Arabidopsis gcsl Mutants and Distinct Fd-GOGAT Genes: Implications for Photorespiration and Primary Nitrogen Assimilation

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Ferredoxin-dependent glutamate synthase (Fd-GOGAT) plays a major role in photorespiration in Arabidopsis, as has been determined by the characterization of mutants deficient in Fd-GOGAT enzyme activity (gcls). Despite genetic evidence for a single Fd-GOGAT locus and gene, we discovered that Arabidopsis contains two expressed genes for Fd-GOGAT (GLU1 and GLU2). Physical and genetic mapping of the gcsl locus and GLU genes indicates that GLU1 is linked to the gcsl locus, whereas GLU2 maps to a different chromosome. Contrasting patterns of GLU1 and GLU2 expression explain why a mutation in only one of the two genes for Fd-GOGAT leads to a photorespiratory phenotype in the gcsl mutants. GLU1 mRNA was expressed at the highest levels in leaves, and its mRNA levels were specifically induced by light or sucrose. In contrast, GLU2 mRNA was expressed at lower constitutive levels in leaves and preferentially accumulated in roots. Although these results suggest a major role for GLU1 in photorespiration, the sucrose induction of GLU1 mRNA in leaves also suggests a role in primary nitrogen assimilation. This possibility is supported by the finding that chlorophyll levels of a gcsl mutant are significantly lower than those of the wild type when grown under conditions that suppress photorespiration. Both the mutant analysis and gene regulation studies suggest that GLU1 plays a major role in photorespiration and also plays a role in primary nitrogen assimilation in leaves, whereas the GLU2 gene may play a major role in primary nitrogen assimilation in roots.

INTRODUCTION

Glutamate synthase (glutamine-oxoglutarate aminotransferase or GOGAT) is a key enzyme involved in the assimilation of inorganic nitrogen in higher plants (Lea and Miflin, 1974; Keys et al., 1978; Miflin and Lea, 1980; Stewart et al., 1980). Functioning coordinately with glutamine synthetase (GS; EC 6.3.1.2), the GS/GOGAT pair provides the primary port of entry for nitrogen in whole-plant metabolism. Inorganic nitrogen, in the form of ammonia, is assimilated via this glutamate synthase cycle into the organic nitrogen compounds glutamine and glutamate, which are the nitrogen donors in essentially all biosynthetic reactions involving nitrogen (e.g., amino acids, nucleic acids, and chlorophyll). Primary nitrogen assimilation requires cofactors, reducing equivalents, and carbon skeletons generated during photosynthesis. Thus, in most plants, assimilation of inorganic nitrogen into organic form occurs predominantly in leaf chloroplasts where these components are readily available (Sechley et al., 1992). In plant species that are able to efficiently transport photosynthate to roots, such as maize and temperate legumes, nitrogen assimilation also occurs at high rates in root plastids (Oaks, 1992).

In addition to its major role in primary nitrogen assimilation, the GS/GOGAT cycle also plays a crucial role in reassembling the large amount of ammonia released during photorespiration (Somerville and Ogren, 1980; Kendall et al., 1986). The amount of ammonia released during photorespiration is up to 10-fold greater than is the amount of primary nitrogen taken up by the plant (Keys et al., 1978). Thus, efficient recapture of this photorespiratory ammonia is essential for survival of the plant. In fact, conditional lethal mutants defective in enzymes required for photorespiratory ammonia reassimilation, such as GS and GOGAT, have been isolated from several plant species (Somerville and Ogren, 1982; Kendall et al., 1986; Blackwell et al., 1987). In the Arabidopsis photorespiratory screens, mutants specifically defective in Fd-GOGAT enzyme activity were the largest class of mutants isolated (gcls, formerly called gluS), but paradoxically, no mutants defective in GS were recovered from Arabidopsis, whereas these mutants were recovered from barley (Somerville and Ogren, 1982; Wallsgrove et al., 1987; Artus, 1988).

Arabidopsis, like all higher plants, contains two types of glutamate synthase enzymes, an NADH-dependent enzyme...
(NADH-GOGAT; EC 1.4.1.14) and a ferredoxin-dependent enzyme unique to photosynthetic organisms (Fd-GOGAT; EC 1.4.7.1). Fd-GOGAT is the predominant form of glutamate synthase found in Arabidopsis, accounting for up to 96% of the total GOGAT activity in leaves and 68% of the total GOGAT activity in roots (Somerville and Ogren, 1980; Suzuki and Rothstein, 1997). Arabidopsis photorespiratory gls mutants containing <5% of the wild-type levels of Fd-GOGAT enzyme activity in leaves become chlorotic and die when grown under conditions enhancing photorespiration (air) but live when grown under conditions suppressing photorespiration. Their "normal" growth in high CO₂ suggests that primary nitrogen assimilation is occurring at normal rates either via the low levels of residual Fd-GOGAT enzyme activity in the gls mutants or via the NADH-GOGAT enzyme, which is present in the gls mutants at the same normal low levels found in wild-type leaves (Somerville and Ogren, 1980; Suzuki and Rothstein, 1997). However, rates of primary nitrogen assimilation were not quantified in the Fd-GOGAT mutants.

