blade outside of the sheath contained a slightly lower abundance of NADH-GOGAT protein than the nongreen portion of the 9th blade on a fresh weight basis. The fully expanded leaf blades at positions lower than the 9th leaf had decreased NADH-GOGAT levels as a function of increasing age, and the oldest, 5th blade contained only 4% of the NADH-GOGAT protein compared with the youngest 10th leaf blade. Fd-GOGAT protein, on the other hand, was the major form of GOGAT in the green tissues, and the highest amount of Fd-GOGAT protein (111 μg protein/g fresh weight) was detected in the 7th leaf blade. In the nongreen 10th leaf blade, the content of Fd-GOGAT protein was approximately 7% of that found in the 7th leaf blade. In addition, the content of NADH-GOGAT protein in the 10th leaf blade was about 4 times higher than that of Fd-GOGAT protein. The content of plastidic glutamine synthetase polypeptide was also the highest in the 7th leaf blade (429 μg/g fresh weight) and lowest in nongreen blades and sheaths. On the other hand, the relative abundance of the cytosolic glutamine synthetase polypeptide was the highest in the oldest leaf blade, decreasing to 10 to 20% of that value in young, nongreen leaves. These results suggest that NADH-GOGAT is important for the synthesis of glutamate from the glutamine that is transported from senescing source tissues through the phloem in the nongreen sink tissues in rice leaves.

The major source of nitrogen for developing leaves and ears of mature rice plants is the nitrogen released from older, senescing leaves. For example, previous studies with ^{15}N showed that remobilized nitrogen accounted for 64% of the total nitrogen in the youngest leaf blades (17). In phloem sap of rice plants, Gln and Asn, which is also synthesized from Gln (16, 19, 21), accounted for 42 and 12%, respectively, of the total amino acids (9). On the other hand, Glu is a major free amino acid in both young and mature leaf blades of rice (12). Thus, because Gln is the major form of remobilized nitrogen in the phloem, it should be converted into Glu in developing sink tissues of rice plants. Our previous studies showed that the tissue localization of the GS1[2] polypeptide was specific to the vascular bundles of rice leaves (11). Studies of transgenic plants by Edwards et al. (6) showed that a promoter for GS of pea nodules was expressed in phloem elements. Thus, GS1 in senescing leaves is apparently responsible for the remobilization of leaf nitrogen. There is, however, no information concerning the origin of Glu in expanding sink leaves.

GOGAT is a likely candidate for the synthesis of Glu in such tissues. In higher plants, there are at least two GOGATs (16, 18, 21), one requiring reduced Fd as reductant (Fd-GOGAT, EC 1.4.7.1) and the other NADH (NADH-GOGAT, EC 1.4.1.14). These two GOGATs are immunologically distinct proteins because antibody raised against NADH-GOGAT purified from rice cell cultures does not recognize Fd-GOGAT purified from rice leaves (8) and, conversely, anti-Fd-GOGAT antibody does not cross-react with NADH-GOGAT (8, 26). Fd-GOGAT is the major form in green tissues (16, 25, 26), and based on immunocytological studies, it is localized in the chloroplast stroma (3). Research with mutants lacking Fd-GOGAT showed that the major function of Fd-GOGAT in green leaves, together with GS2 (27), is in the

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[2] Abbreviations: GS1, cytosolic glutamine synthetase; GS, glutamine synthetase; GS2, plastidic glutamine synthetase; GOGAT, glutamate synthase; IgG, immunoglobulin G; TBS, 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl; TTBS, TBS +0.2% (v/v) Tween-20.

reassimilation of NH_4^+ released during photorespiration (14, 23).

