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Use of Arabidopsis Mutants and Genes To Study Amide Amino Acid Biosynthesis

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INTRODUCTION

Studies of enzymes involved in nitrogen assimilation in higher plants have an impact on both basic and applied plant research. First, basic research in this area should uncover the mechanisms by which plants regulate genes involved in a metabolic pathway. Second, because nitrogen is a rate-limiting element in plant growth (Hageman and Lambert, 1988), it may be possible to increase the yield or improve the quality of crop plants by the molecular or genetic manipulation of genes involved in nitrogen assimilation.

Research on nitrogen assimilation into amino acids has been complicated by the fact that some of these reactions are catalyzed by multiple isoenzymes located in distinct subcellular compartments. With traditional biochemical approaches, it has been impossible to sort out the function of each isoenzyme in plant nitrogen metabolism. The discovery that genes for chloroplastic and cytosolic isoenzymes of glutamine synthetase (GS) are expressed in distinct cell types (Edwards et al., 1990; Carvalho et al., 1992; Kamachi et al., 1992) suggests that traditional biochemical studies, which begin with tissue disruption, artificially mix isoenzymes that may not coexist in the same cell type in vivo. Thus, in vitro biochemical methods commonly used to define the rate-limiting enzyme in a pathway in unicellular microorganisms may lead to erroneous interpretations when employed to study plant metabolic pathways. An alternative way to define the in vivo function of a particular isoenzyme or to define a rate-limiting enzyme in a pathway is by mutant analysis, as shown by studies of *Escherichia coli* and yeast. Plant mutants defective in particular isoenzymes of GS or ferredoxin-dependent glutamate synthase (Fd-GOGAT) have been identified in screens for photorespiratory mutants in Arabidopsis and barley (Somerville and Ogren, 1980, 1982; Wallsgrove et al., 1987). More recently, Arabidopsis mutants with alterations in the activity of additional enzymes of nitrogen assimilation have been identified using a screening method that does not depend on a growth phenotype (Schultz and Coruzzi, 1995). The in vivo role of the mutated isoenzyme can be determined by performing a detailed phenotypic analysis of the nitrogen-flux and growth profile of M_3 progeny plants.

In this article, we review studies of the Arabidopsis genes for and mutants in isoenzymes involved in the assimilation of nitrogen into glutamine, glutamate, aspartate, and asparagine. The structural genes for each isoenzyme have been used to study gene regulation by metabolites and environmental factors such as light and also to characterize Arabidopsis mutants defective in a particular isoenzyme. The combined efforts of molecular and genetic studies in the model plant Arabidopsis aim at (1) constructing a molecular-genetic blueprint of the assimilation of nitrogen into amide amino acids, (2) uncovering the global factors that coordinate the regulation of genes in a metabolic pathway, (3) understanding the distinct physiological role of each encoded isoenzyme, and (4) developing strategies to engineer plants that assimilate or transport nitrogen more efficiently.

THE PATHWAY OF NITROGEN ASSIMILATION

Inorganic nitrogen in the form of nitrate is taken up by plants and reduced to ammonia via the concerted actions of nitrate reductase and nitrite reductase (Lea, 1993; see Crawford, 1995, this issue). The enzymes involved in the assimilation of nitrogen into the nitrogen-transporting amino acids glutamine, glutamate, aspartate, and asparagine are shown in Table 1, along with the nomenclature used for the isoenzymes and genes. These four amino acids play important roles in the nitrogen assimilation pathway in plants. Figure 1 details the roles proposed for each isoenzyme in processes such as primary nitrogen assimilation in leaves and roots, assimilation of photorespiratory ammonia in leaves, and reassimilation of "recycled" nitrogen generated by amino acid deamination. The positions of isoenzymes in the pathways depicted are proposed based on our current understanding of enzymes, mutants, and gene regulation, as described later.

Ammonia is assimilated into glutamine and glutamate through the combined actions of GS (EC 6.3.1.7) and Fd-GOGAT (EC 1.4.7.1) or NADH-dependent glutamate synthase (NADH-

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Table 1. Amide Amino Acid Metabolic Enzymes and Genes Identified in Arabidopsis

Enzymes	Enzyme Acronym	Genes	Mutant Alleles[a]	References
Asparagine synthetase	AS	*ASN1*, *ASN2*, *ASN3*		Lam et al. (1994); H.-M. Lam and G. Coruzzi (unpublished results)
Aspartate aminotransferase[b]	AspAT			
Mitochondrial		*ASP1*		Schultz and Coruzzi (1995)
Plastidic/peroxisomal		*ASP3*, *AAT1*[c]	*aat3*	Schultz (1994); Schultz and Coruzzi (1995); Wilkie et al. (1995)
Cytosolic		*ASP2*, *ASP4*	*aat2-2*, *aat2-4*	Schultz (1994); Schultz and Coruzzi (1995)
Glutamate dehydrogenase	GDH	*GDH*	*gdh-1*	R. Melo-Oliveira and G. Coruzzi (unpublished results)
Glutamate synthase				
Ferredoxin-dependent	Fd-GOGAT	*GLU1*, *GLU2*	*gls* (*gluS*)	Somerville and Ogren (1980); K. Coschigano and G. Coruzzi (unpublished results)
NADH-dependent	NADH-GOGAT	*GLT1*		H.-M. Lam and G. Coruzzi (unpublished results)
Glutamine synthetase				
Cytosolic	GS1	*GLN1* (*gsR1*, *gsR2*, *gsKb6*)		Peterman and Goodman (1991)
Chloroplastic	GS2	*GLN2* (*gsL1*)		Peterman and Goodman (1991)

[a] No complementation test has been performed to pair each mutation to its corresponding gene. However, mapping data show that the mutations of the *gls* mutants obtained by Somerville and Ogren (1980) and the *GLU1* gene are linked (K. Coschigano and G. Coruzzi, unpublished results).
[b] The products of various AspAT genes are thought to be located in different organelles, based on the presence or absence of signal peptides (Schultz, 1994; Schultz and Coruzzi, 1995). Mutants defective in plastidic/peroxisomal (*aat3*) and cytosolic AspAT (*aat2-2*, *aat2-4*) were isolated by activity gel assay (see Figure 3).
[c] The *AAT1* gene obtained by Wilkie et al. (1995) is not related to the AAT1 band identified in the activity gel assay by Schultz (1994), as discussed in the text.