Complementation analysis of 15 of the 58 Fd-GOGAT–deficient mutants identified in the Arabidopsis photorespiratory mutant screen suggested a single genetic locus encoding Fd-GOGAT (glsS, renamed gls1; Somerville and Ogren, 1980; Artus, 1988). The isolation of only one gene encoding Fd-GOGAT in Arabidopsis (Suzuki and Rothstein, 1997) is consistent with the single genetic locus theory. However, here, we report that Arabidopsis in fact contains two expressed genes encoding Fd-GOGAT isoforms (GLU1 and GLU2). These genes show contrasting patterns of gene expression. We also demonstrate that GLU1 and a gls1 allele map to the same local region of chromosome 5, whereas GLU2 maps to a different chromosome. Furthermore, we show that a gls mutant displays defects in primary nitrogen assimilation. That is, chlorophyll levels of a gls mutant are significantly lower than those of the wild-type plants grown under conditions that suppress photorespiration. Our gene expression studies combined with the gls mutant analysis suggest that the GLU1 gene plays a major role in photorespiration as well as a role in primary nitrogen assimilation in leaves. The Fd-GOGAT isoenzyme encoded by GLU2 is proposed to be involved mainly in primary nitrogen assimilation in roots.

RESULTS

GLU1 and GLU2 Encode Distinct Fd-GOGAT Isoenzymes in Arabidopsis

Two classes of cDNA clones with homology to the Fd-GOGAT genes of other plant species were isolated from a cDNA library made from light-grown Arabidopsis. Twelve cDNA clones were identical to each other (except for 3' terminations occurring in several different places) and were named pATGLU1. A thirteenth cDNA clone called pATGLU2 was distinct from the pATGLU1 clones but was homologous to the Fd-GOGAT genes. Because none of the cDNA clones was full length (the mRNA transcript is longer than 5 kb), the complete Fd-GOGAT sequences in this study are composites generated using additionally isolated cDNAs and 5' rapid amplification of cDNA ends (RACE) products (see Methods). The nucleotide sequences of the GLU1 (GenBank accession number U39287) and GLU2 (GenBank accession number U39288) cDNAs are 71% identical, differing primarily in the 5' and 3' untranslated regions. The predicted amino acid sequences encoded by the GLU1 and GLU2 cDNA sequences are shown in Figure 1. For GLU2, the proposed start codon is verified by an in-frame stop codon found 96 bases upstream. For GLU1, the proposed start codon occurs at a similar position; however, no in-frame stop codon was found upstream. The predicted amino acid sequences of the GLU1 and GLU2 peptides are 80% identical, differing primarily at the N and C termini. Both GLU1 and GLU2 cDNAs encode an N-terminal extension to the mature protein that has characteristics of a chloroplast transit peptide (high serine/threonine content and net positive charge; Keegstra et al., 1989).

A comparison of the amino acid sequences encoded by GLU1 and GLU2 with two other plant GOGAT sequences (maize, Sakakibara et al., 1991; alfalfa, Gregerson et al., 1993) is also shown in Figure 1. The Arabidopsis GOGAT proteins encoded by GLU1 and GLU2 are more similar to the Fd-GOGAT sequence of maize (79 and 76% identity, respectively) than to the NADH-GOGAT sequence of alfalfa (41% identity for each). Furthermore, the Fd-GOGAT sequences of Arabidopsis and maize lack the additional 550 amino acids at the C terminus of NADH-GOGAT from alfalfa that encodes the NADH binding domain (Gregerson et al., 1993). Thus, it appears that the Arabidopsis GLU1 and GLU2 genes encode two distinct isoenzymes of Fd-GOGAT.

It should be noted that the GLU1 gene we report here is nearly identical in sequence to the Fd-GOGAT encoding gene reported by Suzuki and Rothstein (1997). In addition to 13 nucleotide differences, which result in eight amino acid differences, the Suzuki and Rothstein sequence includes a 78-bp region (nucleotides 277 to 354, amino acids 94 to 119) that is not in the GLU1 sequence reported here. This 78-bp region, which occurs in the sequence encoding the putative chloroplast transit peptide, is likely to be an intron for the following reasons. (1) The GLU1 sequence reported here was derived from cDNA clones and 5' RACE products. By contrast, the Fd-GOGAT gene sequence reported by Suzuki and Rothstein is a composite derived from a genomic clone (nucleotides −20 to +1110) spanning the 78-bp region in question and a partial cDNA (nucleotides +721 to +5178). (2) The 78-bp sequence found in the genomic sequence and not our cDNA sequence contains a consensus sequence for intron splicing (Sharp, 1994). (3) Removal of
this 78-bp region by RNA splicing would maintain the same reading frame but remove 26 amino acids from the putative chloroplast transit peptide reported by Suzuki and Rothstein (1997). Thus, the GLU1 cDNA sequence reported here is most likely a correction to that published by Suzuki and Rothstein (1997).