Compared with Fd-GOGAT, much less is known about NADH-GOGAT in plant tissues, except in legume nodules (1, 4). For example, neither the precise tissue and subcellular localization of NADH-GOGAT nor molecular genetic studies has yet been carried out (8, 16). A major reason for this is that NADH-GOGAT is much less active than Fd-GOGAT in green tissues (16, 18). However, because barley (14) and *Arabidopsis* (20, 23) mutants lacking Fd-GOGAT grow normally under nonphotorespiratory conditions, NADH-GOGAT could have an important role in the synthesis of Glu for plant growth and development. In fact, *Arabidopsis* mutants lacking Fd-GOGAT contained sufficient NADH-GOGAT activity to account for the primary assimilation of nonphotorespiratory NH_4^+ (20). Recently, we prepared polyclonal antibodies against NADH-GOGAT purified from rice cell cultures (8). Therefore, immunodetection of NADH-GOGAT protein in rice plants is now possible. In this paper, we describe the distribution of the NADH-GOGAT protein in various developing stages of rice leaves using the anti-NADH-GOGAT antibody. The results suggest that NADH-GOGAT is responsible for the synthesis of Glu in expanding nongreen sink leaves.

MATERIALS AND METHODS

Plant Material and Harvest

Rice (*Oryza sativa* L. cv Sasanishiki) plants were grown in hydroponic culture in a greenhouse for 75 d after imbibition, as described previously (12). We chose 12 plants at a nine-leaf stage and at the stage at which leaf blades 5 (the lowest position) to 8 on the main stem were fully expanded. The 9th leaf was cut into three segments, i.e. expanding green blade outside of the 8th leaf sheath, nongreen leaf blade inside of the sheath, and nongreen leaf sheath. The nongreen 10th leaf inside of the 9th leaf sheath, the three segments of the 9th leaf, and all fully expanded leaf blades and leaf sheaths were harvested. These samples were weighed, frozen in liquid nitrogen, and stored at −80°C until extracted.

Preparation of Leaf Extracts

The frozen samples (0.45–1.3 g fresh weight) were homogenized with a mortar and pestle in the presence of washed sand in extraction buffer for GOGAT as described previously (7). The homogenate was centrifuged at 39,100*g* for 30 min. The supernatant fraction was used for the immunoblotting analyses of NADH-GOGAT, Fd-GOGAT, GS1, and GS2. When both GOGAT activities were assayed, the supernatant fraction was desalted by passing through a Sephadex G-25 column, and the filtrate was used as the enzyme solution.

Assays of Enzyme Activity and Soluble Protein

Activities of NADH-GOGAT and Fd-GOGAT were assayed as described previously (7). One unit of enzyme activity was defined as that amount that synthesized 1 μmol of Glu per min at 30°C. Duplicate independent samples were used, and the enzyme assays were performed with triplicate reactions. Soluble protein content was determined by the method of Bradford (2) using BSA as the standard.

Affinity Purification of Anti-NADH-GOGAT IgG and Anti-Fd-GOGAT IgG

Polyclonal antibody raised against the rice cell NADH-GOGAT (8) was further purified with the antigen bound to a nylon membrane (Zeta-probe membrane, Bio-Rad) by modification of the method of Kelly et al. (13). About 10 μg of purified NADH-GOGAT (8) was subjected to SDS-PAGE (7% [w/v] gel) according to the method of Laemmli (15) and electrotransferred to the nylon membrane as described previously (8, 12). Two side strips were cut from the membrane, reacted with the anti-NADH-GOGAT IgG, and visualized with a goat anti-rabbit IgG alkaline phosphatase conjugate (Promega). The region containing NADH-GOGAT on the membrane was excised, and the membrane strips (0.5 × 2.5 cm) were incubated in 50 mL of TBS containing 5% (w/v) skim milk (TBS-milk) for 2 h and then twice in 50 mL of TTBS-milk for 20 min at 37°C. Each strip was then incubated with a 1:20 dilution of the anti-NADH-GOGAT-IgG in 6 mL of TTBS-milk for 12 h at 37°C. The strips were rinsed three times with 50 mL of TTBS-milk for 20 min each and finally with 50 mL of TBS for 5 min at 37°C. The bound IgGs were eluted by incubating the strips with 1 mL of 0.2 M glycine-HCl (pH 2.5) for 2 min at room temperature. The eluates were immediately neutralized by adding 0.17 mL of 1.0 M Tris-HCl (pH 8.8) containing 6.9% (w/v) BSA and stored at 4°C after addition of NaN_3 to give a final concentration of 0.05% (w/v).

