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THE MOLECULAR-GENETICS OF NITROGEN ASSIMILATION INTO AMINO ACIDS IN HIGHER PLANTS

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ABSTRACT

Nitrogen assimilation is a vital process controlling plant growth and development. Inorganic nitrogen is assimilated into the amino acids glutamine, glutamate, asparagine, and aspartate, which serve as important nitrogen carriers in plants. The enzymes glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), aspartate aminotransferase (AspAT), and asparagine synthetase (AS) are responsible for the biosynthesis of these nitrogen-carrying amino acids. Biochemical studies have revealed the existence of multiple isoenzymes for each of these enzymes. Recent molecular analyses demonstrate that each enzyme is encoded by a gene family wherein individual members encode distinct isoenzymes that are differentially regulated by environmental stimuli, metabolic control, developmental control, and tissue/cell-type specificity. We review the recent progress in using molecular-genetic approaches to delineate the regulatory mechanisms controlling nitrogen assimilation into amino acids and to define the physiological role of each isoenzyme involved in this metabolic pathway.

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INTRODUCTION

The assimilation of inorganic nitrogen onto carbon skeletons has marked effects on plant productivity, biomass, and crop yield (45, 64). Nitrogen deficiency in plants has been shown to cause a decrease in the levels of photosynthetic structural components such as chlorophyll and ribulose bisphosphate carboxylase (rubisco), with resulting reductions in photosynthetic capacity and carboxylation efficiency (26). Because enzymes involved in the assimilation of nitrogen into organic form in plants are crucial to plant growth, they are also effective targets for herbicide development (19).

A tremendous amount of biochemical and physiological studies have been performed on nitrogen assimilatory enzymes from a variety of plant species. Summaries of these biochemical studies can be found in several comprehensive reviews (40, 69, 70, 84, 91). The biochemical reactions of nitrogen assimilatory enzymes discussed herein are summarized in Table 1. Although these biochemical studies have provided a solid groundwork for the understanding of nitrogen assimilation in plants, a complete picture of the factors controlling and the enzymes involved in this process in a single plant is still lacking. The existence of multiple isoenzymes for each step in nitrogen metabolism has complicated biochemical purification schemes (95). Because the mechanisms controlling intra- and intercellular transport of inorganic and organic nitrogen in plants are presently unknown, it is impossible to predict the in vivo function of nitrogen assimilatory enzymes localized in distinct cells or subcellular compartments based on in vitro biochemistry.

Recently, molecular techniques and the analysis of plant mutants deficient in a particular isoenzyme have been employed to study nitrogen assimilation and metabolism. These studies have shown that the genes involved in nitrogen assimilation are not constitutively expressed “housekeeping” genes but are

Table 1 Biochemical reactions and mutants of plant nitrogen assimilation enzymes

Enzyme	Reaction	Mutant identification
GS1/GS2	glutamate + NH_4^+ + ATP = glutamine + ADP + Pi	barley (GS2) (143)
Fd-GOGAT	glutamine + 2-oxoglutarate + 2 Fd (red) = 2 glutamate + 2 Fd (ox)	Arabidopsis (116), barley (11), pea (8)
NADH-GOGAT	glutamine + 2-oxoglutarate + NADH = 2 glutamate + NAD	none
GDH	glutamate + H_2O + NAD/NADP = NH_4^+ + 2-oxoglutarate + NADH/NADPH	Arabidopsis (62; R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data)
AspAT	glutamate + oxaloacetate = aspartate + 2-oxoglutarate	Arabidopsis (62, 107)
AS	glutamine + aspartate + ATP = asparagine + glutamate + AMP +PPi	none