The GLU1 Gene and a gls1 Allele Comap in the Arabidopsis Genome

The discovery of two genes for Fd-GOGAT in Arabidopsis outlined above was in contrast to genetic evidence for a single locus for Fd-GOGAT. Arabidopsis mutants deficient in >95% Fd-GOGAT enzyme activity displayed a conditional lethal phenotype and mapped to a single genetic locus, gls1 (Somerville and Ogren, 1980). Several of these gls mutant alleles are shown in Figure 2. When grown in air, the gls mutants are chlorotic and eventually die (cf. Figures 2B to 2E with Figure 2A). When grown in 2% CO₂, the mutants are viable and look similar to the wild type (cf. Figures 2G to 2J with Figures 2A and 2F). Because 15 of the gls mutants have been genetically characterized and all were allelic (Somerville and Ogren, 1980; Artus, 1988), we wanted to determine whether these mutations occurred in one of the cloned structural genes for Fd-GOGAT (GLU1 or GLU2) or elsewhere, perhaps in an undiscovered gene or regulatory locus.

The GLU1 and GLU2 genes were each mapped to Arabidopsis chromosomes by using gene-specific restriction fragment length polymorphisms (RFLPs; Botstein et al., 1980) defined between the Arabidopsis ecotypes Columbia and Landsberg erecta (Figure 3A). The RFLPs detected for GLU1 and GLU2 are distinct, demonstrating that the GLU1 and GLU2 probes are indeed gene specific (Figure 3A, compare GLU1 and GLU2). Segregation analysis of these GLU1 and GLU2 RFLPs with 462 markers in 26 recombinant inbred (RI) lines was used to determine their map positions relative to the other known physical markers (Lister and Dean, 1993). GLU1 mapped to a position at 10.9 centimorgans (cM) on chromosome 5 between the markers ctr1 (9.1 cM) and nga249 (22.3 cM) (Figure 3B). GLU2 mapped to position 99.1 cM on chromosome 2 below the Athb7 marker (94.8 cM) (Figure 3B). Separately, we determined that the gls1-30 allele (formerly called C530; Somerville and Ogren, 1980) mapped to chromosome 5 between the simple sequence length polymorphism (SSLP; Bell and Ecker, 1994) markers ctr1 (four recombination events out of 30 mutants tested) and nga249 (six recombination events out of 29 mutants tested; Figure 3B). These results demonstrate that the gls1-30 mutation and the GLU1 gene map to the same local region of chromosome 5 (Figure 3B), whereas GLU2 maps to a distinct chromosome (Figure 3B). Thus, the gls1 mutations most likely occur in the GLU1 gene, whereas the unaltered GLU2 gene may account for the low level of “residual” (5%) Fd-GOGAT enzyme activity in the gls1 mutants (Somerville and Ogren, 1980).

GLU1 and GLU2 mRNAs Are Differentially Expressed in Leaves and Roots

In an attempt to understand how a mutation in one of the two expressed genes for Fd-GOGAT could lead to the photorespiratory phenotype, we examined the expression pattern of the GLU1 and GLU2 genes. Gene-specific probes were used to detect levels of GLU1 and GLU2 mRNA in leaves and roots of Arabidopsis. The genes show contrasting patterns of tissue-specific mRNA accumulation, as shown in Figure 4. GLU1 mRNA was detectable in leaves of dark-adapted plants but accumulated to highest levels in leaves of light-treated plants (Figure 4, lanes 1 and 2). By contrast, GLU1 mRNA was undetectable in roots (Figure 4, lanes 3 and 4). The leaf-specific expression pattern of GLU1 correlates with its proposed role in photorespiration, as determined genetically using the gls1 mutants. In contrast to GLU1 mRNA, GLU2 mRNA was detectable at low constitutive levels in leaves and accumulated to the highest levels in roots (Figure 4, lanes 1 to 4). The GLU2 gene product most likely accounts for the Fd-GOGAT activity detected in root plastids (Suzuki et al., 1982) and may function in primary nitrogen assimilation in roots.

