Glutamate:Glyoxylate Aminotransferase Modulates Amino Acid Content during Photorespiration

Daisuke Igarashi*, Hiroko Tsuchida, Mitsue Miyao, and Chieko Ohsumi

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In photorespiration, peroxisomal glutamate:glyoxylate aminotransferase (GGAT) catalyzes the reaction of glutamate and glyoxylate to produce 2-oxoglutarate and glycine. Previous studies demonstrated that alanine aminotransferase-like protein functions as a photorespiratory GGAT. Photorespiratory transamination to glyoxylate, which is mediated by GGAT and serine glyoxylate aminotransferase (SGAT), is believed to play an important role in the biosynthesis and metabolism of major amino acids. To better understand its role in the regulation of amino acid levels, we produced 42 GGAT1 overexpression lines that express different levels of GGAT1 mRNA. The levels of free serine, glycine, and citrulline increased markedly in GGAT1 overexpression lines compared with levels in the wild type, and levels of these amino acids were strongly correlated with levels of GGAT1 mRNA and GGAT activity in the leaves. This accumulation began soon after exposure to light and was repressed under high light levels, drought, and salt stress. The results suggest that the photorespiratory amino-transferase reactions catalyzed by GGAT and SGAT are both important regulators of amino acid content.

Photorespiration is a metabolic pathway in which glycolate-2-P is produced by the oxygenase activity of Rubisco (Leegood et al., 1995). At current atmospheric concentrations of O2 and CO2, photorespiration in C3 plants dissipates more than 25% of the carbon fixed by means of photosynthesis (Sharkey, 1988). Thus, photorespiration is often considered to be a wasteful process. However, photorespiration may be a mechanism for mitigation of the photoinhibition that can occur under high light levels, drought, and salt stress (Kozaki and Takeba, 1996; Hoshida et al., 2000; Wingler et al., 2000).

The photorespiratory nitrogen cycle contributes to the metabolism of certain amino acids (Gln, Glu, Ser, and Gly). Nitrogen is incorporated into the photorespiratory C2 cycle through transamination of glyoxylate to Gly. Peroxisomal glyoxylate aminotransferases play a central role within the photorespiratory pathway. The reactions in this pathway are catalyzed by GGAT and SGAT. In Arabidopsis (Arabidopsis thaliana), it was reported that Ala 2-oxoglutarate aminotransferase-like protein (Igarashi et al., 2003; Liepman and Olsen, 2001) function as photorespiratory GGAT and SGAT, respectively. In the conversion of Gly to Ser, ammonium and CO2 are released. Gln synthetase (GS)-2 and ferredoxin (Fd)-dependent GOGAT effectively refix the released ammonium in plastids. It has been estimated that the production of ammonium by photorespiration is an order of magnitude greater than the primary assimilation of nitrogen resulting from nitrate reduction (Keys et al., 1978). That is, photorespiration appears to be an essential process in the control of amino acid biosynthesis and metabolism.

Ser and Gly are involved in protein biosynthesis and serve as precursors in a variety of important biosynthetic pathways, including phospholipid synthesis (Ser) and purine formation (Gly). Ser and Gly are synthesized through two main pathways. The first pathway involves transamination of glyoxylate into Gly, which is in turn converted into Ser. A source of glyoxylate is provided by oxidation of glycolate during the photorespiratory cycle or the conversion of isocitrate to succinate during the glyoxylate cycle involved in the degradation of fatty acids. The second pathway is connected with glycolysis and leads to Ser formation from 3-phosphoglycerate. Gly is obtained from Ser through a Ser hydroxymethyltransferase-catalyzed reaction (Bourguignon et al., 1999). In photosynthetic tissues, photorespiration is assumed to be a major pathway for Ser and Gly synthesis because of the high flux of carbon through this cycle (Gerbaud and Andre, 1979; Tolbert 1980; Somerville and Somerville, 1983). In non-photosynthetic tissues (e.g. seeds or roots), Ser and Gly are synthesized mainly through glycolysis and the glyoxylate cycle. However, detailed regulation mechanisms of biosynthesis of these amino acids are unclear.

