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Arabidopsis *gls* 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 (*gls*). 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 *gls1* locus and *GLU* genes indicates that *GLU1* is linked to the *gls1* 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 *gls1* 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 *gls* 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 reassimilating 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 (*gls*, 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

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(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 (1% CO_2) (Somerville and Ogren, 1980). This conditional lethal phenotype of the *gls* class of mutants suggests that Fd-GOGAT is essential for photorespiration. Their "normal" growth in high CO_2 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 (*gluS*, 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% CO_2, 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 CS30; 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

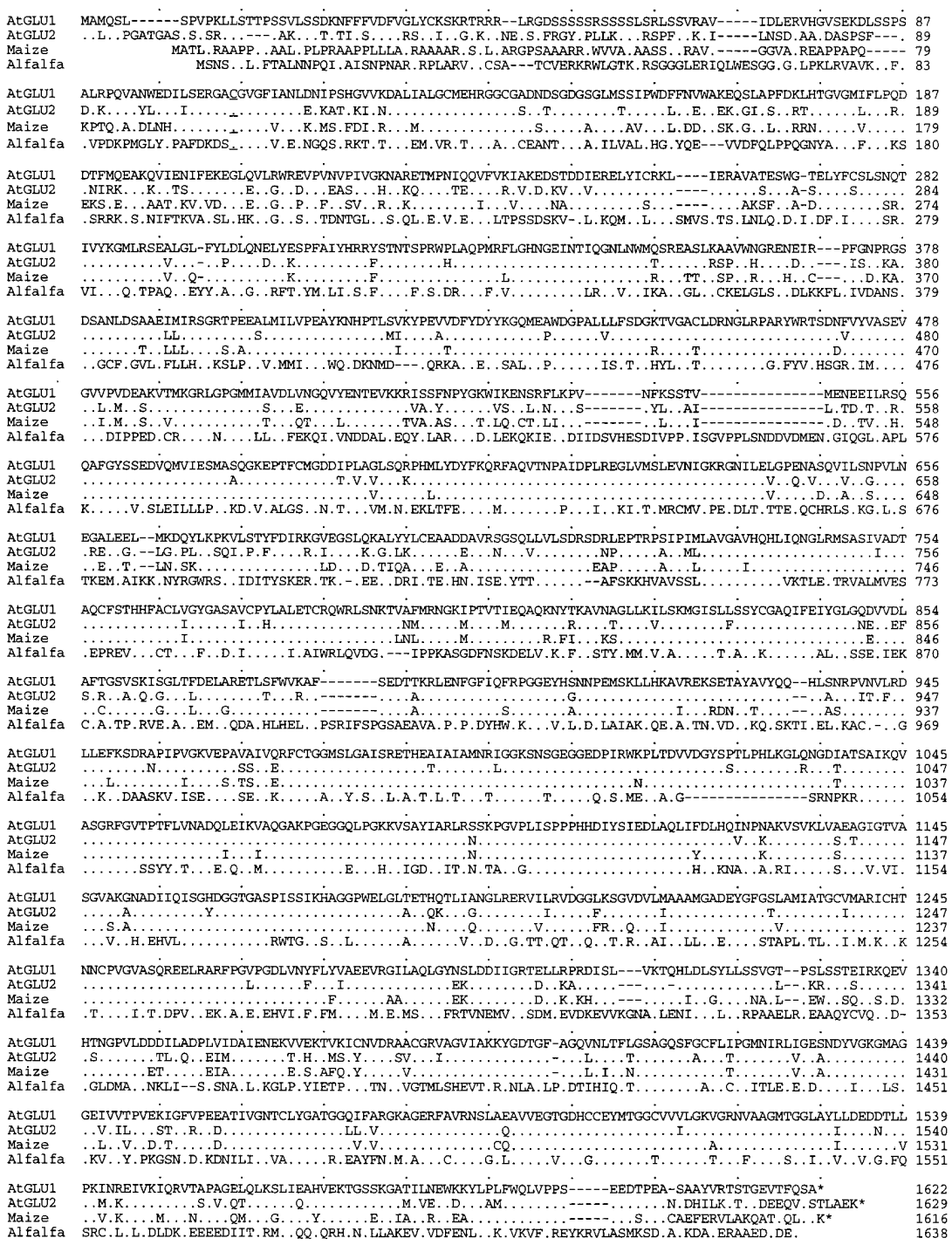


Figure 1. *GLU1* and *GLU2* Encode Distinct Fd-GOGAT Isoenzymes in Arabidopsis.