GOGAT; EC 1.4.1.14). Although glutamate dehydrogenase (GDH; EC 1.4.1.2) is the primary route of nitrogen assimilation in microorganisms, several lines of evidence suggest that GDH in higher plants functions largely in glutamate catabolism (Robinson et al., 1991).

Glutamine and glutamate serve as nitrogen-transport compounds and nitrogen donors in the biosynthesis of an enormous number of compounds (Lea et al., 1989), including essentially all amino acids (see, for example, Radwanski and Last, 1995, this issue), nucleic acids, and other nitrogen-containing compounds such as chlorophyll (see von Wettstein et al., 1995, this issue). Nitrogen may be subsequently channeled from glutamine and glutamate to aspartate by aspartate aminotransferase (AspAT; EC 2.6.1.1) or to asparagine by asparagine synthetase (AS; EC 6.3.5.4). The four nitrogen-transport amino acids generated by this pathway (glutamine, glutamate, aspartate, and asparagine) are the predominant amino acids found in most higher plants. In Arabidopsis, these amino acids represent 64% of the total amino acids found in leaf extracts (Figure 2A; Schultz, 1994). The amide amino acids glutamine and asparagine each carry an extra nitrogen atom in the amide group of their side chains, and as such they play an important role as nitrogen carriers in cellular metabolism (Lea and Miflin, 1980; Urquhart and Joy, 1981). HPLC analyses of phloem exudates of Arabidopsis leaves suggest that asparagine is the predominant amino acid exported from leaves of dark-grown or dark-adapted plants (Figure 2B; Schultz, 1994). In contrast, glutamine is used primarily to transport assimilated nitrogen from roots to shoots, as shown by analysis of xylem exudates of Arabidopsis roots (Figure 2C; Schultz, 1994).

Glutamine Synthetase: Defining Roles for Chloroplastic and Cytosolic Isoenzymes

Nitrogen assimilation into glutamine via GS occurs in a variety of organs and subcellular compartments. In leaves, chloroplastic GS2 functions to assimilate primary ammonia reduced from nitrate and also to reassimilate ammonia released during photorespiration (Figure 1). Because ammonia "lost" via photorespiration exceeds primary nitrogen assimilation by 10-fold (Keys et al., 1978), the ability of GS2 to recapture this ammonia is essential to plant growth. Although most nitrate is transported to leaves for subsequent reduction and assimilation into organic form, some nitrate is reduced and assimilated into glutamine in roots via cytosolic GS1 or via chloroplastic GS2 present in root plastids (Figure 1; Miflin, 1974). GS also functions to reassimilate ammonia released during seed germination in cotyledons and during leaf senescence (Miflin and Lea, 1976; Edwards et al., 1990). All species examined to date, including Arabidopsis, appear to possess a single nuclear gene for chloroplastic GS2 and at least three genes for cytosolic

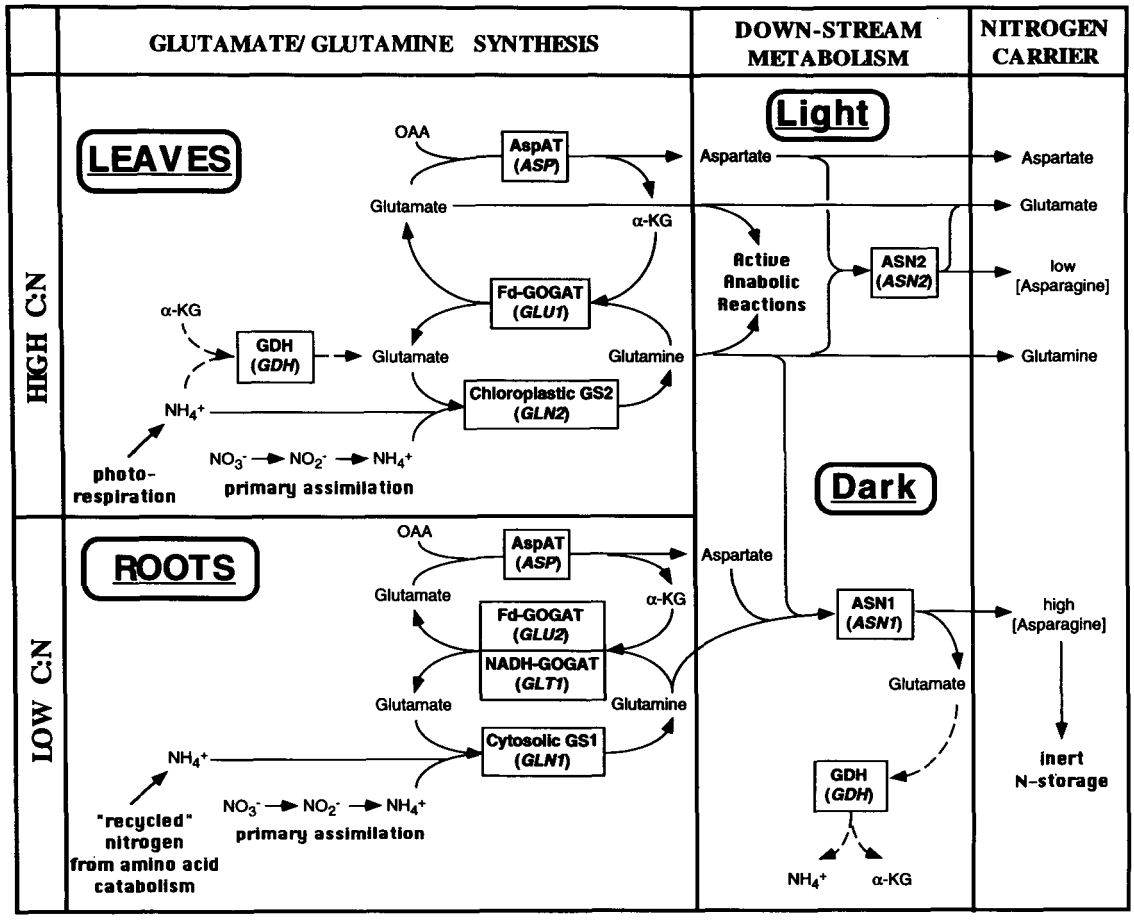

Figure 1. A Model of the Major Pathway of Amide Amino Acid Metabolism in Arabidopsis under Different Physiological Conditions.