Abbreviations: GS1, cytoplasmic glutamine synthetase; GS2, chloroplastic glutamine synthetase; Fd-GOGAT, ferredoxin-dependent glutamate synthase; NADH-GOGAT, NADH-dependent glutamate synthase; GDH, glutamate dehydrogenase; AspAT, aspartate aminotransferase; AS, asparagine synthetase; Fd, ferredoxin; Pi, inorganic phosphate; PPi, pyrophosphate; NH_3, ammonia

carefully regulated by factors such as light, metabolites, and cell type. This review highlights examples where molecular, genetic, and biochemical analyses have begun to define the in vivo roles of individual isoenzymes in plant nitrogen assimilation and to uncover the mechanisms regulating this process. Special attention is paid to the genetically tractable system Arabidopsis, because it enables studies of biochemistry, molecular biology, and genetics of nitrogen assimilation in a single species. We also focus on the metabolism of glutamine, glutamate, aspartate, and asparagine, which are the dominant components in the total free amino acid pool in most legumes and crop plants (69, 88). While most studies of nitrogen metabolism have previously been performed in legumes and crop species, HPLC analyses of Arabidopsis have also demonstrated that these four amino acids can account for 60–64% of the total free amino acids present in leaves and are transported in the vascular tissues (62, 107). Thus, Arabidopsis appears to be a suitable model plant for the study of nitrogen assimilation; the results should have an impact on understanding less genetically tractable plants.

ASSIMILATION OF INORGANIC NITROGEN INTO GLUTAMINE AND GLUTAMATE

In plants, all inorganic nitrogen is first reduced to ammonia before it is incorporated into organic form (21, 50). Ammonia is then assimilated into glutamine

and glutamate, which serve to translocate organic nitrogen from sources to sinks in legumes and nonlegumes, including Arabidopsis (62, 69, 88, 107). The major enzymes involved are glutamine synthetase (GS), glutamate synthase (GOGAT, glutamine-2-oxoglutarate aminotransferase), and glutamate dehydrogenase (GDH). Each of these enzymes occurs in multiple isoenzymic forms encoded by distinct genes (see below). The individual isoenzymes of GS, GOGAT, or GDH have been proposed to play roles in three major ammonia assimilation processes: primary nitrogen assimilation, reassimilation of photorespiratory ammonia, and reassimilation of recycled nitrogen.

Primary Nitrogen Assimilation

In legumes, ammonia can be formed by the direct fixation of atmospheric dinitrogen atoms within root nodules (13, 135, 136). In nonlegumes, ammonia is generated by the concerted reactions of nitrate reductase and nitrite reductase (21, 50). In most tropical and subtropical species, nitrate taken up by the roots is largely transported to leaves where it is reduced to ammonia in plastids (3). Because chloroplastic GS2 and ferredoxin-GOGAT (Fd-GOGAT) are the predominant GS/GOGAT isoenzymes in leaves located in plastids, they have been proposed to function in the assimilation of this primary nitrogen into glutamine and glutamate (84). Because the predominant forms of GS and GOGAT in roots are cytosolic GS1 and NADH-GOGAT, these isoenzymes have been proposed to be involved in primary nitrogen assimilation in roots (84). GDH is less likely to be involved in primary nitrogen assimilation because of its K_m for ammonia (119).

The traditional assignments of GS/GOGAT isoenzyme function based on organ-specific distribution have been challenged by the phenotype of plant mutants defective in these enzymes. For example, although chloroplastic GS2 and Fd-GOGAT are proposed to be important for primary nitrogen assimilation in leaves, plant GS2 or Fd-GOGAT-deficient mutants appear to be competent in primary assimilation and specifically defective in the reassimilation of photorespiratory ammonia (9, 117; see sections on GS and GOGAT). No plant mutants yet exist in cytosolic GS1 or NADH-GOGAT to address whether they in fact are the major isoenzymes involved in primary nitrogen assimilation in leaves and/or roots.