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% CO_2, indicating that Fd-GOGAT plays a major role in photorespiration

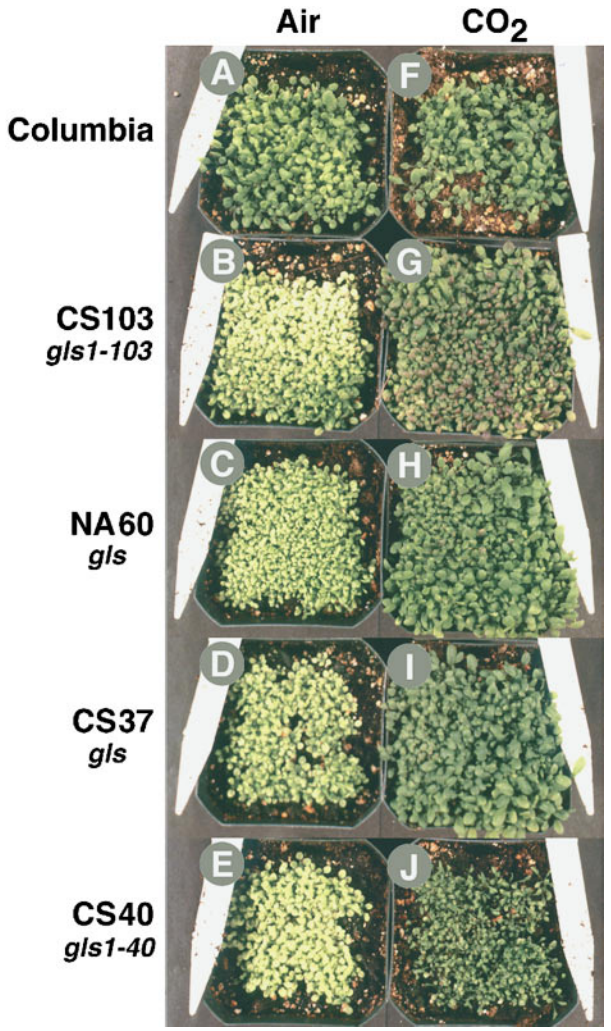


Figure 2. Photorespiratory Phenotype of *gls* Mutants Defective in Fd-GOGAT Activity.

(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% CO_2 in air.

Figure 1. (continued).

(Gregerson et al., 1993; only the N-terminal region that shows identity to the maize Fd-GOGAT and to the large subunit of *Escherichia coli* NAD[P]H-GOGAT is shown).

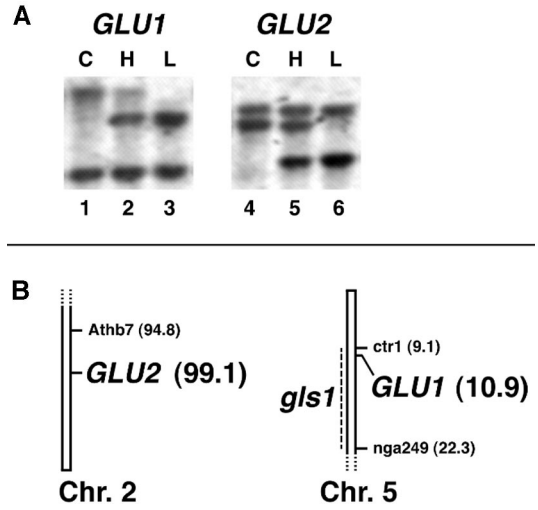


Figure 3. Mapping of the *GLU1* and *GLU2* Genes and a *gls1* Mutant Allele to Arabidopsis Chromosomes.