The enzymes and genes involved in amide amino acid metabolism are summarized in Table 1. As discussed in the text, different isoenzymes in this pathway may play different roles in nitrogen assimilation under different environmental conditions and in different tissue types. Chloroplastic GS2 (GLN2) and one type of Fd-GOGAT (GLU1) seem to play a major role in recapturing the ammonia lost during photorespiration and perhaps in the primary assimilation of ammonia in leaves under light conditions (high C:N ratio). Cytosolic GS1 (GLN1), NADH-GOGAT (GLT1), and another Fd-GOGAT (GLU2) may be responsible for the primary assimilation of ammonia in roots and also for the recapture of ammonia released during amino acid catabolism. Under light conditions (high C:N ratio), glutamine and glutamate generated from the GS/GOGAT cycle are used as the major nitrogen donors in various cellular anabolic reactions or are metabolized into other nitrogen carriers, such as aspartate and asparagine, by the action of AspAT and ASN2, respectively. The role of ASN2 in Arabidopsis is still not clear, because asparagine is maintained at a very low level in the light. Under dark conditions (or low C:N ratio), ASN1 functions to redirect the flow of nitrogen from glutamine into asparagine, which acts as a shunt for storage and/or long-distance transport of nitrogen. The level of asparagine increases dramatically in the dark (see also Figure 2). The role(s) of GDH is not yet clear, as indicated by the dashed line. GDH may act to recapture ammonia lost during photorespiration or it may function in the catabolism of glutamate. α-KG, α-ketoglutarate; OAA, oxaloacetate.

GS1 (Cullimore et al., 1984; Tingey et al., 1987; Peterman and Goodman, 1991).

The in vivo function of chloroplastic GS2 has been elucidated by both molecular studies on the genes and genetic studies of plant GS2 mutants. Based on these studies, chloroplastic GS2 has been proposed to serve two functions in leaves. One is in the assimilation of ammonia reduced from nitrite in plastids, and the second is in the reassimilation of photorespiratory ammonia (Figure 1). The latter role of plastid-localized GS2 is not immediately obvious, because photorespiratory ammonia is released in mitochondria. Based on criteria such as mitochondrial localization and K_m data, it has been suggested in the past that GDH, or even cytosolic GS1, might reassimilate photorespiratory ammonia (Keys et al., 1978; Yamaya and Oaks, 1987). To gain a better understanding of the photorespiratory process, mutant plants were isolated whose growth was inhibited under photorespiratory conditions (Somerville and Ogren, 1982; Wallsgrove et al., 1987). Those

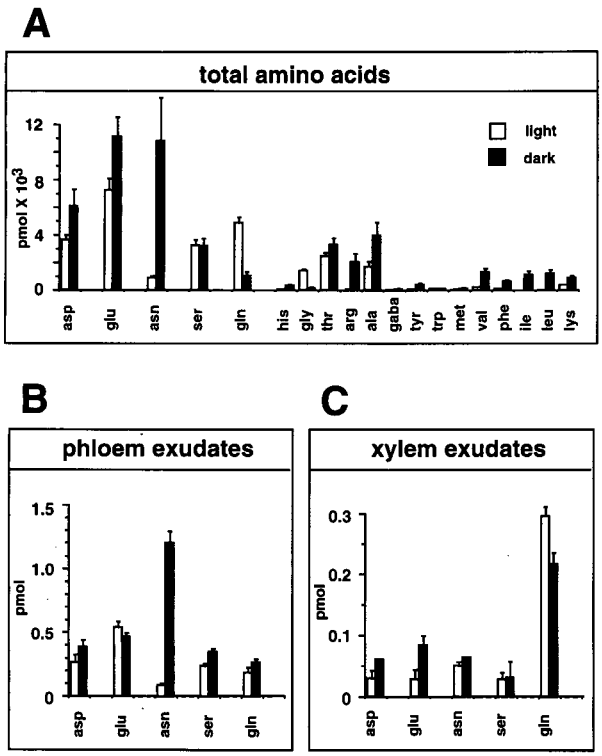

Figure 2. Amino Acid Levels in Light-Grown and Dark-Adapted Wild-Type Arabidopsis Plants.

Amino acids were extracted from Arabidopsis plants grown in light (empty boxes) or subsequently dark adapted for 24 hr (filled boxes), derivatized, and separated by reverse phase HPLC. The standard three letter code is used for all amino acids.

(A) Average total amino acid content. Each sample represents the average of three different plants (two leaves/plant). gaba, γ-amino butyric acid.

(B) Average amino acid content in phloem exudates of three independent plants (one leaf/plant).

(C) Average amino acid content of xylem sap collected from cut hypocotyls of three independent plants.

Data are from Schultz (1994).

mutants lack the ability to reassimilate ammonia lost during photorespiration. Some photorespiratory mutants isolated in barley are specifically deficient in chloroplastic GS2 (Wallsgrove et al., 1987). Paradoxically, these GS2 mutants are unable to reassimilate photorespiratory ammonia, even though they contain normal levels of cytosolic GS1 (Wallsgrove et al., 1987). This paradox was resolved by studies of the cell-specific expression patterns of genes for chloroplastic and cytosolic GS. Promoter–β-glucuronidase fusions and subsequent immunolocalizations showed that cytosolic GS1 genes are not expressed in mesophyll cells, where photorespiration occurs (Forde et al., 1989; Edwards et al., 1990; Carvalho et al., 1992). Thus, cytosolic GS1 cannot compensate for the loss of chloroplastic GS2 in mesophyll cells of photorespiratory mutants of barley because cytosolic GS1 is most likely expressed only in phloem cells.