Reassimilation of Photorespiratory Ammonia

Photorespiration is thought to be a wasteful process occurring predominantly in C3 plants that is initiated by rubisco oxygenase activity (41, 61). Thus, in plants grown in air, the oxygenation by rubisco results in the diversion of a portion of ribulose bisphosphate from the Calvin cycle and its conversion to two molecules of phosphoglycolate. The photorespiratory enzymes in plants

catalyze a series of metabolic conversions of phosphoglycolate that occur sequentially in chloroplasts, peroxisomes, and mitochondria. These reactions lead to the release of carbon dioxide and photorespiratory ammonia. In C3 plants, the ammonia released through photorespiration may exceed primary nitrogen assimilation by 10-fold (61). Therefore, to survive, a plant must be able to reassimilate this photorespiratory ammonia into glutamine or glutamate. Plant mutants defective in enzymes of the photorespiratory pathway have been identified by a conditional lethal phenotype screen (9, 117; also see below). The existence of photorespiratory mutants specifically defective in chloroplastic GS2 or Fd-GOGAT countered the suggestion that GDH, located in mitochondria, played a major role in reassimilation of photorespiratory ammonia (148). Thus, although the biochemical data and subcellular localization studies suggested that GDH played a major role in the reassimilation of photorespiratory ammonia, genetic data suggested otherwise.

Assimilation of Recycled Nitrogen

Ammonia is released during biochemical processes such as protein catabolism, amino acid deamination, and some specific biosynthetic reactions such as those involving methionine, isoleucine, phenylpropanoid, and lignin biosynthesis (66, 84). For plants to efficiently utilize nitrogen assimilated from the soil, they must be able to recycle nitrogen released during various catabolic reactions. While ammonia recycling occurs at all times in a plant, there are two major times when massive amounts of recycled ammonia must be reassimilated into glutamine or glutamate for transport. The first is during germination, when seed storage proteins are broken down and nitrogen is transported as glutamine to the growing seedling (69). Later, proteins in senescing leaves are degraded, and the nitrogen is reassimilated as glutamine for transport to the developing seed (83). Increased activities for cytosolic GS1, NADH-GOGAT, and GDH during these processes have suggested the involvement of these particular isoenzymes (70, 119).

GLUTAMINE SYNTHETASE

Biochemistry Background of Glutamine Synthetase

Two classes of glutamine synthetase (GS: E.C.6.3.1.2) isoenzymes that are located in the cytosol (GS1) or chloroplast (GS2) have been identified by ion-exchange chromatography. Although there are multiple forms of cytosolic GS, we refer to all cytosolic forms of GS as GS1 for simplicity. The distinct physiological roles of GS2 and GS1 have been implicated by their organ-specific distributions. For instance, because GS2 is the predominant isoenzyme in leaves, it has been proposed to function in primary assimilation of ammonia

reduced from nitrate in chloroplasts and/or in the reassimilation of photorespiratory ammonia (84). Because cytosolic GS1 is predominant in roots, it has been proposed to function in root nitrogen assimilation, although root plastid GS2 has also been implicated in this process (82). The finding that cytosolic GS1 is the predominant GS isoenzyme expressed during senescence in different plant species suggests that this GS isoenzyme plays a role in the mobilization of nitrogen for translocation and/or storage (56–58). The localization of GS1 in vascular bundles further supports the notion that cytosolic GS functions to generate glutamine for intercellular nitrogen transport (16, 55).

Despite the numerous studies on GS isoenzymes performed at the biochemical level, the exact in vivo role of each GS isoenzyme in plant metabolism is equivocal. The GS isoenzymes are encoded by a gene family in all plant species examined to date. A thorough characterization of the members of the GS gene family found in pea (127, 141), rice (106), Arabidopsis (90), *Phaseolus* (22, 37, 73), maize (72, 104, 115), and soybean (49, 102) showed that each species appears to possess a single nuclear gene for chloroplastic GS2 and multiple genes for cytosolic GS1. These studies have demonstrated that several members of the GS gene families are regulated differently by cell type, light, and metabolites as outlined below.