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In this study, we investigated photorespiratory transamination using GGAT1 overexpression and knockout lines and estimated the function of GGAT in the regulation of amino acid metabolism linked to photorespiration.

RESULTS

Expression Analysis

Genomic sequence information indicated that there are four Ala aminotransferase-like genes in Arabidopsis (Igarashi et al., 2003). Two of them (GGAT1 [At1g23310] and GGAT2 [At1g70580]), which encode proteins that contain peroxisome-targeting signal 1 (PTS1), were identified as encoding photorespiratory GGAT proteins (Igarashi et al., 2003; Liepman and Olsen, 2003). To better characterize the function of these genes, we examined the tissue-specific expression patterns of two GGAT genes (GGAT1 and GGAT2) using the promoter-β-glucuronidase (GUS) system. We also examined the diurnal changes in mRNA levels.

GUS stain was strongly detected in leaves of both GGAT1- and GGAT2-GUS seedlings (Fig. 1A), which suggests that GGAT1 and GGAT2 function as photorespiratory GGAT enzymes. In GGAT1-GUS seedlings, GUS signals were also strongly detected in roots (Fig. 1A), suggesting that the role of GGAT1 is not restricted to photorespiration. The function of root peroxisomes has not been identified, but in nonphotorespiratory tissue (roots) and in darkness, GGAT1 may catalyze the aminotransferase reaction. At night, Ser content increased in a GGAT1-knockout line (Igarashi et al., 2003), supporting this hypothesis.

After light exposure, the levels of GGAT1 mRNA decreased and those of GGAT2 mRNA increased rapidly (Fig. 1B). We previously reported that the GGAT1 knockout line (ggat1-1) cannot grow normally in air and recovered under high CO2 conditions and concluded that GGAT1 is a major pathway (Igarashi et al., 2003). However, the enzymatic activity of GGAT remained constant during the day (Fig. 2A), suggesting that GGAT1 and GGAT2 may function redundantly in leaf photorespiration.

Generation of GGAT1 Overexpression Lines

Detailed analysis of GGAT1 overexpression and knockout plants is necessary for characterization of the regulatory system for amino acid metabolism that depends on photorespiration. We previously isolated a GGAT1 knockout line (ggat1-1) from tag-insertion lines (Igarashi et al., 2003). In this study, we generated 42 GGAT1 overexpression lines by introducing the GGAT1 genomic region. To characterize the relationship between GGAT activity and amino acid metabolism, it is essential to isolate many GGAT1 overexpression lines that have different GGAT activities. We selected the native GGAT1 promoter rather than a constitutive promoter to control GGAT1 expression. Furthermore, by transferring the 5′-region of GGAT1 to the left-border side of T-DNA, we successfully isolated many transgenic lines that expressed various levels of GGAT1 mRNA. Levels of GGAT1 mRNA and GGAT activities in the GTox-17 GGAT1 overexpression line were higher than those in the wild type (Fig. 2, A and B). The diurnal changes in mRNA levels (Fig. 1) remained apparent (Fig. 2B). Diurnal GGAT activities remained stable in both wild-type and GTox-17 lines (Fig. 2A).

Correlation of Amino Acid Content, GGAT Activity, and GGAT1 mRNA Levels

We selected nine transgenic lines that expressed a wide range of GGAT1 mRNA levels and determined their amino acid content, GGAT activity, and GGAT1 mRNA levels. As shown in Figure 3, there was a strong
correlation between GGAT activity and the level of GGAT1 mRNA (R = 0.84). In addition, the Ser (R = 0.97), Gly (R = 0.74), and citrulline (R = 0.94) contents were also strongly correlated with GGAT activity. Table 1 presents the correlation values between GGAT1 activity and amino acid content. Other than Ser, Gly, and citrulline, no significant correlations were observed. The results suggest that GGAT activity is the limiting factor for synthesis of Ser, Gly, and citrulline.