Interestingly, screens for photorespiratory mutants in Arabidopsis have failed to uncover any mutants defective in GS, either chloroplastic GS2 or cytosolic GS1. There are several possible explanations for this finding: (1) The Arabidopsis photorespiratory screen was not saturating. This is unlikely, because multiple alleles for many enzymes in the photorespiratory pathway were isolated in that screen, including 58 mutants affecting Fd-GOGAT (Artus, 1988). (2) Both chloroplastic GS2 and cytosolic GS1 are expressed in mesophyll cells so that a mutation in one gene is masked. Again, this is unlikely, because cytosolic GS1 is not expressed in mesophyll cells, at least in tobacco and rice (Carvalho et al., 1992; Kamachi et al., 1992). (3) There is more than one gene for chloroplastic GS2 in Arabidopsis. (4) A mutation in chloroplastic or cytosolic GS is lethal in Arabidopsis, preventing the isolation of mutants.

The finding that the nonlegume plant Arabidopsis contains at least three genes for cytosolic GS1 is surprising, because the possession of multiple GS genes was believed to be important for nitrogen fixation in legumes. Genes for all three cytosolic GS1 isoenzymes of Arabidopsis are expressed at high levels in roots (Peterman and Goodman, 1991). The use of gene-specific probes has revealed that the three genes for cytosolic GS1 have subtle differences in their expression patterns. In addition, two genes for cytosolic GS1 are both expressed at high levels in germinated seed, suggesting that these genes may play a role in the synthesis of glutamine for transport of nitrogen out of cotyledons (Peterman and Goodman, 1991). This is consistent with previous data showing that individual genes for cytosolic GS1 of pea and bean are likely to serve this function (Cullimore et al., 1984; Tingey et al., 1987). However, as evidenced by the barley GS2 mutants, the only true way to know the in vivo function of a GS isoenzyme is to determine the phenotype of plants specifically defective in a single isoenzyme. Currently, several approaches are being employed to isolate or create mutants in chloroplastic or cytosolic GS in Arabidopsis (I. Oliveira and G. Coruzzi, unpublished results).

Glutamate Synthase: Using Genes and Mutants To Probe the Functions of Fd-GOGAT and NADH-GOGAT

Glutamate, the nitrogen donor for most transamination reactions in amino acid metabolism, is synthesized by glutamate synthase (GOGAT) in conjunction with GS in a cyclic manner (Figure 1; Lea, 1993). In Arabidopsis, glutamate represents a major constituent of the total amino acids extracted from mature leaves (Figure 2A; Schultz, 1994). Biochemical studies have revealed two distinct forms of the GOGAT enzyme, a ferredoxin-dependent form (Fd-GOGAT) and an NADH-dependent form (NADH-GOGAT; Suzuki and Gadal, 1984). Fd-GOGAT is more abundant in mature photosynthetic tissues, whereas NADH-GOGAT is more abundant in immature nonphotosynthetic tissues (Matoh and Takahashi, 1982). Fd-GOGAT and

Table 2. Differential Expression of Arabidopsis GS and GOGAT Genes

Enzyme (Gene Designation)	Tissue Specificity		Light Induction	References
	Shoot	Root		
Chloroplastic GS2 (*GLN2*)	+ + +[a]	+/−	Yes	Peterman and Goodman (1991)
Fd-GOGAT (*GLU1*)	+ + +	−	Yes	K. Coschigano and G. Coruzzi (unpublished results)
Cytosolic GSR1 (*GLN1*)	+	+ + +	No	Peterman and Goodman (1991)
NADH-GOGAT (*GLT1*)	+	+ + +	No	H.-M. Lam and G. Coruzzi (unpublished results)
Fd-GOGAT (*GLU2*)	+	+ + +	No	K. Cochigano and G. Coruzzi (unpublished results)

[a] mRNA levels range from +/− (detectable but not abundant) to + + + (much greater abundance); −, not detectable.

NADH-GOGAT are both plastidic enzymes (Suzuki and Gadal, 1984; Chen and Cullimore, 1989). Biochemical analysis of GOGAT enzymes is complicated by the fact that both Fd-GOGAT and NADH-GOGAT each exist as multiple isoenzymes (Suzuki et al., 1982; Chen and Cullimore, 1989). It has been difficult to assign roles to these isoenzymes based solely on the biochemical studies conducted in a number of different species. Molecular-genetic analyses of GOGAT genes and mutants in a single species, Arabidopsis, have begun to clarify the roles of these different GOGAT isoenzymes, as outlined below.

In wild-type Arabidopsis leaves, Fd-GOGAT contributes ~95% of the total GOGAT activity; the remaining 5% of GOGAT activity can be attributed to NADH-GOGAT (Somerville and Ogren, 1980). Photorespiratory mutants, termed *gls* mutants, have been identified in Arabidopsis in which leaf Fd-GOGAT activity is nearly undetectable, yet the constitutively low levels of NADH-GOGAT remain unchanged (Somerville and Ogren, 1980). The fact that these *gls* mutants grow normally in high CO_2 but die in air suggested that the major role for Fd-GOGAT in Arabidopsis leaves is to reassimilate photorespiratory ammonia and that this role is "dispensable" for primary assimilation. Thus, the Fd-GOGAT isoenzyme and the chloroplastic GS2 isoenzyme appear to be linked in a cycle that functions to reassimilate photorespiratory ammonia (Figure 1). This coupling of enzyme activity is borne out at the molecular level: a gene for Arabidopsis Fd-GOGAT (*GLU1*) and a gene for chloroplastic GS2 (*GLN2*) are both expressed primarily in leaves, and their steady state mRNA levels increase with light treatment (Table 2; Peterman and Goodman, 1991; K. Coschigano and G. Coruzzi, unpublished results).

cDNAs for Fd-GOGAT have been cloned from maize (Sakakibara et al., 1991), tobacco (Zehnacker et al., 1992), barley (Avila et al., 1993), and spinach (Nalbantoglu et al., 1994). Only a single cDNA was isolated in each case. Two distinct Fd-GOGAT cDNAs were recently cloned from Arabidopsis, indicating the existence of two functional Fd-GOGAT genes, *GLU1* and *GLU2* (K. Coschigano and G. Coruzzi, unpublished results). It is curious that there are two expressed Fd-GOGAT genes in Arabidopsis, given that mutants lacking Fd-GOGAT activity (that is, the *gls* mutants) were isolated in the screen for photorespiratory mutants conducted by Somerville and Ogren (1980, 1982), suggesting one genetic locus. However, it should be noted that the leakiness of the *gls* mutant phenotype (C. Somerville, personal communication) may be accounted for by the existence of two Fd-GOGAT genes.