Molecular and Genetic Studies of Chloroplastic GS2

The in vivo function of chloroplastic GS2 has been elucidated by both molecular studies on the genes and genetic studies of plant GS2 mutants. The GS2 gene is primarily expressed in green tissues in all species examined (20, 30, 72, 104). Indeed, the developmental onset of GS2 gene expression coincides with the maturation of chloroplasts in pea (30, 141) and the development of photosynthetic cotyledons in *Phaseolus* (20). Studies performed in pea (30), maize (104), *Phaseolus* (29), and Arabidopsis (90) demonstrated that GS2 gene expression is tightly regulated by light, and in several cases this has been shown to be mediated at least in part by phytochrome activation (30). GS2 gene expression can also be regulated by metabolic control in response to carbohydrate and amino acid supplementation in tobacco and Arabidopsis (33; I Oliveira & G Coruzzi, unpublished data). In addition, GS2 mRNA accumulation has been reported to increase in leaves of plants cultivated under photorespiratory conditions (20, 30), a finding in line with one of the proposed functions of GS2, the reassimilation of photorespiratory ammonia (82).

Although screens for plant mutants unable to survive in photorespiratory conditions were conducted in Arabidopsis and later in barley, mutants specifically defective in GS2 were identified only in the barley screen (116, 143). The barley GS2 mutants lack the ability to reassimilate ammonia lost during photorespiration. These mutants die not because of a toxic buildup of ammonia

but because of the drain on the organic nitrogen pool (8), as the decrease of photosynthetic rate in GS2 mutants can be rescued by supplementation of alanine, asparagine, and glutamine (67). A dramatic result of this mutant study is the finding that a GS isoenzyme located in the chloroplast is essential for the reassimilation of photorespiratory ammonia released in mitochondria. Because the parameters regulating the intra- and intercellular transport of inorganic and organic nitrogen are presently unknown, this is a dramatic example of how a mutant deficient in a particular subcellular isoenzyme can be used to define the true in vivo role of an isoenzyme.

Paradoxically, the barley mutants deficient in chloroplast GS2 were unable to reassimilate photorespiratory ammonia released in the mitochondria even though they contained normal levels of GS1 in the cytosol (143). This apparent paradox has been resolved by studies on the cell-specific expression patterns of genes for chloroplastic GS2 and cytosolic GS1. Studies of GS-promoter-GUS fusions revealed that chloroplastic GS2 is expressed predominantly in leaf mesophyll cells, where photorespiration occurs, whereas cytosolic GS1 is expressed exclusively in the phloem (31, 34). Although this observation contradicts previous biochemical data that suggested that a large portion of cytosolic GS1 activity was located in mesophyll protoplasts of pea (142), these promoter-GUS fusion results were later confirmed by in situ immunolocalization studies of the native cytosolic GS1 proteins in rice and tobacco (16, 55). This vascular-specific expression pattern may explain why cytosolic GS1 cannot compensate for the loss of chloroplastic GS2 in mesophyll cells of the barley GS2 mutants.

One piece of the GS isoenzyme puzzle that is outstanding is the fact that the screens for photorespiratory mutants in Arabidopsis 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 (4). 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 (16, 55). 3. There is more than one gene for chloroplastic GS2 in Arabidopsis. 4. A mutation in chloroplastic or cytosolic GS is lethal in Arabidopsis and prevents the isolation of mutants.

Molecular Studies of Cytosolic GS1

Because cytosolic GS is an enzyme involved in the assimilation of ammonia fixed by *Rhizobium,* the early studies on genes for cytosolic GS1 were conducted in legumes such as *Phaseolus,* soybean, pea, and alfalfa (126). In each

case, multiple genes for cytosolic GS1 existed, certain members of which were expressed at highest levels in nodules. On the basis of the identification of the "nodule-specific" or "nodule-enhanced" expression of certain GS genes, it was proposed that the multiplicity of genes for cytosolic GS1 has evolved in legumes for this purpose (20, 102, 126, 141). The finding that Arabidopsis also contains at least three genes for cytosolic GS1 (90) suggests that the GS gene family has evolved independently of its role in the nitrogen-fixation process in legumes. Genes for all three cytosolic GS1 isoenzymes of Arabidopsis are expressed at high levels in roots (90). The use of gene-specific probes has revealed that the three genes for cytosolic GS1 of Arabidopsis have subtle differences in their expression patterns. Two genes for cytosolic GS1 are both expressed at high levels in germinated seeds, which suggests that these genes may play a role in the synthesis of glutamine for transport of nitrogen out of cotyledons (90). This is consistent with previous data showing that individual genes for cytosolic GS1 of pea and bean are likely to serve this function in legumes (22, 128). Promoter-GUS fusion studies (31, 34) and subsequent immunolocalization analyses (16, 55) also demonstrated that cytosolic GS1 is expressed in phloem in several species. This further supports the role of GS1 in generating glutamine for intercellular transport.