Amino Acid Accumulation

All 42 GGAT1 overexpression lines accumulated Ser, Gly, and citrulline (Fig. 4). Most of the increase in total amino acid level compared with the wild type was accounted for by increased Ser in the GGAT1 overexpression lines and by increased Glu in the knockout line (gat1-1) because the ratios of Ser and Glu to total amino acid in the wild type were 0.1 and 0.2, respectively. The following two results strongly suggest that the alteration of amino acid content resulted directly from overexpression of the GGAT1 genes. First, there were strong correlations between GGAT activity and the levels of GGAT1 mRNA, Ser, Gly, and citrulline (Table 1). Second, all transgenic lines accumulated Ser, Gly, and citrulline (Fig. 4). Furthermore, the accumulation of Ser suggested that active conversion of Gly to Ser is occurring and that SGAT activity is limited. The increased total amino acid content in the overexpression lines reveals the possibility of improving nitrogen-use efficiency by creating GGAT1 overexpression lines.

Figure 2. Diurnal changes in GGAT enzymatic activities and mRNA levels in the GGAT1 overexpression line of Arabidopsis. Seedlings of Col-0 and the GTox-17 GGAT1 overexpression line were grown on PNS-agar plates for 2 weeks. Aboveground tissues were harvested at each sampling time. A, Diurnal change in GGAT enzymatic activities. Methods of enzyme assay are described in the text. B, Relative amounts of mRNA were measured by means of real-time PCR and calculated using the ΔCt method (same as described in legend of Fig. 1).

Figure 3. Correlations between amino acid content, GGAT activity, and GGAT1 mRNA levels. Nine independent GGAT1 overexpression lines of Arabidopsis that exhibited a wide range of levels of GGAT1 mRNA were grown on PNS-agar plates for 2 weeks. Aboveground tissues were harvested at midday and amino acid content, GGAT activity, and GGAT1 mRNA levels were determined. Assay methods are described in the text. Data are only presented for correlations that were significant in Table 1. Values represent the relative amounts compared with the value for the wild type (Col-0). R, Correlation coefficient.
processes may be similar between dicots and monocots. Acetyl-CoA metabolic regulation by means of photorespiration (Fig. 5). This suggests that basic mechanisms for amino acid accumulation in leaves, as was the case in Arabidopsis, did not lead to overproduction of Ser in transgenic rice (Oryza sativa; data not shown). Instead, use of the rice Cab promoter, which is known to direct high levels of expression in photosynthetic tissues (Sakamoto et al., 1991), successfully increased GGAT1 mRNA levels and Ser content in leaves, as was the case in Arabidopsis (Fig. 5). This suggests that basic mechanisms for amino acid metabolic regulation by means of photorespiration processes may be similar between dicots and monocots.

Ser Accumulation in the GGAT1 Overexpression Line of Rice

We generated transgenic rice that expressed the Arabidopsis GGAT1 cDNA. Expression of GGAT1 cDNA under the control of the Arabidopsis GGAT1 promoter did not lead to overproduction of GGAT1 in transgenic rice (Oryza sativa; data not shown). Instead, use of the rice Cab promoter, which is known to direct high levels of expression in photosynthetic tissues (Sakamoto et al., 1991), successfully increased GGAT1 mRNA levels and Ser content in leaves, as was the case in Arabidopsis (Fig. 5). This suggests that basic mechanisms for amino acid metabolic regulation by means of photorespiration processes may be similar between dicots and monocots.

Amino Acid Correlation

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Ser Accumulation in the GGAT1 Overexpression Line of Rice

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Diurnal Changes in Amino Acid Levels

To investigate the relation of amino acid metabolism with photorespiration, we analyzed diurnal changes in amino acid.