Because the Fd-GOGAT mutants were the largest class of photorespiratory mutants obtained in the Arabidopsis screen (Somerville, 1984; Artus, 1988), it is possible that mutation of either one of the two Fd-GOGAT genes results in a phenotype. Alternatively, if one Fd-GOGAT gene is expressed at high levels and the other at low levels, a mutation in the highly expressed gene could give rise to a phenotype. This latter explanation has been shown to be the case for the duplicated tryptophan synthase β genes of Arabidopsis (Last et al., 1991; also see Radwanski and Last, 1995, this issue) and may also be true for the Fd-GOGAT genes, because one (*GLU1*) is highly expressed in shoots and the other (*GLU2*) is highly expressed in roots (Table 2; K. Coschigano and G. Coruzzi, unpublished results). Genetic mapping experiments should indicate whether it is the *GLU1* gene, the Fd-GOGAT gene most likely to be involved in photorespiration based on its expression pattern, that is affected in the photorespiratory *gls* mutants. Analysis of Fd-GOGAT genes and *gls* mutants will provide the ultimate proof that the product of *GLU1*, together with the chloroplastic GS2, is responsible for recapturing the nitrogen lost as ammonia during photorespiration (Figure 1).

A single cDNA for NADH-GOGAT has been cloned from two plant species, alfalfa (Gregerson et al., 1993) and Arabidopsis (H.-M. Lam and G. Coruzzi, unpublished results). The structure of plant NADH-GOGAT resembles that of the *Escherichia coli* enzyme, which is composed of two polypeptides, a large and a small subunit (Miller and Stadtman, 1972). In plants, the two subunits of the NADH-GOGAT enzyme are contained in one polypeptide, with the NADH binding domain near the C terminus (Oliver et al., 1987; Gregerson et al., 1993). Biochemical data has shown that NADH-GOGAT activity is low in mature leaves and high in roots (Matoh and Takahashi, 1982) and cotyledons (Hecht et al., 1988). These data are consistent with expression studies in Arabidopsis, which show high levels of NADH-GOGAT (*GLT1*) mRNA in roots and low levels in leaves (Table 2; H.-M. Lam and G. Coruzzi, unpublished results). This organ-specific pattern of enzyme activity and gene expression indicates that NADH-GOGAT, possibly together with the root-specific Fd-GOGAT (*GLU2*), most likely functions in a cycle with cytosolic GS1 to generate glutamine and glutamate

for intracellular transport. The coordinated functions of NADH-GOGAT and cytosolic GS1 may act primarily on the initial incorporation of ammonia into root cells, the recapturing of ammonia lost by catabolism of amino acids, and the remobilization of ammonia released through processes such as the breakdown of seed storage proteins in cotyledons during germination (Figure 1).

Asparagine Synthetase: Exploring the Physiological Functions of Asparagine Metabolism

Asparagine serves as a major nitrogen transport and storage compound in many higher plants (Lea and Miflin, 1980; Urquhart and Joy, 1981). In certain legume species, asparagine can account for up to 86% of the transported nitrogen (Lea and Miflin, 1980). Because it is relatively inert and has a high nitrogen:carbon ratio (compared with glutamine), asparagine is an ideal compound for nitrogen transport and storage, especially under conditions in which the carbon supply is limited (Lea and Miflin, 1980; Urquhart and Joy, 1981; Sieciechowicz et al., 1988; Lam et al., 1994). This hypothesis is supported by the observation that asparagine levels are elevated in dark-grown or dark-adapted pea plants (Urquhart and Joy, 1982). Similarly, asparagine levels in Arabidopsis are also dramatically modulated by light (Schultz, 1994). Asparagine is a major component of the total amino acids in extracts of leaves from dark-adapted plants but a minor component of light-grown plants (Figure 2A; Schultz, 1994). Similar observations were obtained in analyses of amino acid constituents of phloem exudates in these plants (Figure 2B; Schultz, 1994).

The enzyme primarily responsible for the biosynthesis of asparagine in plant cells is AS. In *E. coli,* the *asnA* (Nakamura et al., 1981) and *asnB* (Scofield et al., 1990) genes encode AS enzymes that use ammonia and glutamine, respectively, as substrates. In higher plants, all the AS enzymes characterized thus far are glutamine dependent and most likely have a high K_m for ammonia (Sieciechowicz et al., 1988; Richards and Schuster, 1992). The instability of the AS enzyme, the presence of enzyme inhibitors, and the rapid turnover of asparagine by asparaginase (Streeter, 1977; Joy et al., 1983; Huber and Streeter, 1985) have severely hampered standard biochemical studies of asparagine biosynthesis in plants. More recently, molecular biological approaches have been employed to study directly the structure and regulation of AS genes in pea (Tsai and Coruzzi, 1990, 1991), asparagus (Davis and King, 1993), and Arabidopsis (Lam et al., 1994).

In Arabidopsis, the first AS gene (*ASN1*) was isolated by heterologous hybridization with the pea *AS1* gene probe (Lam et al., 1994). Like its pea counterpart, the Arabidopsis *ASN1* gene is negatively regulated by light (Tsai and Coruzzi, 1990, 1991; Lam et al., 1994). This light repression of *ASN1* gene expression is at least partially mediated through the photoreceptor phytochrome (Tsai and Coruzzi, 1990; Lam et al., 1994). Besides acting as a direct signal, light can also exert its effect on *ASN1* expression indirectly by changing the metabolic state of the plant. For instance, light provides the energy source for photosynthetic processes which lead to the accumulation of carbon skeletons. It is thus important to find that *ASN1* mRNA levels are also under metabolic regulation. Sucrose represses the dark induction of *ASN1*, and this effect is relieved by addition of a nitrogen source such as glutamine, glutamate, or asparagine (Lam et al., 1994). These observations support a model in which, when the carbon pool is low relative to the nitrogen pool, the *ASN1* gene product functions to redirect the flow of nitrogen into asparagine, which then acts as a shunt for nitrogen storage and/or long-distance nitrogen transport (Figure 1; Lam et al., 1994). More recently, two more Arabidopsis cDNA clones for glutamine-dependent AS genes (*ASN2* and *ASN3*) have been cloned from Arabidopsis by complementation of yeast mutants. Preliminary analyses show that the expression pattern of *ASN2* is different from that of *ASN1* (H.-M. Lam and G. Coruzzi, unpublished results). Studies of these additional *ASN* genes may help in understanding other functions of AS in Arabidopsis.