Expression of genes encoding cytosolic GS1 is under different modes of regulation. For instance, ammonia supplementation has been reported to induce mRNA accumulation of a soybean GS1 gene (49). Treatment with either ammonia or nitrate has also been shown to elicit an increase in the steady state levels of one of the maize GS1 isoforms (GS1-1) in roots and another isoform in shoots (GS1-2). In contrast, another isoform of cytosolic GS in maize (GS1-3) was not affected by the same treatment (121). Studies of transgenic plants transformed with a soybean GS1 promoter-GUS fusion construct revealed that ammonia could induce the accumulation of β-glucuronidase in *Lotus* but not in tobacco plants, although the tissue specificity was conserved in both species (81). In addition, studies of soybean GS1 promoter deletion constructs in transgenic *Lotus* plants demonstrated that the elements responsible for tissue specificity and the induction by ammonia are located in two distinct regions of the GS1 promoter (78, 79). Together, the data accumulated on the expression of the different forms of GS enzymes further illustrate the complexity of the biological function of the GS genes as reflected by the diversity and the differential pattern of expression of each isoenzyme.

GLUTAMATE SYNTHASE

Biochemistry Background of Glutamate Synthase

In higher plants, there are two antigenically distinct forms of glutamate synthase (GOGAT) that use NADH (NADH-GOGAT: E.C.1.4.1.14) or ferredoxin

(Fd-GOGAT: E.C.1.4.7.1) as the electron carrier (70, 110, 119, 122). NADH-GOGAT is located primarily in plastids of nonphotosynthetic tissues such as roots (80, 122). In root nodules of legumes, NADH-GOGAT is involved in the assimilation of nitrogen fixed by *Rhizobium* (2, 17). It has been hypothesized that NADH-GOGAT catalyzes the rate-limiting step of ammonia assimilation in these root nodules (43). In nonlegumes, NADH-GOGAT may function in primary assimilation or reassimilation of ammonia released during amino acid catabolism (84).

In contrast with NADH-GOGAT, Fd-GOGAT is located primarily in the leaf chloroplast where light leads to an increase in Fd-GOGAT protein and activity (70, 110). These findings suggested that the physiological role(s) of Fd-GOGAT is related to light-inducible processes in leaves such as photosynthesis and photorespiration. Fd-GOGAT may also play a smaller role in nonphotosynthetic tissues, because some Fd-GOGAT activity is associated with roots (123). The molecular and genetic studies outlined below have helped to clarify the relative in vivo roles of NADH- vs Fd-GOGAT.

Molecular Studies of NADH–Glutamate Synthase

cDNA clones of NADH-GOGAT were successfully isolated from the legume alfalfa (43) and the nonlegume Arabidopsis (H-M Lam & G Coruzzi, unpublished data). Both the alfalfa and Arabidopsis NADH-GOGAT genes encode putative functional domains within the mature protein that are highly homologous to the large and small subunits of *Escherichia coli* NADPH-GOGAT (43; H-M Lam & G Coruzzi, unpublished data). A putative NADH-binding motif, contained in the small subunit of *E. coli* NADPH-GOGAT, is also found in the corresponding C-terminal domain of both the alfalfa and Arabidopsis NADH-GOGAT enzymes (43; H-M Lam & G Coruzzi, unpublished data).