Kinetics of Light Induction of GLU1 mRNA Transcripts

Because the previous set of experiments indicated that extended dark or light treatments (48 hr) led to changes in levels of GLU1 mRNA, we examined whether this regulation might occur in the course of a 16-hr day. As seen previously, GLU1 mRNA levels were low in mature green plants that were dark-adapted for 64 hr (Figure 5, lane 1). Subsequent exposure to light dramatically induced GLU1 mRNA accumulation in as little as 3 hr, reaching a peak at 24 hr (Figure 5, lanes 2 to 5). GLU1 mRNA also accumulated to high levels in plants grown in continuous light with no period of dark adaptation (Figure 5, lane 6). We also examined light control of GLU1 expression in etiolated seedlings exposed to light for various times (Figure 5, lanes 7 to 11). GLU1 mRNA was expressed at nearly undetectable levels in etiolated seedlings (Figure 5, lane 7), and this low level of mRNA expression was induced by light in 3 hr, peaking at 24 hr (Figure 5, lanes 8 to 11). In contrast to GLU1 mRNA, GLU2 mRNA levels appeared to have higher levels of basal accumulation in the dark-adapted mature green plants (Figure 5, lane 1), and exposure to light caused only a modest increase in levels (Figure 5, lanes 2 to 6). In etiolated seedlings, GLU2 mRNA levels were present at high basal levels and were not induced by light (Figure 5, lanes 7 to 11).

Sucrose Induces GLU1 mRNA Levels in Dark-Adapted Plants

The light induction of GLU1 mRNA is consistent with a role of GLU1 in primary nitrogen assimilation and photorespiration
Figure 1. GLU1 and GLU2 Encode Distinct Fd-GOGAT Isozymes in Arabidopsis.

A comparison of the predicted amino acid sequences of the GLU1 (GenBank accession number U39287) and GLU2 (GenBank accession number U39288) cDNAs of Arabidopsis is shown relative to plant cDNAs for Fd-GOGAT or NADH-GOGAT. Dots indicate identity with the first sequence. Dashes indicate gaps introduced to maximize the alignment. The start of the mature peptide is underlined. Asterisks denote the C termini of the proteins. AtGLU1 is the predicted translation product of Arabidopsis cDNA GLU1 (this study). AtGLU2 is the predicted translation product of Arabidopsis cDNA GLU2 (this study). Maize is the maize Fd-GOGAT (Sakakibara et al., 1991). Alfalfa is the alfalfa NADH-GOGAT.
in leaves. Because other light-regulated genes involved in primary nitrogen assimilation can also be induced independently by sucrose (nitrate reductase, nitrite reductase, and glutamine synthetase; Cheng et al., 1992; Vincentz et al., 1993; Faure et al., 1994), we decided to determine whether carbon availability could induce the accumulation of GLU1 mRNA, independent of light, in Arabidopsis.

For these experiments, plants were initially grown for 12 days on Murashige and Skoog (MS) medium plus 3% sucrose in a normal day/night cycle (16-hr day and 8-hr night). Plants were then transferred to media without sucrose and dark-adapted for 3 days. After this treatment, plants were transferred to new media containing 0% sucrose, 3% sucrose, or 3% mannitol and incubated in either continuous dark (Figure 6, lanes 1 to 3) or continuous light (Figure 6, lanes 4 to 6) for an additional 3 days. In the plants kept in the dark, GLU1 mRNA was expressed at very low levels when grown on 0% sucrose (Figure 6, lane 1), and the addition of sucrose (3%) induced accumulation of GLU1 mRNA in the absence of light (Figure 6, lane 2). As a control, we showed that the addition of mannitol (a nonmetabolizable sugar) had no inductive effect on levels of GLU1 mRNA (Figure 6, lane 3). Light treatment was able to induce maximally the accumulation of GLU1 mRNA in the dark-adapted, carbon-starved plants (Figure 6, lane 4). Sucrose treatment could not superinduce the accumulation of GLU1 mRNA in the light-treated plants (Figure 6, lane 5). Thus, GLU1 mRNA was maximally induced by light, but sucrose could at least partially replace light for induction in dark-grown plants.

In contrast to GLU1 mRNA, GLU2 mRNA was expressed at constitutive levels in the dark-adapted and the light-treated plants (Figure 6, lanes 1 and 4). The addition of sucrose had no dramatic effect on the accumulation of GLU2 mRNA in either the dark- or light-treated plants (Figure 6, lanes 2 and 5). Thus, GLU2 may provide a constitutive low level of Fd-GOGAT enzyme for primary nitrogen assimilation in leaves. The induction of GLU1 mRNA by sucrose may provide additional levels of Fd-GOGAT enzyme required for nitrogen assimilation under conditions of high carbon skeleton availability.

A gls Photorespiratory Mutant Displays a Defect in Primary Nitrogen Assimilation

The gls photorespiratory mutants deficient in Fd-GOGAT enzyme activity were originally isolated based on their conditional chlorosis in air and recovery in 1% CO2, indicating that Fd-GOGAT plays a major role in photorespiration.