The levels of Ser, Gly, and citrulline increased during the morning, remained relatively constant during the afternoon, and then declined after sunset in the wild type (Fig. 7A). In contrast, the levels of these amino acids continued to increase throughout the daytime in the GGAT1 overexpression lines. Ser increased most dramatically, to 20 times the level in the wild type. The increase in Ser depended on GGAT activity in the GGAT1 overexpression line during the day (Figs. 3 and 4). Therefore, it is possible that GGAT activity is a limiting factor for Ser biosynthesis in the wild type.

Because GGAT activity did not change during the daytime (Fig. 2A), it is concluded that the substrate supply is the limiting factor for Ser biosynthesis in the GGAT1 overexpression lines. This hypothesis is supported by the observation that overexpression line GTox-4, which expressed the highest level of GGAT1 mRNA, and two other lines (GTox-17 and GTox-24) with levels nearly as high showed a similar rate of increase in Ser (Fig. 7B).

Glu and Ala decreased in the GGAT1 overexpression lines and increased in the knockout line (ggat1-1), suggesting that these amino acids act as amine donors for the GGAT reaction. Gln and Asp accumulation in the knockout line would result from excess amino groups that were transferred into pools of these amino acids.

Differences in Nitrogen Source Affect Amino Acid Profiles

Amino acid profiles in higher plants are modulated by the composition of the nitrogen source exogenously supplied to the growth medium (Raab and Terry, 1995). We therefore compared the amino acid content between the wild-type and the GGAT1 overexpression lines grown in different nutrient solutions.

As shown in Table II, the GGAT1 overexpression lines grown in plant nutrient and sugar (PNS) medium or in PNS+NH₄NO₃ medium both accumulated Ser, Gly, and citrulline compared with the wild type. Gln and Asn accumulated in plants grown in the PNS+NH₄NO₃ medium, but not in plants grown in the PNS medium. Although Arg was not detected in plants grown in the PNS medium, it accumulated in the GGAT1 overexpression lines grown in the PNS+NH₄NO₃ medium. Other than these amino acids, the different nitrogen sources did not appear to affect amino acid content. These modulations in the amino acid profiles appear to reflect the efficiency of ammonium use.

Amino Acid Accumulation in Sink Tissues

We investigated the effects of GGAT1 overexpression on amino acid profiles in sink tissues. In the GGAT1 overexpression lines, Ser and Gly both accumulated much more in sink tissues than was the case in the wild type and knockout lines (ggat1-1; Fig. 8). However, Ser and Gly levels in the ggat1-1 appeared to be comparable to those in the wild type in both stems and...
siliques. Although the mechanism is unclear, it appears that alteration of metabolic regulation by photorespiratory GGAT improved nutrient levels in the sink tissues.

DISCUSSION

Accumulation of Ser, Gly, and Citrulline in GGAT1 Overexpression Lines

More than 25% of the carbon fixed during photosynthesis may be used up by photorespiration (Sharkey, 1988). In addition, leaf mitochondria generate ammonium at an extraordinary rate during photorespiration, as much as 50 times the rate of nitrate reduction (Ogren, 1984). This toxic ammonium is efficiently re-assimilated into Gln and Glu by GS-2 and Ed-GOGAT, and these are then used to produce nitrogenous compounds needed for plant growth. Thus, photorespiratory amino acid metabolic pathways appear to be important for nitrogen metabolism in plants.

GGAT1 overexpression lines accumulated high levels of Ser, Gly, and citrulline compared with the wild type. High positive correlations between levels of these amino acids, GGAT1 expression, and GGAT enzymatic activities were observed under photosynthetic conditions (Fig. 3). These amino acids accumulated after transfer of the plants into the light (Fig. 7). In contrast, the rate of accumulation decreased under high CO₂ (Fig. 6). These results demonstrate that GGAT1 overexpression lines accumulate these amino acids at rates determined by the rate of photorespiration. Although the level of GGAT1 mRNA declined from morning to midday in both the wild-type and the GTox-17 GGAT1 overexpression line (Fig. 2B), the decrease was much larger in GTox-17 and GGAT activity did not change in either group of plants. This might result from GGAT2 accumulation after light irradiation and the stability of GGAT proteins. Throughout the day, Ser, Gly, and citrulline increased in the GTox-17 GGAT1 overexpression line (Fig. 7A) even though GGAT activity did not increase (Fig. 2A), suggesting that shortly after light exposure, the limiting factor for accumulation of these amino acids is not GGAT activity but rather the supply of substrate for the GGAT reactions during photorespiration. Thus, when the rate of photorespiration is high, GGAT activity may be a limiting factor in the photorespiratory cycle.