Measurements of mRNA levels and promoter-GUS fusions of the NADH-GOGAT genes in alfalfa and *Lotus* have shown the tight relationship of the regulated expression of NADH-GOGAT to the nodulation process in legumes (137). It was found that the NADH-GOGAT gene is expressed primarily in cells of effective nodules and is maintained at low or undetectable levels in other tissues. In the nonlegume Arabidopsis, mRNA levels of NADH-GOGAT are enhanced in roots as opposed to leaves (H-M Lam & G Coruzzi, unpublished data). Preliminary studies also show that the expression of the Arabidopsis NADH-GOGAT gene increases during the early stages of seed germination (H-M Lam & G Coruzzi, unpublished data). Because the expression patterns of the genes for cytosolic GS1 and NADH-GOGAT appear coordinated, they may function together in processes such as the primary assimilation of nitrate-derived ammonia in root cells, the reassimilation of ammonia released during catabolic reactions, and/or remobilization of ammonia released

during germination. Because plant mutants in NADH-GOGAT have not been identified, its true in vivo role remains conjectural.

Molecular and Genetic Studies of Ferredoxin–Glutamate Synthase

Fd-GOGAT is uniquely found in photosynthetic organisms. Fd-GOGAT genes have been cloned from six plant species: maize (105), tobacco (149), barley (5), spinach (85), Scots pine (36), and Arabidopsis (K Coschigano & G Coruzzi, unpublished data). A single gene was identified in every species except Arabidopsis, which has been shown to contain two expressed genes (*GLU1* and *GLU2*), each encoding a distinct form of Fd-GOGAT.

Fd-GOGAT mRNA accumulates primarily in leaf tissue in response to light, as has been shown in maize, tobacco, and Arabidopsis (*GLU1*) (105, 149; K Coschigano & G Coruzzi, unpublished data). Involvement of phytochrome in the light induction of Fd-GOGAT has been demonstrated at the mRNA level in tomato (6) and observed at the protein level in mustard cotyledons and Scots pine seedlings (32, 48). In Arabidopsis, Fd-GOGAT mRNA accumulation (*GLU1*) can also be induced in the absence of light by exogenous sucrose applications (K Coschigano & G Coruzzi, unpublished data).

In addition to the highly expressed *GLU1* gene for Fd-GOGAT in Arabidopsis described above, a second expressed gene encoding Fd-GOGAT (*GLU2*) was isolated in Arabidopsis. The discovery of the second gene encoding a distinct form of Fd-GOGAT is consistent with the observance of two antigenically distinct Fd-GOGAT isoforms in rice (123). In contrast with *GLU1* mRNA, accumulation of *GLU2* mRNA is low in leaves but high in roots. *GLU2* mRNA expression does not appear to be significantly influenced by light or sucrose but instead is observed at constitutive, low levels (K Coschigano & G Coruzzi, unpublished data). The expression pattern of the Arabidopsis *GLU2* gene for Fd-GOGAT is very similar to that seen for the gene encoding NADH-GOGAT (see above).

The roles of the various GOGAT isoenzymes are being elucidated through the isolation of plant mutants. Photorespiratory mutants specifically lacking Fd-GOGAT enzyme activity have been isolated from three plant species: Arabidopsis (116), barley (11), and pea (8). In the three Arabidopsis *gluS* mutants initially characterized, leaf Fd-GOGAT activity was reduced to <5% of wild-type levels, whereas NADH-GOGAT (which contributes about 5% of the total GOGAT activities in normal conditions) remained unchanged (roots were not analyzed) (116). In the photorespiratory barley mutants, both leaf and root Fd-GOGAT activity was reduced to <6% of wild type, which suggests that these activities are under the control of the same gene (11). All of the Fd-GOGAT-deficient mutants isolated from the three plant species were chlo-

rotic and eventually died when grown in atmospheric conditions promoting photorespiration (air), which thus established an essential role for Fd-GOGAT in photorespiration. However, because the Fd-GOGAT-deficient mutants recovered and were viable when grown in conditions where photorespiration was suppressed (high CO_2 or low O_2), Fd-GOGAT appeared at first glance to be dispensable for nonphotorespiratory roles, such as in primary nitrogen assimilation. This conclusion was paradoxical because most primary assimilation probably occurs in leaves, where Fd-GOGAT activity predominates (95% of total GOGAT activity) and NADH-GOGAT is a minor component (5% of total GOGAT activity).