Figure 2. Photorespiratory Phenotype of gls Mutants Defective in Fd-GOGAT Activity.
(A) and (F) Arabidopsis wild-type Columbia. (B) and (G) Photorespiratory gls mutant CS103. (C) and (H) NA60. (D) and (I) CS37. (E) and (J) CS40.
In (A) to (E), plants were grown in air; in (F) to (J), plants were grown in 2% CO2 in air.
However, although the gls mutants were reported to grow "normally" in 1% CO₂, the nitrogen assimilatory capacity of these mutants was not quantified. Because Fd-GOGAT normally accounts for up to 96% of leaf GOGAT activity in wild-type plants, we decided to determine whether a gls mutant with as little as 5% of wild-type Fd-GOGAT activity displayed any quantifiable defects in primary nitrogen assimilation when compared with the wild type.

We measured the ability of a gls mutant (NA60) and wild-type Columbia to assimilate inorganic nitrogen by quantifying total chlorophyll levels in plants grown on low (2 mM ammonium and 4 mM nitrate) versus high (20 mM ammonium and 40 mM nitrate) concentrations of inorganic nitrogen (Delgado et al., 1994). Plants were grown on these media in either air (Figure 7A) or 1% CO₂ to suppress any photorespiratory effects (Figure 7B). Chlorophyll measurements obtained in each atmospheric condition were then examined separately using a two-way analysis of variance (ANOVA; Figure 7C).

In air, the chlorophyll levels in the gls mutant were significantly lower than those of the wild type at either concentration of inorganic nitrogen examined (P < 0.0001; Figures 7A and 7C). This confirms that the chlorotic photorespiratory phenotype observed for gls mutants grown in soil (Somerville and Ogren, 1980) is also significant when the gls mutants are grown on MS medium containing 0.5% sucrose, even though sucrose is known to suppress photosynthesis (Cseplo and Medgyesy, 1986).

When grown in 1% CO₂, the wild type and NA60 each accumulated significantly higher levels of chlorophyll compared with air under either nitrogen concentration. These results demonstrate that the CO₂ supplementation used in our studies was effective in suppressing photorespiration in the tissue culture–grown plants (cf. Figures 7A and 7B). Moreover, we were able to monitor specifically primary nitrogen assimilation in plants grown in 1% CO₂, in which photorespiration was suppressed. As shown in Figure 7B, when grown in 1% CO₂, wild-type plants showed a significant increase in chlorophyll accumulation in response to increasing concentrations of inorganic nitrogen (P < 0.0001 in a posteriori comparisons). By contrast, in the NA60 mutant, chlorophyll levels were dramatically reduced compared with the wild type (P < 0.0001; Figures 7B and 7C), and no significant increase in chlorophyll levels occurred in response to an increase of inorganic nitrogen (P = 0.3654 in a posteriori comparisons). This analysis indicates that the gls mutant...
exhibits a defect in primary nitrogen assimilation that is specifically observed when photorespiration is suppressed. Conversely, under photorespiratory conditions (air), there was no significant difference between the wild type and NA60 in their response to nitrogen concentration ($P = 0.7029$; Figures 7A and 7C).

**DISCUSSION**

Here, we have used molecular and genetic approaches to define the roles of individual GOGAT genes in Arabidopsis. Previous studies have shown that Fd-GOGAT accounts for 96% of GOGAT activity in leaves, whereas NADH-GOGAT accounts for 4% (Somerville and Ogren, 1980; Suzuki and Rothstein, 1997). GOGAT plays two assimilatory roles in leaves: one in primary nitrogen assimilation and the other in reassimilation of photorespiratory ammonia. Genetic analysis of photorespiratory mutants isolated in Arabidopsis suggested a single genetic locus ($gls1$) specifying Fd-GOGAT activity (Somerville and Ogren, 1980; Artus, 1988). Consistent with this finding, Suzuki and Rothstein (1997) reported the isolation of a single Arabidopsis gene encoding Fd-GOGAT. In this study, we report that Arabidopsis contains two expressed genes for Fd-GOGAT ($GLU1$ and $GLU2$) and detail the molecular genetic experiments used to determine an in vivo function for each gene, taking advantage of available gls photorespiratory mutants in Fd-GOGAT.

The $GLU1$ gene appears to be the major expressed gene for Fd-GOGAT, because $GLU1$ mRNA is predominant in leaves. The second Fd-GOGAT gene, $GLU2$, is expressed at much lower levels in leaves and at highest levels in roots. It is surprising that two Fd-GOGAT genes were discovered in Arabidopsis, because single genes for Fd-GOGAT have been reported for plants with much larger genomes. These include maize, tobacco, barley, spinach, and Scots pine (Sakakibara et al., 1991; Zehnacker et al., 1992; Avila et al., 1993; Nalbantoglu et al., 1994; García-Gutiérrez et al., 1995). Because the Arabidopsis $GLU2$ gene was only discovered after sequencing 13 independent cDNAs (only one of which was $GLU2$), it is likely that other species contain a second gene for Fd-GOGAT that may have been missed in other cDNA screens because of low expression levels.