(A) Gene-specific RFLPs for *GLU1* and *GLU2*. Genomic DNA was extracted from Columbia (C; lanes 1 and 4), Landsberg *erecta* (L; lanes 3 and 6), or both (H; mixture of equal proportions of Landsberg *erecta* and Columbia DNA; lanes 2 and 5) and restricted with BamHI. Replicate blots were hybridized with *GLU1* or *GLU2* gene-specific probes. Mapping of *gls1* is as described in Methods.

(B) Enlargement of regions of chromosomes 2 and 5 showing the mapping results of the *GLU1* and *GLU2* genes and the *gls1* mutant allele. Markers for the RI mapping (Lister and Dean, 1993) are indicated to the right of each chromosome (Chr.) diagram. The numbers in parentheses are the positions of the markers (in centimorgans) according to the RI line map of Lister and Dean (as of May, 1995). The dashed line to the left of chromosome 5 indicates the region to which the *gls1* mutant was mapped using SSLP markers (Bell and Ecker, 1994). Dashed extensions of chromosomes indicate that this diagram shows only a region of each chromosome.

(Somerville and Ogren, 1980). However, although the *gls* mutants were reported to grow "normally" in 1% CO_2, 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% CO_2 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% CO_2, the wild type and NA60 each accumulated significantly higher levels of chlorophyll compared with air under either nitrogen concentration. These results demonstrate that the CO_2 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% CO_2, in which photorespiration was suppressed. As shown in Figure 7B, when grown in 1% CO_2, 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

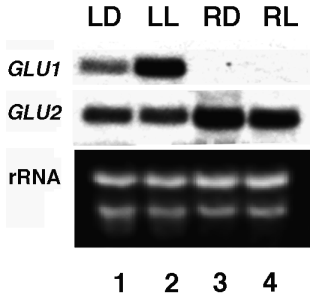


Figure 4. Differential Expression of *GLU1* and *GLU2* mRNAs in Arabidopsis Leaves and Roots.

Twenty-five-day-old plants initially grown semihydroponically with a 16-hr-light and 8-hr-dark cycle (see Methods) were switched for 48 hr to either continuous darkness (lanes 1 and 3) or continuous light (lanes 2 and 4) before harvesting. Total RNA was extracted from leaves (lanes 1 and 2) or roots (lanes 3 and 4). LD, lane 1, leaves from dark-adapted plants; LL, lane 2, leaves from light-treated plants; RD, lane 3, roots from dark-adapted plants; RL, lane 4, roots from light-treated plants. Digoxigenin-labeled *GLU1* and *GLU2* gene-specific probes were hybridized with replicate blots of 20 μg of total RNA per lane. rRNAs are shown by ethidium bromide staining.

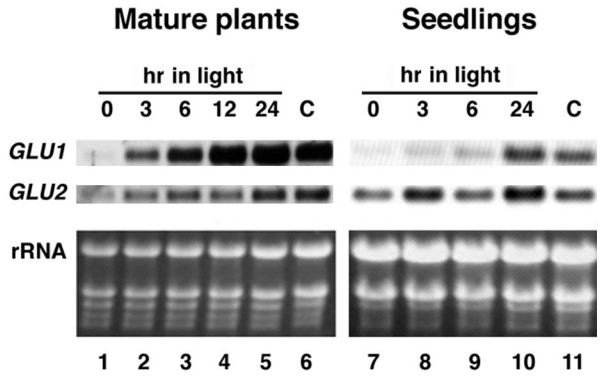


Figure 5. *GLU1* mRNA Accumulation Is Induced by Light.

Twenty-five-day-old plants grown in continuous light were dark-adapted for 64 hr (lane 1) and then transferred to light for 3, 6, 12, or 24 hr (lanes 2 to 5) before harvesting the whole plant for RNA extraction. Control plants (C), which were grown in continuous light, were collected at the last time point (lane 6). Three-day-old etiolated seedlings (lane 7) were transferred to light for 3, 6, or 24 hr (lanes 8 to 10) before harvesting the entire seedling for RNA extraction. Control seedlings (C) were grown in continuous light for 3 days (lane 11). Replicate RNA gel blots containing 10 μg (lanes 1 to 6) or 20 μg (lanes 7 to 11) of total RNA were hybridized with digoxigenin-labeled *GLU1* and *GLU2* gene-specific probes, as described previously. Ethidium bromide staining of rRNAs is shown at bottom.