Aspartate Aminotransferase: Defining Distinct Roles for Isoenzymes in Different Subcellular Compartments

AspAT catalyzes the reversible transamination reaction: oxaloacetate + glutamate ↔ aspartate + α-ketoglutarate (Figure 1). AspAT can be considered one of the most important aminotransferases because of its role in shuttling reducing equivalents between organelles and the cytosol via the malate/aspartate shuttle (Givan, 1980; Ireland and Joy, 1988). In pea, biochemical and genetic studies have shown that there are at least three distinct forms of AspAT isoenzymes; these are localized to the chloroplast, mitochondria, and cytosol (Weeden and Marx, 1987). AspAT isoenzymes have also been detected in the peroxisomes and/or glyoxysomes of maize and spinach (Scandalios et al., 1975; Huang et al., 1976; Liu and Huang, 1977). Although AspAT plays a role in transamination reactions in each of these compartments, the question remains as to the exact physiological role of each isoenzyme.

AspAT genes have been cloned from several plant species, including alfalfa, *Panicum,* soybean, carrot, and lupin (Gantt et al., 1992; Taniguchi et al., 1992; Turano et al., 1992; Wadsworth et al., 1993). Although only one or two cDNAs have been isolated from each of these other plant species, five different AspAT cDNAs have been cloned from Arabidopsis (Schultz and Coruzzi, 1995; Wilkie et al., 1995). *ASP1* encodes a mitochondrial form of AspAT, *ASP3* encodes a peroxisomal or plastidic form of AspAT, and *ASP2* and *ASP4* each encode a cytosolic form of AspAT (Schultz and Coruzzi, 1995). Still another cDNA, *AAT1*, encodes a chloroplastic form of AspAT (Wilkie et al., 1995). *ASP1* and *ASP3* are expressed at similar levels in most of the tissues examined. Of the two genes encoding cytosolic AspAT, the *ASP2* gene is expressed at high levels in roots, whereas *ASP4* mRNA is of low abundance and cannot be detected by RNA gel blot analyses (Schultz and Coruzzi, 1995).

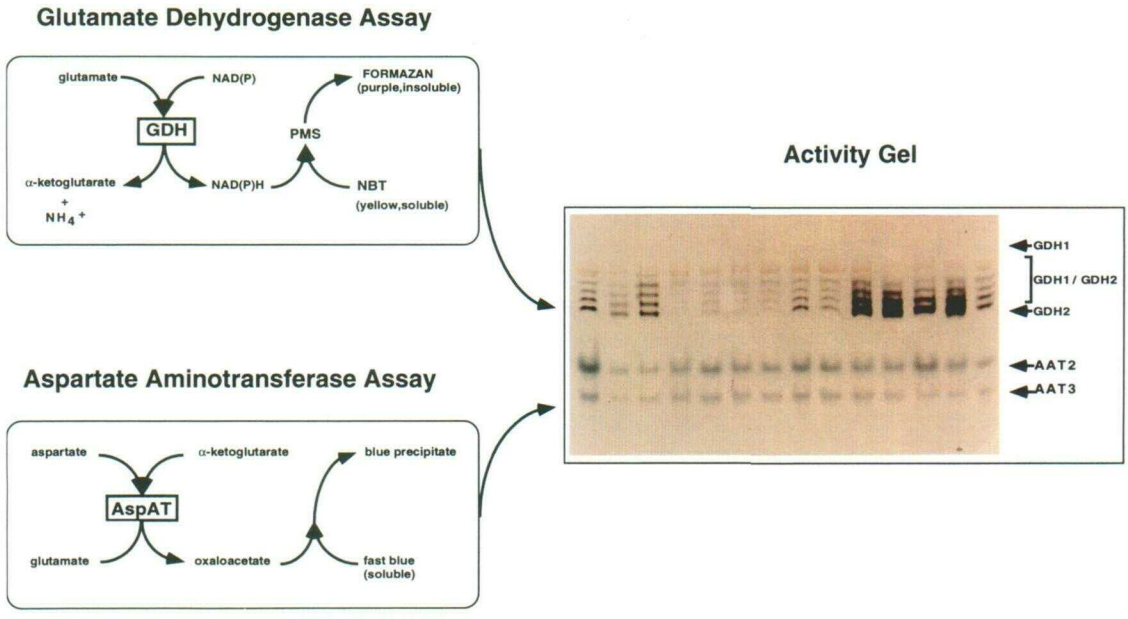

Figure 3. Arabidopsis GDH and AspAT Activity Gels.

GDH and AspAT activities were detected in leaf extracts run on nondenaturing polyacrylamide gels after the indicated coupled color reactions (Wendel and Weeden, 1989). The bands on the gel represent the migration points of different GDH and AspAT holoenzymes. It is possible to see four to seven bands of GDH activity that are due to the association of two differently charged peptides to form the active GDH hexamer. Two AspAT isoenzymes are visible, AAT2 and AAT3. Data are from Schultz (1994) and R. Melo-Oliveira and G. Coruzzi (unpublished results). NBT, nitro blue tetrazolium; PMS, phenazine methosulfate.