Polyclonal antibody raised against the Fd-GOGAT purified from rice leaves (7) was also affinity purified by the same methods as described above.

Quantitation of Two GOGATs and GS Isoforms by Immunoblotting Analyses

The soluble proteins from rice tissues were separated by SDS-PAGE (7% [w/v] for GOGAT and 12.5% [w/v] for GS) as described above. The separated polypeptides were transferred either to a Zeta-probe membrane for NADH-GOGAT detection or to a nitrocellulose membrane for Fd-GOGAT, GS1, and GS2 by electroblotting as described above. The polypeptides were then reacted with either the affinity-purified anti-NADH-GOGAT IgG, the affinity-purified anti-Fd-GOGAT IgG, anti-GS1 IgG raised against synthetic peptide-ovalbumin conjugate (10), or anti-GS2 IgG raised against rice leaf GS2 (12). The reacted polypeptides were visualized as described above. The intensity of the developed color on the membrane was quantitated using a reflective densitometer (Shimadzu CS-930) to scan the bands at 530 nm.

When the actual amounts of NADH-GOGAT protein were estimated, the soluble proteins prepared from leaf blades and sheaths were separately run on SDS-PAGE having larger volume (about 60 μL) sample wells because of the enzyme's low content in the soluble protein fraction. One lane of each slab gel was loaded with 50 ng of purified NADH-GOGAT (8) as a standard. Various amounts of the purified NADH-GOGAT and 20 μg of soluble protein prepared from the

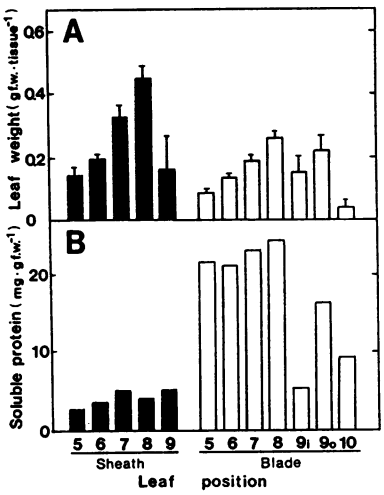

Figure 1. Determination of tissue fresh weight (f.w.) (A) and soluble protein content (B) at various leaf blade (white bars) and leaf sheath (black bars) positions in rice plants. Numbers indicate leaf positions. The expanding, outer green portion and nongreen, inner portion of the 9th leaf blade are indicated by 9o and 9i, respectively. Error bars, ±SD.

nongreen portion of the 9th leaf blade were also run, and the amounts of NADH-GOGAT protein in all samples were calibrated. Similarly, 30 ng of the purified Fd-GOGAT (7) and 20 ng of the purified GS2 (12) were run when actual amounts of Fd-GOGAT and GS2 in extracts from various tissues were estimated, respectively. The densitometric determination was performed in a linear range determined with those standards. Because we lack homogeneous GS1, the contents of GS1 polypeptide in the samples were indicated as the percentage of maximum peak area after the densitometric scanning of the bands, as described previously (10). Independent duplicate analyses were performed for immunoblotting of each enzyme, and essentially the same results were obtained.

RESULTS

Changes in Fresh Weight and Soluble Protein Content of Leaf Blades and Sheath Tissues at Different Leaf Positions of Rice Plants

Leaf blades and sheaths at positions from the oldest (5th) to the youngest (10th) leaves on the main stem of 12 rice plants were harvested individually and weighed (Fig. 1A). At this sampling time, the 10th leaf was totally inside of the 9th leaf sheath, and nearly half of the 9th leaf blade had emerged. Soluble protein contents in fully expanded green leaf blades were relatively constant at about 20 mg per g fresh weight, whereas nongreen blades of the 10th leaf and the nongreen portion of the 9th leaf blade contained about 9 and 5 mg of protein, respectively (Fig. 1B). Leaf sheaths at all positions contained less than 5 mg of protein per g fresh weight.