SGAT also plays a major role in controlling the flux of carbon toward Gly and in supplying the substrate for Ser synthesis (Somerville and Ogren, 1980). In this way, GGAT and SGAT could function cooperatively in the production of the photorespiratory amino acid Gly.

Conversion of Gly into Ser and Energy Consumption

The positive correlation between Ser content and GGAT activity (Fig. 3) was stronger than that for Gly, which is synthesized directly from the GGAT reaction. The absolute Ser content was also much higher than that of Gly (Table II). The conversion of Gly into Ser is catalyzed by Gly decarboxylase and Ser hydroxymethyltransferase and is accompanied by the generation of

![Figure 4](image-url)  
Figure 4. Amino acid content in the GGAT1 overexpression line of Arabidopsis. Forty-two independent GGAT1 overexpression lines that expressed various levels of GGAT1 mRNA, as well as a knockout line (ggat1-1) and the wild type (Col-0), were grown on PNS-agar plates for 2 weeks. Aboveground tissues were harvested at midday and amino acid content was determined. In the graph of total amino acid content, black and white bars indicate Ser and other protein composition amino acids with citrulline, respectively.

![Figure 5](image-url)  
Figure 5. Levels of GGAT1 mRNA and Ser accumulation in a GGAT1 overexpression line of rice. Ten independent T2 transgenic plants derived from four independent GGAT1 overexpression lines (lines 27-6, 31-4, 35-3, and 39-3) and the wild type were grown on soil for 6 weeks. The middle sections of the leaves were harvested at midday, and GGAT1 mRNA levels and Ser content were determined. Relative amounts of mRNA were measured by means of real-time PCR and calculated using the ΔCt method. Within each graph, values are normalized by expressing them as a multiple of the lowest value (line 27-6) recorded in the GGAT1 overexpression line of rice. ND, Not detected; WT, wild type.
NH₃, CO₂, and NADH (Oliver and Raman, 1995). Gly decarboxylase is extremely abundant in mitochondria isolated from leaves (Douce and Neuburger, 1999). Therefore, the conversion of Gly into Ser should be a stable and active reaction. NADH generated by this reaction might be translocated to the peroxisomes, where it would serve as a substrate for the reduction of hydroxypyruvate to glycerate by hydroxypyruvate reductase (Raghavendra et al., 1998). In addition, recent research has suggested that ammonium and NADH generated during Gly conversion for Gln and citrulline synthesis is catalyzed by GS-2, carbamoylphosphate synthetase, and Orn carbamoyltransferase in the mitochondria (Taira et al., 2004; Linka and Weber, 2005). Gly-driven oxidative phosphorylation should yield roughly three ATP per Gly consumed. In the conversion of Orn to citrulline, one ATP is consumed for Gln synthesis and two ATP are consumed for carbamoylphosphate synthesis, allowing tight coupling of the phosphorylation and synthesis reactions (Taira et al., 2004). Citrulline is converted into Arg (Ludwig, 1993). Because citrulline content increased greatly after transfer of plants into the light in GGAT1 overexpression lines and in the wild type (Fig. 7A), Arg content in the GGAT1 overexpression line grown in a medium with supplemental ammonium was higher than that in the wild type (Table II). Our results are thus consistent with reported pathways for the metabolism of nitrogen released by photorespiration. The absolute content of citrulline and Arg that accumulated in the GGAT1 overexpression line was low compared with that of Gln and Glu, demonstrating that mitochondrial reassimilation of ammonium is a minor pathway.