To determine the role of each AspAT isoenzyme, Arabidopsis mutants that are lacking an AspAT isoenzyme have been isolated by directly assaying leaves of ethyl methanesulfonate (EMS)–mutagenized M_2 seedlings for AspAT isoenzyme activity (Schultz, 1994). Individual AspAT isoenzymes can be visualized by AspAT activity analysis following nondenaturing gel electrophoresis of crude leaf extracts (Figure 3). Leaves contain two prominent AspAT isoenzymes: AAT2, which is likely to be localized in the cytosol, and AAT3, which is chloroplast localized (Schultz and Coruzzi, 1995). A third band of AspAT activity, AAT1, which is not related to the *AAT1* gene isolated by Wilkie et al. (1995), is rarely observed in crude extracts but is detected in preparations of purified mitochondria (Schultz and Coruzzi, 1995). Four classes of AspAT mutants were identified in a screen of 8000 EMS-mutagenized lines. Two classes were designated "loss-of-activity" mutants because they are missing either the AAT2 (cytosolic) or the AAT3 (chloroplastic) isoenzymes (Table 1; Schultz, 1994). The third and fourth classes were designated "altered-mobility" mutants because the mobility of either AAT2 or AAT3 is altered within the gel (Schultz, 1994). The allelic series of AspAT mutants is being characterized for altered growth phenotype, as well as for the level of each amino acid in the free amino acid pool, to determine the relative strength of the different alleles. Because the ability of the five AspAT clones to complement these mutants has yet to be tested, a direct correlation between a mutant and its corresponding gene is still missing. Further analysis of these mutants defective in either cytosolic or plastidic AspAT and their growth characteristics should make it possible to assign a role to each AspAT gene/isoenzyme and to determine whether the multiple forms of AspAT play distinct or overlapping roles in aspartate metabolism in Arabidopsis.

Glutamate Dehydrogenase: Determining Catabolic versus Biosynthetic Roles

In vitro studies have shown that plant GDH can catalyze two distinct biochemical reactions: the amination of α-ketoglutarate (biosynthetic), and the deamination of glutamate (catabolic) (Lea et al., 1991). Originally, GDH was thought to be the primary route of ammonia assimilation in plants, a role previously described for this enzyme in bacteria (Srivastava and Singh, 1987) and yeast (Rhodes et al., 1989). However, the biosynthetic role of GDH was challenged by the discovery of an alternative pathway for ammonia assimilation, that is, the GS/GOGAT cycle (Figure 1). Furthermore, the GDH enzyme has a high K_m for ammonia, a characteristic that argues against an assimilatory role and suggests a catabolic role for this enzyme (Stewart et al., 1980). Another possible biological role for GDH is in the ammonia detoxification process (Figure 1). This role has been suggested based on the observation that

this enzyme is induced by high levels of ammonia (Cammaerts and Jacobs, 1985). Mitochondrial GDH has been proposed to be involved in the assimilation of high concentrations of photorespiratory ammonia released in mitochondria (Yamaya and Oaks, 1987). However, the isolation of photorespiratory mutants defective in chloroplastic GS2 and Fd-GOGAT (Somerville and Ogren, 1980, 1982; Wallsgrove et al., 1987) argues against a major role for GDH in this process (Wallsgrove et al., 1987). However, it is still possible that GDH assimilates a portion of photorespiratory ammonia to generate catalytic amounts of glutamate to initiate the GS/GOGAT cycle (Figure 1). In conclusion, despite the bulk of information on the biochemistry of plant GDH, the actual function of this enzyme in higher plants has remained elusive.

To understand the actual physiological role of GDH in plants, Arabidopsis mutants that are deficient in GDH are being isolated in the M2 generation of EMS-mutagenized Arabidopsis using GDH activity staining of crude plant protein extracts after nondenaturing gel electrophoresis (R. Melo-Oliveira and G. Coruzzi, unpublished results). In Arabidopsis extracts, the GDH enzyme can be resolved into seven bands; these result from the random association of two differently charged subunits in a hexameric complex (Cammaerts and Jacobs, 1985; see Figure 3). It has been suggested that two nonallelic genes are responsible for the synthesis of these two types of subunits (Cammaerts and Jacobs, 1983, 1985). Thus far, only a single Arabidopsis GDH mutant, *gdh-1*, with an altered pattern of GDH activity (it possesses only a single holoenzyme corresponding to a homohexamer activity) has been identified (R. Melo-Oliveira and G. Coruzzi, unpublished results). Interestingly, a similar GDH mutant has been described in maize that is null for the *GDH1* gene product (Pryor, 1979). Studies of the maize GDH mutant have shown that it has a lower shoot/root ratio and a substantial sensitivity to low temperatures (Rhodes et al., 1989; Magalhaes et al., 1990; Pryor, 1990). However, the small number of GDH mutants available for analysis makes it difficult to explore fully the actual biological role of GDH. The further isolation and characterization of Arabidopsis GDH mutants will provide a unique opportunity to analyze the contribution of the GDH enzyme to the nitrogen metabolism pathway in the context of the whole plant. Furthermore, the characterization of the primary structure of the plant *GDH* gene(s) will allow a comparative analysis of its spatial and temporal expression.

LIGHT AND METABOLIC CONTROL OF AMIDE AMINO ACID BIOSYNTHESIS

Light and metabolic status are the two major signals that govern the regulation of amide amino acid metabolism. The regulation by light of the *ASN1* and *GLN2/GLU1* genes is a striking example of reciprocal gene regulation (Figure 4A and Table 2). Light induces *GLN2/GLU1* expression and represses *ASN1* expression. The physiological significance of changes in expression of these genes in response to these environmental signals is reflected in corresponding changes in the amino acid profile of Arabidopsis leaf extracts: asparagine levels are high in the dark, and glutamine levels are high in the light (Figure 4B). This reciprocal pattern of accumulation/synthesis coincides with the proposed functions of glutamine and glutamate versus asparagine (Figure 1). In the light, when photosynthesis occurs and carbon skeletons are abundant, nitrogen is assimilated and transported as glutamine; levels of mRNA for genes involved in glutamate and glutamine synthesis (*GLU1* and *GLN2*) are accordingly induced by both light and sucrose (Figure 4A and Table 2; K. Coschigano and G. Coruzzi, unpublished results; I. Oliveira and G. Coruzzi, unpublished results). In contrast, light represses the synthesis of asparagine, which therefore accumulates only in tissues of dark-adapted plants. Levels of *ASN1* mRNA are dramatically induced in dark-adapted plants (Figure 4B), and this induction is repressed by light or high levels of sucrose (Figure 4A; Lam et al., 1994). Thus, under conditions of carbon limitation or nitrogen excess, plants activate genes for asparagine biosynthesis.