Distribution of NADH-GOGAT and Fd-GOGAT in Rice Leaves

Polyclonal antibodies against NADH-GOGAT (8) and Fd-GOGAT (7) were affinity purified to eliminate nonspecific binding of these antibodies. Because our anti-NADH-GOGAT IgG was prepared against NADH-GOGAT purified from rice cell cultures (8), the cross-reactivity and specificity of the IgG against NADH-GOGAT in rice leaves were first tested with crude extracts prepared from the nongreen (mixture of the 9th and 10th leaves) and green leaves (Fig. 2A). A single band at the same position as rice cell NADH-GOGAT was clearly detected by immunoblotting after SDS-PAGE when the extracts from the nongreen leaves were tested. A much fainter band was also detected at the same position when the extracts from green leaf blades and green sheaths were tested. Thus, the purified anti-NADH-GOGAT IgG monospecifically recognized NADH-GOGAT in rice leaves as well as the rice cell enzyme. In addition, the relative content of NADH-GOGAT protein on a unit soluble protein basis in the nongreen leaves was higher than that in green

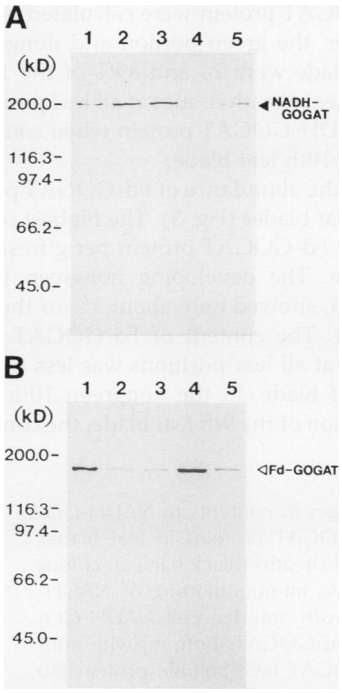

Figure 2. Monospecificity of anti-NADH-GOGAT IgG and anti-Fd-GOGAT IgG to GOGAT proteins in rice tissue extracts. A, Immunoblotting of the purified rice cell NADH-GOGAT (lane 1, 60 ng of protein) and crude extracts of rice cells (lane 2, 15 μg of protein), nongreen rice leaves (lane 3, 50 μg of protein), green leaf blades (lane 4, 50 μg of protein), and green leaf sheaths (lane 5, 50 μg of protein) with anti-rice cell NADH-GOGAT IgG after SDS-PAGE. B, Immunoblotting of the purified rice leaf Fd-GOGAT (lane 1, 30 ng) and crude extracts of rice cells (lane 2, 50 μg), nongreen leaves (lane 3, 10 μg of protein), green leaf blades (lane 4, 5 μg of protein), and green leaf sheaths (lane 5, 5 μg of protein) with anti-rice leaf Fd-GOGAT IgG. The nongreen leaves were a mixture of the 9th and 10th leaves, whereas the green leaves were a mixture of the 7th and 8th leaves.

leaves and sheaths of rice plants. Similarly, the affinity-purified anti-Fd-GOGAT IgG was monospecific to Fd-GOGAT in crude extracts from green leaf blades and sheaths, nongreen leaves, and rice cell cultures (Fig. 2B). As confirmed with NADH-GOGAT purified from rice cell cultures in our previous work (8), NADH-GOGAT and Fd-GOGAT in the extracts from rice leaves were immunologically distinct proteins.