Analysis of the Function of GGAT in the Monocot

Transgenic rice that expressed Arabidopsis GGAT1 also accumulated Ser. Rice also has predicted GGAT-like genes in both the japonica (BAC20747) and indica
AAO84040) cultivars (International Rice Genome Sequencing Project, 2005). The amino acid sequence of the protein encoded by Arabidopsis GGAT1 is 80% similar to that of the rice GGAT1-like proteins. These two groups of proteins contain a Ser-Arg-Met group at the C terminus that is a conserved PTS1. These sequence characteristics support the hypothesis that rice GGAT1-like proteins function as photorespiratory GGATs. This suggests that the regulation of photorespiratory glyoxylate aminotransferase is similar in a dicot (Arabidopsis) and a monocot (rice). However, the degree of Ser accumulation in rice was not remarkable compared with that in Arabidopsis, and, other than Ser, we detected no marked differences in amino acid accumulation between the wild-type and the GGAT1 overexpression line of rice. This could result from instability in GGAT1 originating in a heterologous expression system, slight differences in the metabolite flux during photorespiration, and small absolute levels of GGAT1 mRNA.

Photorespiration and Nitrogen

Nitrogen, an essential nutrient for plant growth, is primarily taken up by roots in the form of nitrate and is reduced to ammonium by nitrate reductase and nitrite reductase. In most higher plants, ammonium is assimilated into Gln through the cooperative activity of GOGAT and GS. These reactions represent the major pathway for the assimilation of ammonium into amino acids. At elevated CO2 concentrations, photorespiration is limited and the assimilation of nitrogen is inhibited in the shoots of dicotyledons (e.g. Arabidopsis) and monocotyledons (e.g. wheat [Triticum aestivum]; Bloom et al., 2002; Rachmilevitch et al., 2004). Thus, under normal conditions, photorespiration is assumed to contribute to effective use of nitrogen.

Our results demonstrated that, in an ammonium-rich medium, levels of Gln, Asn, and Arg increased in the both of the wild-type and the GGAT1 overexpression lines (Table II), suggesting that these amino acids

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Figure 8. Ser and Gly accumulation in stem and silique. Arabidopsis plants of the wild type (Col-0), GGAT1 knockout line (ggat1-1), and GGAT1 overexpression line (GTox-17) were grown in soil for 6 weeks. Stems were harvested 15 cm from the base. Siliques were harvested 9 to 11 d after flowering. All samples, including leaves, were harvested at midday.
form a nitrogen pool under nitrogen-rich conditions. However, the increase in levels of these amino acids in the GGAT1 overexpression line in response to supplemental ammonium was much higher than the increase in the wild type. This suggests that, with increased release of ammonia during the conversion of Gly into Ser and uptake of ammonium by the roots, ammonium increases to excessive levels and would be assimilated into less-toxic Gln, Asn, and Arg. The high accumulation of total amino acids in the GGAT1 overexpression line may show that improvement in GGAT activity in this line can enhance photorespiration and the efficiency of nitrate and ammonium assimilation.

Substrates for GGAT Reaction

The analysis of GGAT- and SGAT-deficient mutants and of recombinant proteins indicated that Ala is a substrate for both GGAT and SGAT (Murray et al., 1987; Liepman and Olsen, 2001, 2003; Igarashi et al., 2003). GGAT catalyzes an AGT reaction (Noguchi and Hayashi, 1981; Nakamura and Tolbert, 1983; Yu et al., 1984; Igarashi et al., 2003; Liepman and Olsen, 2003). However, a high $K_m$ value of SGAT for Ala (101.2 mM) indicates that Ala may not be a physiological substrate for SGAT (Liepman and Olsen, 2001).