The presence of two expressed Fd-GOGAT genes in Arabidopsis is curious because a single gene mutation affecting Fd-GOGAT activity had been isolated in the phenotypic screen for photorespiratory mutants (116). Thus, although there were two genes for Fd-GOGAT, a mutation in one gene produced a photorespiratory-deficient phenotype. However, it appears that a mutation in the highly expressed *GLU1* gene results in a photorespiratory defect as the *GLU1* gene maps to the region of the *gluS* photorespiratory mutation (K Coschigano & G Coruzzi, unpublished data). The *GLU2* gene, which is expressed at constitutively low levels in leaves and at higher levels in roots, maps to a different chromosome and thus may be involved in the primary assimilation process. Interestingly, Fd-GOGAT was also implicated in playing a role in primary assimilation in maize by observance of a rapid, transient, and cycloheximide-independent accumulation of Fd-GOGAT transcripts in maize roots after treatment with nitrate (98). Arabidopsis mutants null for GLU1 activity would be quite valuable to elucidate the role of the *GLU1* gene, and thus a comprehensive analysis of all of the *gluS* alleles is being performed (K Coschigano & G Coruzzi, unpublished data). These *GLU1* mutants could in turn be used to isolate mutations in *GLU2*. The phenotype of a Fd-GOGAT null mutant (*GLU1*, *GLU2* double mutant) could be used to distinguish between Fd-GOGAT roles and NADH-GOGAT roles.

GLUTAMATE DEHYDROGENASE

Biochemistry Background of Glutamate Dehydrogenase

Two major forms of glutamate dehydrogenase (GDH) have been reported: an NADH-dependent form (NADH-GDH: E.C.1.4.1.2) found in the mitochondria (25, 74) and an NADPH-dependent form (NADPH-GDH: E.C.1.4.1.4) localized to the chloroplast (71). The GDH enzyme is abundant in several plant organs (15, 70, 76). Moreover, the GDH isoenzymatic profile can be influenced by dark stress, natural senescence, or fruit ripening (15, 75, 118). These studies suggest that GDH may play a specific or unique role in assimilating ammonia or catabolizing glutamate during these processes.

Although GDH enzyme activity exists in plant tissues at high levels, there is an ongoing debate about its physiological role in higher plants. Originally, GDH was proposed to be the primary route for the assimilation of ammonia in plants. However, this biosynthetic role of GDH has been challenged by the discovery of an alternative pathway for ammonia assimilation via the GS/GOGAT cycle. Moreover, the fact that the GDH enzyme has a high K_m for ammonia argues against a role in primary nitrogen assimilation (119). Studies have shown that GDH enzyme activity can be induced in plants exposed to high levels of ammonia (15), and as such GDH has been proposed to be important specifically for ammonia-detoxification purposes. Mitochondrial GDH has been proposed to be involved in the assimilation of high levels of photorespiratory ammonia released in mitochondria (148). However, the isolation of photorespiratory mutants defective in chloroplastic GS2 (in barley) (143) or Fd-GOGAT (in barley and in Arabidopsis) (8, 59, 116) suggests that GDH is not important in photorespiration (143). Furthermore, treatment of plants with the GS inhibitor MSO prevents the incorporation of ammonia into glutamate and glutamine, even though both GDH activity and ammonia levels remain high (70). Together, these results may be used to argue against a biosynthetic role for GDH. Instead, a catabolic role for GDH has been invoked, which is supported by the fact that GDH activity is induced during germination and senescence, two periods where amino acid catabolism occurs (70, 119).

Molecular and Genetic Studies of Glutamate Dehydrogenase

Studies of plant GDH genes and mutants have begun to shed some light on the role of GDH in plants. In both Arabidopsis and maize there appear to be two genes for GDH based on Southern analysis and mutant analysis. The predicted peptide sequences encoded by cDNAs for maize and Arabidopsis *GDH1* reveal high identity to the GDH enzymes of other organisms (103; R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data). Furthermore, the predicted protein sequences of Arabidopsis and maize GDH suggest that they encode NADH-dependent enzymes that are likely to be associated with the mitochondria (103; R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data).