Determining that there are two Fd-GOGAT genes in Arabidopsis ($GLU1$ and $GLU2$) conflicted with genetic evidence for a single Fd-GOGAT locus defined by the gls photorespiratory mutants. This observation raised two questions: (1) which GOGAT gene is affected in the $gls1$ photorespiratory mutants, and (2) do the $GLU1$ and $GLU2$ genes play distinct roles in photorespiration and primary assimilation? We have addressed these questions with the series of molecular and genetic experiments, as discussed below.

As a first step to determine which GOGAT gene is affected in the $gls1$ photorespiratory mutants, we mapped both the $gls1$
The differences in level of expression of GLU1 and GLU2 mRNA can also explain why a mutation in only one of two expressed genes for Fd-GOGAT would cause a photorespiratory-dependent phenotype. A mutation in the highly expressed GLU1 gene could cause the phenotype because the GLU2 gene is expressed only at low constitutive levels. As such, GLU2 could not compensate for the missing GLU1 activity when photorespiration was occurring. The presence of the second GLU2 gene may also explain the “leakiness” of the gls mutants in Arabidopsis as compared with other photorespiratory mutants (C. Somerville, personal communication). This duplicated gene situation is reminiscent of the finding that a mutation in the more highly expressed of two genes for tryptophan synthase causes auxotrophy (Last et al., 1991). A contrasting situation exists for GS, the enzymatic partner for Fd-GOGAT in Arabidopsis. The existence of multiple genes for GS (Peterman and Goodman, 1991) may explain why GS photorespiratory mutants were not isolated in Arabidopsis, as they were in barley (Wallsgrove et al., 1987).

Because this study shows that Arabidopsis contains two genes encoding distinct but similar isoforms of Fd-GOGAT, the role of each isoenzyme in primary nitrogen assimilation needs to be addressed. Because primary assimilation occurs predominantly in leaves, Fd-GOGAT, which accounts for 96% of total leaf GOGAT activity, is likely to play a major role in this process, in addition to its proposed role in photorespiration. The GLU1 isoform of Fd-GOGAT may be expected to play a role in primary nitrogen assimilation because its mRNA is expressed at highest levels in leaves, and light or sucrose can each induce its accumulation. Paradoxically, however, the gls1 photorespiratory mutants, which are missing as much as 95% leaf Fd-GOGAT activity, were seemingly unimpaired in primary nitrogen assimilation. They were reported to grow normally and flower when grown in high CO₂, at which time photorespiration is suppressed (Somerville and Ogren, 1980).

Although residual Fd-GOGAT activity or the NADH-GOGAT activity remaining in the gls mutants might enable the plants to assimilate sufficient nitrogen to survive, we decided to determine whether gls mutants are significantly impaired in primary nitrogen assimilation. We showed that a gls mutant failed to respond to exogenously supplied inorganic nitrogen, as measured by chlorophyll accumulation when photorespiration was suppressed. This inability of the mutant to respond to exogenously supplied inorganic nitrogen suggests that the affected Fd-GOGAT gene (GLU1) is involved in primary nitrogen assimilation in addition to its major role in photorespiration, and its loss cannot be compensated by the presence of the GLU2 isoform which is expressed at low levels in leaves and predominates in roots.

The combined analysis of the GLU1 and GLU2 genes and the gls mutants has enabled us to make solid predictions as to the in vivo roles of the two distinct genes for Fd-GOGAT in Arabidopsis. The results presented here strongly suggest that GLU1 plays a major role in photorespiration and also
plays a major role in primary nitrogen assimilation in leaves, as supported by the genetic linkage to the gls mutation. Its putative role in primary assimilation is also supported by the fact that GLU1 mRNA is highly expressed in leaves in a light-dependent manner. This light regulation is probably mediated at least in part by phytochrome, as has been demonstrated for Fd-GOGAT in other species such as tomato, mustard, and Scots pine (Hecht et al., 1988; Elminger and Mohr, 1991; Becker et al., 1993).

We have also shown here that light induction of GLU1 may act via changes in carbon metabolites. That is, sucrose can induce GLU1 gene expression in the absence of light. It has been shown previously that other genes involved in primary nitrogen assimilation (e.g., nitrate reductase, nitrite reductase, and chloroplastic glutamine synthetase) are induced by sucrose in the absence of light (Cheng et al., 1992; Vincentz et al., 1993; Faure et al., 1994). Based on similar patterns of gene expression in response to light and metabolites, we propose that the GLU1 gene product functions in concert with chloroplastic GS2 in leaves. Thus, these gene regulation studies are consistent with the gls mutant studies indicating that the GLU1 gene may function in primary nitrogen assimilation and in photorespiration.