The diurnal Glu and Ala profiles differed between the knockout line (ggat1-1) and the overexpression line (Fig. 7A). From morning (8 AM) to night, these amino acids increased in ggat1-1 and decreased in the overexpression line. Both Glu and Ala are used as amine donors for transamination to glyoxylate in the GGAT reaction. Estimates based on whole-leaf labeling analysis suggested that Ala contributed three times more amino groups to photosynthetic Gln formation than Glu did (Betsche, 1983), whereas another study based on feeding metabolites to purified peroxisomes concluded that Glu contributed more to glyoxylate transamination than Ala did (Yu et al., 1984). The cooperative change of Glu and Ala in the GGAT1 knockout line and the GGAT1 overexpression line suggests that both amino acids contribute to transamination for Gly formation.

Amino Acid Accumulation in Sink Tissues

We showed that Ser and Gln content increased in sink tissues (Fig. 8). Although this may be a result of photorespiration in stems and siliques, the accumulation level of Ser was higher than that in leaves, suggesting that amino acids in the source tissues would be directly transported to sink tissues and concentrated. Because photorespiration is involved in photosynthesis and metabolism of nitrogen and amino acid and protection against stress, it is a proposed target for genetic engineering to improve crop productivity. However, further analysis of the flux of photorespiratory metabolites and of the transport and accumulation mechanisms between source and sink tissues will be necessary to permit effective improvement of crop production by engineering of the genes involved in photorespiration.

In summary, our analysis of the GGAT1 knockout and GGAT1 overexpression lines suggested that GGAT activity is directly involved in the regulation of Gln and Ser levels. Furthermore, the total amino acid content and levels of other major amino acids differed markedly between these two lines, suggesting that GGAT may regulate the biosynthesis and metabolism of fundamental amino acids.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana L. Col-0) cultures were grown at photo-synthetically active radiation (PAR) of 70 μmol m$^{-2}$ s$^{-1}$ on a 16-h-light/8-h-dark photoperiod at 22°C unless otherwise indicated. A growth chamber (EYELA FLI-301NH-CL) was used for the analysis of plants grown under high CO2 concentrations. Plants were grown on sterile PNS medium (Haughn and Somerville, 1986) in petri dishes containing 0.8% (w/v) agar.

To compare the amino acid content between the wild-type and the GGAT1 overexpression lines grown under different nitrogen sources, these plants were grown on PNS medium with or without supplementation with 10 mM NH$_4$NO$_3$. PNS medium contains 5 mM KNO$_3$, and 2 mM Ca(NO$_3$)$_2$ as nitrogen sources and an additional 10 mM NH$_4$NO$_3$ almost doubled nitrogen in the medium. The plants were grown for 2 weeks on PNS medium or on PNS + NH$_4$NO$_3$ medium, and the aboveground tissues were collected for amino acid analysis.

To determine the amino acid content in the plants grown under high CO$_2$ conditions or in the siliques and stems, plants were grown in soil watered with PNS medium at PAR of 120 μmol m$^{-2}$ s$^{-1}$. Arabidopsis siliques (9-11 d after flowering), stems (cut 15 cm above the roots), and fully expanded leaves were harvested for analysis.

Rice (Oryza sativa L. cv Kitaake) was planted in soil watered with 1,000-fold diluted Hyponex (Hyponex) and grown under natural light conditions on a 14-h-light/10-h-dark photoperiod at a 23°C day/18°C night cycle in a greenhouse (Tsuchida et al., 2001).

Expression Analysis of GGAT

For reverse transcription (RT)-PCR, total RNA was isolated from leaves using an RNeasy plant mini kit (Qiagen). First-strand cDNA synthesis was conducted with an oligo(dT)$_{12,18}$ primer (GE Healthcare) and reverse transcription SuperScript II (Invitrogen). Real-time PCR reactions were performed using an Applied Biosystems Prism 7700 sequence detector. Specific primers for GGAT1 (5’-TCCTTCCGAGCAAGCATTGAG-3’ and 5’-GAATACGGCAAGAAGAAAGTGTG-3’) and for GGAT2 (5’-CACTCTTCCTTCTTCTTCCACGACA-3’ and 5’-GACGGATTTGACACAGAGTAGGACCA-3’) were used. For rice analysis, specific primers for GGAT1 (5’-TGAACCAAGGGGTAGCTTCG-3’ and 5’-GACGTTCATTTGACATGTA-3’) were used for quantification of the introduced gene.