The amounts of NADH-GOGAT protein were estimated from rice leaves of various ages by immunoblotting (Fig. 3). The contents of Fd-GOGAT protein were also determined in the same tissue extracts with the anti-Fd-GOGAT IgG. The highest contents of NADH-GOGAT protein were detected in nongreen developing leaf blades, i.e. the 10th leaf blade before its emergence contained 33.1 μg of NADH-GOGAT protein per g fresh weight. The nongreen portion of the 9th leaf blade inside of the 8th leaf sheath contained 17.7 μg of NADH-GOGAT protein per g fresh weight, which was 2- to 13-fold higher than that detected in the fully expanded leaf blades. The expanding green portion of the 9th leaf blade outside of the 8th sheath showed a slightly lower content of NADH-GOGAT protein than the nongreen portion of the same blade on a unit fresh weight basis. When the contents of NADH-GOGAT protein were calculated on a unit protein basis, however, the green portion and nongreen portion of the 9th leaf blade were 24 and 89% of the 10th leaf blade, respectively. Leaf sheath tissues at all leaf positions contained much less NADH-GOGAT protein when compared with the content in the 10th leaf blade.

In contrast, the abundance of Fd-GOGAT protein was high in the green leaf blades (Fig. 3). The highest content detected was 111 μg of Fd-GOGAT protein per g fresh weight in the 7th leaf blade. The developing nongreen leaf blades, on the other hand, showed only about 7% of the content in the 7th leaf blade. The content of Fd-GOGAT protein in leaf sheath tissues at all leaf positions was less than 10% of that in the 7th leaf blade. In the nongreen 10th blade and the nongreen portion of the 9th leaf blade, the content of NADH-GOGAT protein was 4.1- and 2.6-fold higher than that of Fd-GOGAT protein, respectively. In other green blades and green sheaths of rice leaves, however, Fd-GOGAT protein was the predominant GOGAT form.

Table I. *Activities of NADH- and Fd-Dependent GOGATs at Various Leaf Blade and Sheath Positions of Rice Plants*

Values of activity are the means of triplicate assays with independent duplicate samples.

Leaf Position from Base	NADH-GOGAT	Fd-GOGAT	Ratio of Fd-GOGAT/ NADH-GOGAT
	munit·(g fresh weight)$^{-1}$		
Leaf blade			
5th	25.2	7250	288
6th	37.0	7350	199
7th	31.6	8350	264
8th	45.6	5660	124
9th inside (nongreen)	89.2	349	3.9
9th outside (green)	31.7	5190	164
10th	155	297	1.9
Leaf sheath			
5th	28.2	658	23.3
6th	19.5	705	36.2
7th	29.0	570	19.7
8th	21.7	278	12.8
9th	21.8	127	5.8

The activities of NADH-GOGAT and Fd-GOGAT changed nearly in parallel with the corresponding GOGAT protein levels in the tissues (Table I), although the relative content of NADH-GOGAT protein was slightly higher than its relative activity in the outer green portion of the 9th leaf blade (Fig. 3B). When the ratio of Fd-GOGAT activity to NADH-GOGAT activity was calculated, the green leaf blades and leaf sheaths contained about 10^2 and 10^1 higher levels of Fd-