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 *GLU* gene is affected in the *gls* 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 *GLU* gene is affected in the *gls1* photorespiratory mutants, we mapped both the *gls1*

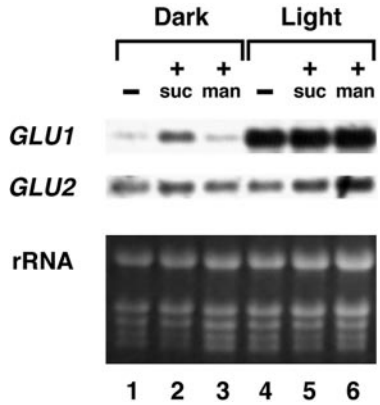
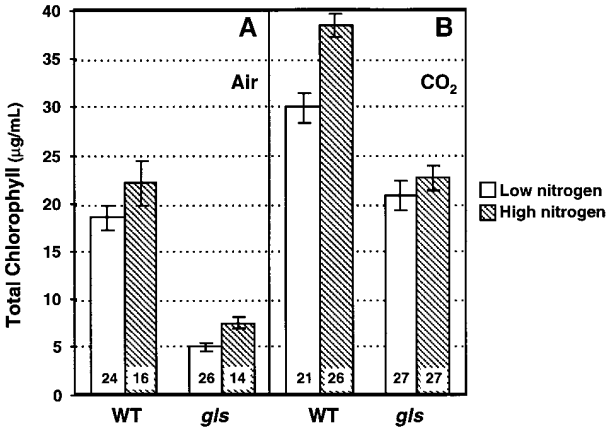


Figure 6. Carbon Metabolites Induce *GLU1* mRNA Accumulation in the Absence of Light.

Twelve-day-old plants grown semihydroponically on MS medium plus 3% sucrose (see Methods) were transferred to medium without sucrose and dark-adapted for 3 days. Plants were then transferred to fresh media minus (−) sucrose (lanes 1 and 4), plus (+) 3% sucrose (suc; lanes 2 and 5), or plus 3% mannitol (man; lanes 3 and 6) and then left in the dark (lanes 1 to 3) or transferred to light (lanes 4 to 6) for 3 days before harvesting the whole plant for RNA extraction. Replicate blots of 10 μg of total RNA were hybridized as described previously. Ethidium bromide staining of the rRNAs is also shown.



C

Effect	Air P	CO_2 P
1 - WT vs. NA60	<0.0001 *	<0.0001 *
2 - low vs. high nitrogen	0.0145 *	0.0005 *
3 - 1 x 2 interaction	0.7029	0.0191 *

Figure 7. A *gls* Mutant Displays a Defect in the Assimilation of Inorganic Nitrogen under Nonphotorespiratory Conditions.

(A) and **(B)** Plants grown in air and plants grown in 1% CO_2, respectively. Wild-type Columbia (WT) and a *gls* mutant (NA60) were grown for 12 days on nitrogen-free MS media supplemented with either low levels of inorganic nitrogen (2 mM ammonium and 4 mM nitrate; open bars) or high levels of inorganic nitrogen (20 mM ammonium and 40 mM nitrate; hatched bars). Mean values of total chlorophyll measurements ±SE are shown. The total number of plants analyzed is indicated within each bar.

(C) Two-way ANOVA of (1) the wild type (WT) versus a *gls* mutant in air and CO_2, (2) low versus high nitrogen conditions in air and CO_2, and (3) interaction of 1 and 2 as given above. Statistically significant results are indicated by an asterisk.

mutation and the *GLU1* and *GLU2* genes onto the Arabidopsis chromosomes, relative to linked physical markers (RFLPs and SSLPs). These studies showed that the *GLU1* gene and a *gls1* allele each map to the same local region of chromosome 5. By contrast, the *GLU2* gene maps to an unlinked location on chromosome 2. These results indicate that *GLU2* is unaffected in the *gls* mutants and that *GLU1* is likely to be the affected gene in the *gls* mutants. Other data support the possibility that *gls* mutants are affected in the *GLU1* gene: here, we show that *GLU1* is the major *GLU* gene expressed in leaves, and Suzuki and Rothstein (1997) showed a twofold decrease in levels of *GLU1* mRNA in leaves of a *gls* mutant.