Phytochrome is thought to be the primary receptor for the light signal (Quail, 1991). Possible intermediate effector molecules (such as COP and DET) of the light signal transduction pathway have been proposed (Chory, 1993; Li et al., 1994). However, a direct linkage between gene expression and the light-signal transduction pathway is still absent. Some nitrogen assimilation gene promoters, including those of pea *GS2* and *AS*, are known to possess light-responsive elements (Tjaden et al., 1995; F.-Y. Tsai, N. Ngai, and G. Coruzzi, unpublished results).

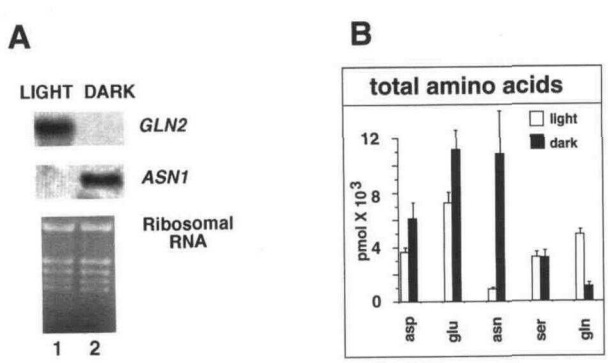

Figure 4. Reciprocal Effect of Light on Arabidopsis *GLN2* and *ASN1* Expression.

(A) The mRNA level of *GLN2* is elevated in light-grown plants (lane 1) but severely repressed in dark-adapted plants (lane 2). In contrast, the mRNA level of *ASN1* is nearly undetectable in light-grown plants (lane 1) but is strongly enhanced in dark-adapted plants (lane 2; Lam et al., 1994).

(B) A portion of Figure 2A is enlarged to show that the changes in *GLN2* and *ASN1* mRNA levels under light and dark conditions are paralleled by changes in glutamine and asparagine levels (also see Table 2 and Figure 1).

The mechanism by which plant genes respond to metabolic signals is also unknown. In contrast, metabolic control in microorganisms has been thoroughly studied (Hinnebusch and Fink, 1983; Neidhardt, 1987). For example, the mechanism that coordinates the catabolic repression of a set of affected genes is well documented (Celenza and Carlson, 1986; Magasanik and Neidhardt, 1987). The effect of carbon skeletons and nitrogen pools on nitrogen assimilation in microorganisms has also been studied thoroughly (Hinnebusch and Fink, 1983; Celenza and Carlson, 1986; Magasanik and Neidhardt, 1987; Neidhardt, 1987). In higher plants, supplementation with exogenous sugars leads to repression of a set of promoters involved in the photosynthetic process (Sheen, 1990). A homolog of the yeast catabolic repression *trans*-acting factor SNF1 has been identified in rye using yeast complementation techniques (Alderson et al., 1991). An SNF1-related gene was also isolated in Arabidopsis (Le Guen et al., 1992). It will be interesting to determine the mechanisms by which light and sucrose affect reciprocal control of *GLN2/GLU1* and *ASN1* in Arabidopsis.

METABOLIC ENGINEERING OF NITROGEN ASSIMILATION

Nitrogen is a rate-limiting element in plant growth. Although plants do not appear to be limited by their ability to take up or reduce nitrate (see Crawford, 1995, this issue), the ability of some crop plants to incorporate nitrogen into protein does appear to be limited. GS is likely to be a critical and possibly rate-limiting enzyme in this process, because it catalyzes the first major step converting nitrogen into an organic form for use in growth and development (Figure 1). To test this possibility, tobacco plants were engineered in which pea chloroplastic or cytosolic GS genes were expressed under the control of a cauliflower mosaic virus 35S promoter (T. Brears and G. Coruzzi, unpublished results). Tobacco lines that overexpress cytosolic GS1 or that are cosuppressed for GS were derived from these experiments. Cytosolic GS1 is normally expressed only in phloem cells of tobacco, but the transgenic GS1-overexpressing plants ectopically express cytosolic GS1 in mesophyll cells, where it may assist in assimilating primary nitrogen or in reassimilating photorespiratory nitrogen. Growth analyses suggest that plants that ectopically express cytosolic GS have a considerable growth advantage when compared with wild-type plants. In contrast, plants cosuppressed for both chloroplastic and cytosolic GS have decreased GS activity and reduced fresh weight in comparison with controls (T. Brears and G. Coruzzi, unpublished data). There is a linear correlation between GS activity and fresh weight in these transgenic plants. Because inhibiting GS activity causes plants to grow poorly, and increasing GS activity via ectopic expression results in plants with increased mass, it appears that GS is a rate-limiting enzyme in plant growth and nitrogen use.

In parallel with the GS experiments, transgenic tobacco plants were created that ectopically express the pea *AS1* gene (Brears et al., 1993). In certain high-expressing transformants, a 100-fold increase in the levels of free asparagine was observed in leaves. The preliminary studies of these GS and AS transgenic plants suggest that it should be possible to increase nitrogen use efficiency in transgenic crop plants by the molecular manipulation of one or several genes involved in nitrogen assimilation into amino acids. Attempts have been made to construct plants that ectopically express both GS and AS, and the growth phenotype of these transgenic plants are being subjected to thorough studies (G. Coruzzi, unpublished results). It is expected that plants with an increased capacity to assimilate nitrogen into glutamine and convert it to asparagine may be able to accumulate and store more organic nitrogen.

CONCLUSIONS

In this review, we have discussed the power of using the combined approaches of molecular biology and genetics to study nitrogen assimilation into amino acids in plants. Using the model plant Arabidopsis, it is now possible to thoroughly delineate the mechanism of genetic regulation of this pathway as well as to determine the in vivo role of each isoenzyme in the nitrogen assimilation pathway. These studies should help to define the rate-limiting enzyme/step of nitrogen assimilation in higher plants. Consequently, it may be possible to manipulate nitrogen assimilation in plants by such molecular and genetic techniques as ectopic expression and transgenic approaches. These basic studies could then be applied to and tested in crop plants, where there are obvious advantages of increasing the efficiency with which nitrogen is used, such as decreasing the reliance on nitrogen-based fertilizers in agriculture.

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