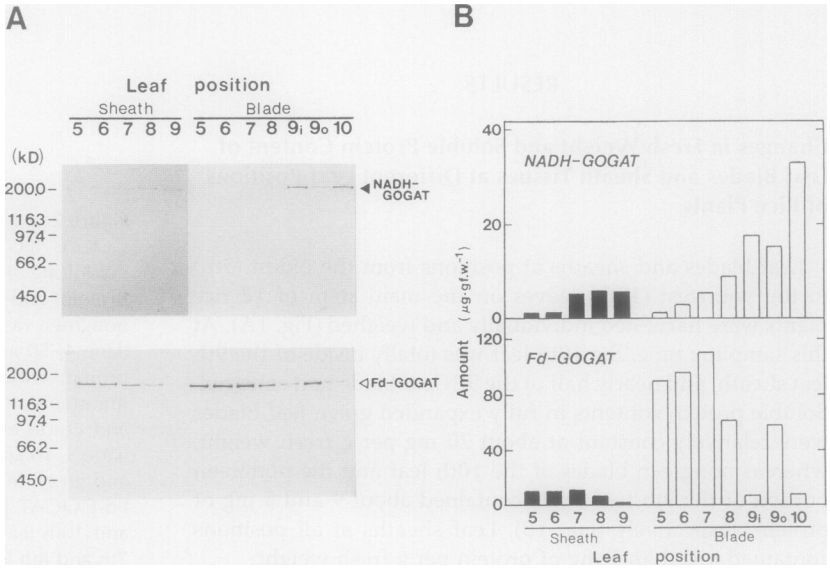

Figure 3. Changes in contents of NADH-GOGAT and Fd-GOGAT proteins in leaf blades (white bars) and sheaths (black bars) at various leaf positions. A, Immunoblotting of NADH-GOGAT (top) with anti-rice cell NADH-GOGAT IgG and Fd-GOGAT (bottom) with anti-rice leaf Fd-GOGAT IgG. Soluble protein (30 μg) from leaf blades and protein (10 μg) from leaf sheaths (for NADH-GOGAT) or 5 μg of protein from either the blades or sheaths (for Fd-GOGAT) were separated by SDS-PAGE at a 7.0% (w/v) gel concentration. B, The intensity of developed color on the membranes in (A) was measured by a densitometer, and peak areas were expressed as micrograms of GOGAT protein per gram fresh weight (f.w.) using purified NADH-GOGAT (8) or Fd-GOGAT (7) as the standard. The expanding, outer green portion and nongreen, inner portion of 9th leaf blade are indicated by 9o and 9i, respectively.

GOGAT than NADH-GOGAT, respectively. Thus, Fd-GOGAT was more active than NADH-GOGAT in green tissues of rice plants. On the other hand, nongreen blades contained approximately the same levels of the two activities. The turnover number of NADH-GOGAT purified from rice cell cultures was 1920 mol of Glu formed min^{-1} mol^{-1} of enzyme protein (8). This value is about one-quarter of the turnover number estimated for Fd-GOGAT purified from rice leaves (25). The finding that the nongreen leaves contained higher NADH-GOGAT protein but the activity was similar to Fd-GOGAT could partly be caused by such differences in the turnover number of each GOGAT.

Distribution of GS1 and GS2 in Rice Leaves

The distribution profile of the GS2 polypeptide was similar to that of Fd-GOGAT protein among the various tissues (Fig. 4). The nongreen leaf blades and leaf sheaths contained much less GS2 polypeptide on a unit fresh weight basis. These two enzymes are located in mesophyll cell chloroplasts (11) and are thought to function in the reassimilation of NH_4^+ released during photorespiration (16).

The relative content of GS1 polypeptide was the highest in the oldest, 5th leaf blade and gradually declined toward the youngest nongreen blade (Fig. 4). Leaf sheaths at all positions contained GS1 polypeptide at levels similar to the 8th and 9th green leaf blades. Because GS1 is specifically localized in vascular bundles of rice leaf blades (11), the results obtained in the current study suggest that the relative content of vascular bundles on a unit fresh weight basis is higher in older leaf blades than the younger tissues.