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 CO_2, 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; Elmlinger 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 GS 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% CO_2 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% CO_2 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–isoamylalcohol (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 λ 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 subcloned using the pCR-Script kit (Stratagene). Sequencing was performed using the TN1000 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 × 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 10 × SSC. DNA was cross-linked to the membrane by using the automatic setting (1200 μE) of the UV Stratalinker 2400 (Stratagene).

Probes for *GLU1* (nucleotides 1096 to 5188) and *GLU2* (nucleotides 2088 to 5390) were labeled with digoxigenin by PCR, using a slightly modified version of the protocol in the Genius System User's Guide to Membrane Hybridization (version 3.0; Boehringer Mannheim). In short, deoxynucleotide concentrations were increased, and both oligonucleotides were used in the labeling reaction. Hybridization was performed as described for the Genius System, except that the SDS concentration was increased to 1%. The signal from the digoxigenin-labeled probes was visualized on autoradiography film by using the chemiluminescent LumiPhos substrate (Boehringer Mannheim). Gene specificity was achieved by washing twice with 0.05 × SSC and 0.1% SDS at 65°C.

Although several enzymes displayed polymorphisms, BamHI, which displayed RFLPs for both *GLU1* and *GLU2*, was chosen for the mapping analysis. Genomic DNA was isolated from 26 recombinant inbred (RI) lines obtained from the Ohio State University Arabidopsis Stock Center (Columbus, OH; Lister and Dean, 1993). After restriction with BamHI, the DNA was analyzed as described above. The parental patterns (Columbia or Landsberg *erecta*) for the *GLU1* and *GLU2* probes were determined for each RI line. The segregation data were sent to C. Lister (John Innes Centre, Norwich, UK), who mapped the results with respect to 462 markers and determined the positions of the *GLU1* and *GLU2* genes in the Arabidopsis genome.

To map the *gls1* mutation, the CS30 allele of *gls1* (*gls1-30*; Columbia ecotype background) was used to pollinate wild-type Landsberg *erecta* plants. Seeds resulting from the cross were planted (F_1 generation), grown to maturity, and allowed to self-pollinate. Seeds of the next generation (F_2) were plated in a single line on a square tissue culture plate of MS medium with 0.9% agar and 3% sucrose and grown in an environmental growth chamber until the second pair of true leaves had emerged. Seedlings were transferred to soil and grown with CO_2 supplementation (2%) for 1 week. A single leaf was ground in a buffer of 50 mM KH_2PO_4, pH 7.5, 5 mM EDTA, and 12.5 mM β-mercaptoethanol, and the soluble extract was assayed for Fd-GOGAT activity essentially as described by Wallsgrove et al. (1977) by using methyl viologen (Sigma) as the reducing agent. The reaction was terminated by boiling for 1 min. Glutamate production was quantified by HPLC (Shimadzu 10A system [Kyoto, Japan], attached to a Perkin-Elmer LS30 luminescence spectrophotometer [Beaconsfield, UK]) on a C18 reverse-phase column (Martin et al., 1982) after derivitization with an *o*-phthaldialdehyde reagent solution (Sigma). Mutants were identified as producing nearly the same amount of glutamate in the presence and absence of reductant (wild-type plants produced four- to 10-fold more glutamate in the presence of the reductant). Genomic DNA was isolated from the mutants, and the mutation was mapped by simple sequence length polymorphism (SSLP) analysis (Bell and Ecker, 1994).

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% CO_2 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 $Ct = 8.24\ A_{666} + 23.97\ A_{647} - 16.64\ A_{603}$, 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% CO_2 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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