The role of GLU2 in nitrogen assimilation is also somewhat clarified by our experiments. The low, relatively constitutive levels of GLU2 mRNA in leaves suggest that GLU2 could be a housekeeping Fd-GOGAT gene that may be used for synthesizing basal levels of glutamate used for protein biosynthesis. The lower level of expression of GLU2 mRNA in leaves and its higher expression in roots are also reminiscent of the regulation of NADH-GOGAT (Lam et al., 1995). In roots, the GLU2 gene product (and possibly NADH-GOGAT) could function in a cycle with cytosolic GS2 isoenzymes, which show root-specific patterns of expression (Peterman and Goodman, 1991).

In conclusion, the characterization of two distinctly regulated genes for Fd-GOGAT and the gls mutants suggests nonoverlapping roles for GLU1 and GLU2 in primary nitrogen assimilation in leaves and roots and in photorespiration. Elucidating the in vivo functions of GLU1 and GLU2 may provide insights into the rate-limiting steps of nitrogen assimilation in leaves and roots that are essential to plant growth and development.

METHODS

Plant Material and Growth Conditions

All experiments were performed using material from Arabidopsis thaliana ecotype Columbia. The Landsberg erecta ecotype was also used for physical and genetic mapping procedures. Plants shown in Figure 2 were grown in soil for 18 days in a growth chamber (Percival Manufacturing Co., Boone, IA) in continuous high light (130 μE m⁻² sec⁻¹) and either in air or in air supplemented with 2% CO₂ as indicated. For RNA isolation from dark-adapted versus light-treated leaves and roots, plants were initially grown in a sterile semihydroponic system on specially modified Murashige and Skoog (MS) medium from Sigma (lacking ammonium nitrate, potassium nitrate, and glycine) supplemented with inorganic nitrogen (2 mM ammonium and 4 mM nitrate), 0.5% sucrose, and 0.5% agar in an environmental growth chamber for 25 days with a 16-hr-light (45 μE m⁻² sec⁻¹) and 8-hr-dark cycle.

Mature plants used in the light induction experiment were initially grown in MetroMix 200 soil (Scotts-Sierra Horticultural Products Co., Marysville, OH) for 25 days in a Percival growth chamber in continuous light (55 μE m⁻² sec⁻¹). Etiolated seedlings used in the light induction experiment were sterilized, pipetted onto water-saturated Whatman No. 1 filter paper in Petri dishes, and then placed in a humid chamber, as described by Schultz and Coruzzi (1995). Plants used in the carbon metabolite experiment were initially grown in a sterile semihydroponic system on MS medium (Gibco BRL) supplemented with 0.4% agar and 3% sucrose for 12 days in an environmental growth chamber on a 16-hr-light (45 μE m⁻² sec⁻¹) and 8-hr-dark cycle. The plants used for the chlorophyll measurements were grown on specially modified MS media from Sigma (lacking ammonium nitrate, potassium nitrate, and glycine) supplemented with 0.5% sucrose, 0.9% agar, and either low inorganic nitrogen (2 mM ammonium and 4 mM nitrate) or high inorganic nitrogen (20 mM ammonium and 40 mM nitrate) in a Percival growth chamber for 12 days with a 16-hr-light (70 μE m⁻² sec⁻¹) and 8-hr-dark cycle either in air or in air supplemented with 1% CO₂ as indicated.

Nucleic Acid Isolation Procedures

Genomic DNA was isolated from whole plants essentially as described by Cone (1989). RNA was isolated using a phenol-chloroform extraction method as follows. Plant tissue was collected, frozen in liquid nitrogen, and stored at −80°C. Tissue was ground in a mortar chilled to −80°C, suspended in extraction buffer (Jackson and Larkins, 1976), and extracted twice with phenol-chloroform-isooamyl alcohol (25:24:1). Nucleic acids were precipitated with ethanol, and then total RNA was preferentially precipitated with 3 M sodium acetate. The RNA pellet was dissolved in water and then precipitated a final time with ethanol.

Isolation of Fd-GOGAT cDNAs

Thirteen partial ferredoxin-dependent glutamate synthase (Fd-GOGAT) clones were isolated from an Arabidopsis cDNA library made from light-grown plant tissue (Gasch et al., 1990) by using a 1-kb polymerase chain reaction (PCR) product obtained from the pea Fd-GOGAT gene (G.M. Coruzzi and R. McGrath, unpublished results). Hybridization occurred overnight in 5 × Denhardt’s solution (1 × Denhardt’s solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 6 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 1 mM EDTA, 0.1% SDS, and 0.05 mg/mL salmon sperm DNA at 50°C. Washes were performed to a final stringency of 1 hr at 42°C in 0.1 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS. Clones were obtained in the vector pBluescript SK⁻ by in vivo excision of the ZAP clones (Stratagene, La Jolla, CA). Longer cDNA clones were obtained by screening a library made from etiolated seedlings (Schindler et al., 1992) by using clones obtained from the first library as probes.
Full-length cDNA sequence at the 5’ end was generated from cDNA fragments obtained using PCR (GLU2 only) and the Gibco BRL 5’ rapid amplification of cDNA ends (RACE) system (both GLU1 and GLU2). Products were cloned using the pCR-Script kit (Stratagen). Sequencing was performed using the T71000 Advanced Nested Set Technology (Gold Biotechnology, St. Louis, MO) and the dideoxy sequencing method with Sequenase DNA polymerase (United States Biochemical). Sequences were managed and identities calculated using GeneWorks (IntelliGenetics Inc., Mountain View, CA), aligned using PileUp in the Genetics Computer Group (Madison, WI) GCG7 software package, and displayed using Maligned (S. Clark, Ontario Cancer Institute, Toronto, Canada).