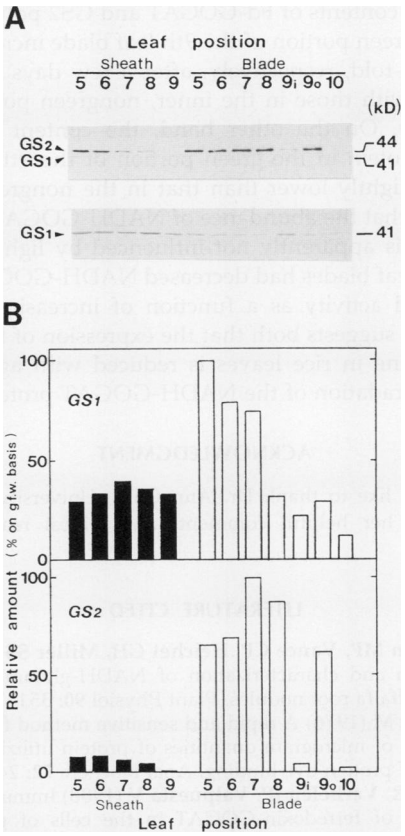

Figure 4. Changes in the polypeptide content of GS1 and GS2 in leaf blades (white bars) and sheaths (black bars) at various leaf positions. A, Immunoblotting of GS isoforms with anti-rice leaf GS2 IgG (top) and anti-GS1 IgG (bottom). Protein (2 μg) from both blade and sheath tissues (for GS2) or 5 μg of protein (for GS1) were separated by SDS-PAGE at a 12.5% (w/v) gel concentration. B, The immunoblots in A were scanned with a densitometer, and the peak areas were expressed as the percentage of maximum on a fresh weight (f.w.) basis. The maximum (=100%) value for GS2 content detected in the 7th leaf blade was 429 μg of GS2 polypeptide/g fresh weight using purified GS2 (10) as the standard. Because homogeneous GS1 was not available, GS1 contents were expressed only as relative values. The expanding, outer green portion and nongreen, inner portion of 9th leaf blade are indicated by 9o and 9i, respectively.

DISCUSSION

This is the first paper describing the distribution of NADH-GOGAT protein in various tissues of rice plants. Because barley (14) and *Arabidopsis* (20, 23) mutants lacking Fd-GOGAT grow normally under nonphotorespiratory conditions, NADH-GOGAT could be important for other aspects of Glu metabolism, not only in those mutants (20) but also in normal plants. However, there is little information available concerning the function of NADH-GOGAT.

A high abundance of NADH-GOGAT protein, as well as activity, was detected in nongreen and growing leaf blades of rice. On the other hand, Fd-GOGAT protein was the major form of GOGAT in green leaves. This complementary distribution of GOGAT proteins in rice leaves likely reflects the different functions of these two enzymes. The high content of NADH-GOGAT in such sink leaves suggests that this GOGAT catalyzes the conversion of Gln, which is transported from senescing source tissues through the phloem (9), to Glu, a major free amino acid in rice leaves (12). GS1 protein is located specifically in vascular bundles of both mature and senescing rice leaves (11), whereas GS2 and Fd-GOGAT proteins, two enzymes involved in photorespiratory nitrogen metabolism (14, 20, 23, 27), are detected in mesophyll cells of the leaf blades (11). If our interpretation of the function of GOGAT proteins is correct, it is possible that the intercellular localization of NADH-GOGAT protein differs from that of Fd-GOGAT. Tissue-print immunoblot analysis for NADH-GOGAT protein is now in progress. It should be noted that Fd-GOGAT activity in young, nongreen blades was still higher than NADH-GOGAT activity, even though Fd-GOGAT in such tissues was 1 order of magnitude less active than that in the green leaf blades. Therefore, participation of Fd-GOGAT in Glu synthesis in nongreen leaves cannot be ruled out at present.

Sakakibara et al. (22) recently obtained a cDNA clone of maize leaf Fd-GOGAT. They showed that accumulation of Fd-GOGAT mRNA was increased considerably by illumination of etiolated leaves. A significant increase in Fd-GOGAT protein was also shown during greening of maize leaves (24). Gene expression of GS2 has also been shown to be enhanced by illumination (5). Our results support these findings in that

the relative contents of Fd-GOGAT and GS2 polypeptides in the outer, green portion of the 9th leaf blade increased about 10- and 15-fold, respectively, after a few days in sunlight, compared with those in the inner, nongreen portion of the same blade. On the other hand, the content of NADH-GOGAT protein in the green portion of the 9th leaf blade was only slightly lower than that in the nongreen portion, suggesting that the abundance of NADH-GOGAT protein in rice leaves is apparently not influenced by light. The fully expanded leaf blades had decreased NADH-GOGAT protein content and activity as a function of increasing age. This observation suggests both that the expression of the NADH-GOGAT gene in rice leaves is reduced with age and that there is degradation of the NADH-GOGAT protein.

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