Real-time PCR reactions were performed in duplicate using 0.9 μl of each primer and 1 × SYBR green PCR master mix (Applied Biosystems) in a 50-μl volume. Relative differences were determined using the $\Delta\Delta_Ct$ method described by the manufacturer.

Construction of Plasmids and Transformation of Arabidopsis

The promoter regions were isolated by means of PCR using specific primers for GGAT1 (5’-CAATGAACTCAGAAGTTAAGATGCTGCCAAC-3’ and 5’-GACGGATGACCCCTATGTTCACTCAGACTCAC-3’) and for GGAT2 (5’-GCAACCTTTGTTGATTCCAGACGTTCTCA-3’ and 5’-CACTCTTCGATTTACCTTCTTCACT-3’). The amplified fragments were digested by BamHI (GGAT1) or HindIII/BamHI (GGAT2) and the fragments (1,843 bp for GGAT1 and 2 kb for GGAT2) were cloned into the 5’-end of the GUS gene in vector pBI101.
For generation of a GGAT1 overexpression line of Arabidopsis, a 5,089-bp genomic fragment of GGAT1, which extends 5’ upstream (1,843 bp) from the translational initiation and 3’ downstream (612 bp) from the point of translational termination, was isolated by means of PCR using specific primers (5’-CAGATTGCGAAGATGTTGCATTGCGATC-3’ and 5’-GGCTCTATTCTCGAACCATGCTGACC-3’). The amplified fragment was cloned into vector pHBlI between the HindIII and EcoRI sites and introduced into the wild type (Col-0) or an GGAT1 knockout line (ggat1-1) by means of Agrobacterium tumefaciens-mediated transformation. Two of 42 lines are ggat1-1 background and others are wild-type background.

Generation of a GGAT1 Overexpression Line of Rice
Arabidopsis GGAT1 cDNA was isolated by means of RT-PCR using specific primers (5’-GGCTCTTACAGCTCCTCAAGGGCATTT-3’ and 5’-GGCGACGCTCTCCCATTTTCGATAA-3’). The amplified fragment was fused to the CaMV promoter (Sakamoto et al., 1991) and cloned into the vector pGCl2Hm. The resultant plasmid was introduced into calli derived from rice by means of A. tumefaciens-mediated transformation.

Histochemical Staining for GUS Activity
Histochemical staining for GUS activity was carried out as described by Jefferson et al. (1987), but with the following modifications. After transgenic plants had been grown for 2 weeks on the PNS-agar plates, plants were harvested and incubated overnight in GUS staining solution (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 1 mM X-Gluc) at 37°C. After staining, samples were treated with 70% ethanol to remove chlorophyll from the tissues.

Enzyme Assays
Aminotransferase activity was assayed as described by Hörder and Rej (1983), but with the following modifications. The aboveground tissues of seedlings grown on PNS-agar plates, plants were harvested and incubated overnight in GUS staining solution (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 1 mM X-Gluc) at 37°C. After staining, samples were treated with 70% ethanol to remove chlorophyll from the tissues.

Measurement of Amino Acid Content
Amino acids were extracted from the aboveground tissues of the plants in 80% ethanol at 80°C. After evaporation, dried samples were dissolved in 0.2 N HCl. Amino acid content was determined using an L-8800 amino acid analyzer (Hitachi). Briefly, amino acids, separated by cation-exchange chromatography, were detected spectrophotometrically after postcolumn reaction with ninhydrin reagent (Noguchi et al., 2006).

Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers AF429639 (GGAT1) and AF429640 (GGAT2).

LITERATURE CITED