Physical/Genetic Mapping of the GLU1 and GLU2 Genes and the gls1-30 Allele

Restriction fragment length polymorphisms (RFLPs; Botstein et al., 1980) were identified for GLU1 and GLU2 by comparing restriction digest patterns of genomic DNA isolated from the Columbia and Landsberg erecta ecotypes by DNA gel blot analysis. After restriction of the genomic DNA with 19 different restriction endonucleases, 1 μg of each sample DNA was electrophoresed through duplicate 0.8% agarose, 1 M Tris–borate–EDTA gels containing 1 μg/mL ethidium bromide. The gels were then treated with 0.25 N HCl for 10 min; with 0.5 N NaOH, 1.5 M NaCl for 30 to 60 min; and with 1.5 M NaCl, 1 M Tris-HCl, pH 7.5, for 30 to 60 min. The DNA was transferred to a Hybond-N membrane (Amersham) by capillary blotting overnight with 0.05 N NaOH and 0.01 M NaCl for 30 min; 0.1 M Tris-HCl, pH 7.5, for 30 min; and 10 × SSC for 30 min. RNA was transferred to a Hybond-N membrane and cross-linked to the membrane, as described above. Antisense, digoxigenin-labeled, gene-specific DNA probes spanning nucleotides 38 to 457 of GLU1 (419 nucleotides) and nucleotides 21 to 557 of GLU2 (536 nucleotides) were made by PCR (Myerson, 1991). Hybridizations with the high SDS hybridization buffer, washes, and detections were performed as described in the Genius System User’s Guide for Membrane Hybridization (version 3.0; Boehringer Mannheim). LumiPhos was the substrate used for the detection.

RNA Gel Blot Analyses

Total RNA (quantity as indicated) was electrophoresed through 0.8% agarose gels containing 1% formaldehyde. Ethidium bromide was added to each RNA sample to a final concentration of 31 μg/mL before denaturation and loading to allow visualization of the RNA without affecting the efficiency of RNA transfer to the membrane (Ogretmen et al., 1993). After electrophoresis, the gels were treated with 0.05 N NaOH and 0.01 M NaCl for 30 min; 0.1 M Tris-HCl, pH 7.5, for 30 min; and 10 × SSC for 30 min. RNA was transferred to a Hybond-N membrane and cross-linked to the membrane, as described above. Antisense, digoxigenin-labeled, gene-specific DNA probes spanning nucleotides 38 to 457 of GLU1 (419 nucleotides) and nucleotides 21 to 557 of GLU2 (536 nucleotides) were made by PCR (Myerson, 1991). Hybridizations with the high SDS hybridization buffer, washes, and detections were performed as described in the Genius System User’s Guide for Membrane Hybridization (version 3.0; Boehringer Mannheim). LumiPhos was the substrate used for the detection.

Chlorophyll Measurements and Statistical Analyses

Wild-type Columbia and a gls mutant (NA60) were grown in air or 1% CO2 on specially modified MS medium from Sigma (lacking ammonium nitrate, potassium nitrate, and glycine) supplemented with 0.5% sucrose, 0.9% agar, and either low inorganic nitrogen (2 mM ammonium and 4 mM nitrate) or high inorganic nitrogen (20 mM ammonium and 40 mM nitrate) in a Percival growth chamber for 12 days with a 16-hr-light (70 μE m−2 sec−1) and 8-hr-dark cycle. Total chlorophyll was extracted from leaves using N,N-dimethylformamide and quantitated using the equation C_{t} = 8.24 A_{666} + 23.97 A_{647} − 16.64 A_{603}, as described by Moran (1982), where Ct stands for total chlorophyll. Primary statistical comparisons were performed independently for the plants grown in air and for the plants grown in 1% CO2 by using a two-way analysis of variance (ANOVA) of the chlorophyll measurements. In each analysis, the two variables were strain (wild type versus mutant) and nitrogen concentration (low versus high). When a significant interaction (P < 0.05) was found, the Fischer’s Protected LSD test (an a posteriori post hoc test using the accumulated error determined by the ANOVA) was used for comparing individual means to indicate which pairs showed significant differences (Statistica for Windows, version 5; StatSoft, Tulsa, OK).
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