Studies have also been performed on GDH gene regulation. The transcripts for maize GDH have been shown to be predominant in roots and present in the bundle sheath cells in leaf tissues (103). This evidence agrees with results at the level of NADH-GDH activity in maize. In contrast, the level of *GDH1* mRNA in Arabidopsis, a C3 plant, is higher in leaves than in roots (R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data). *GDH1* mRNA also accumulates to high levels in dark-adapted plants, and this accumulation is repressed by light or sucrose (R Melo-Oliveira, I Oliveira & G Coruzzi,

unpublished data). This observation is consistent with previous biochemical data that showed that GDH activity increased in response to carbon limitation in maize (87). It appears that the *GDH1* and *GLN2* (GS2) genes of Arabidopsis are reciprocally regulated by light and sucrose (R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data) as shown previously in lupine at the level of enzyme activity (97). These gene expression data suggest that GDH1 and GS2 play nonoverlapping roles in Arabidopsis nitrogen metabolism.

An Arabidopsis mutant deficient in GDH was identified in the M2 generation of EMS-mutagenized Arabidopsis using a GDH activity stain on crude leaf protein extracts following electrophoresis on native gels (62, 145; R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data). The GDH enzymes of Arabidopsis can be resolved into seven isoenzymes in this manner (14, 62, 107). These seven GDH activity bands are the result of the random association of two types of subunits into a hexameric complex (15). It has been proposed that two nonallelic genes are responsible for the synthesis of the GDH1 and GDH2 subunits (14, 15). A single Arabidopsis GDH mutant, *gdh1-1*, has been identified that has an altered pattern of GDH activity: It possesses a single GDH2 holoenzyme and is missing the GDH1 holoenzyme as well as the heterohexamers (R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data). The Arabidopsis *gdh1-1* mutant displays an impaired growth phenotype compared with wild type specifically when plants are grown in media containing exogenous inorganic nitrogen. This conditional phenotype suggests a nonredundant role for GDH in the assimilation of ammonia under conditions of inorganic nitrogen excess. A similar GDH-deficient mutant has been previously described in *Zea mays*, a C4 plant, which also appears to be affected in the *GDH1* gene product (92, 93). Preliminary studies showed that the maize GDH mutant displays a growth phenotype only under low night temperatures (94). Moreover, it has been reported that the maize *GDH1* mutant shows a 10- to 15-fold lower total GDH activity when compared with wild-type maize (77). Because the photorespiratory rate is very low or nonexistent in a C4 plant, the maize *GDH1* mutant cannot be used to assess the role of GDH in photorespiration. Therefore, the Arabidopsis *GDH1* mutant will be valuable to assess the function of this enzyme in photorespiration in a C3 plant. It should be noted that neither the maize nor the Arabidopsis *GDH1* mutants are null for GDH, because they each possess a second *GDH2* gene. Isolation of *GDH2* mutants and creation of *GDH1/GDH2* double mutants will be needed to define the role of GDH unequivocally.

DOWNSTREAM METABOLISM OF GLUTAMINE AND GLUTAMATE

Following the assimilation of ammonia into glutamine and glutamate, these two amino acids act as important nitrogen donors in many cellular reactions,

including the biosynthesis of aspartate and asparagine (40, 66). Aspartate contributes an integral part of the malate-aspartate shuttle that allows the transfer of reducing equivalents from mitochondria and chloroplast into the cytoplasm (52). In C4 plants, aspartate shuttles carbon between mesophyll cells and bundle sheath cells (47). Asparagine is thought to be an important compound for transport and storage of nitrogen resources because of its relative stability and high nitrogen to carbon ratio. Asparagine is a major nitrogen-transport compound in both legumes and nonleguminous plants. In seeds of *Lupinus albus*, 86.5% of the nitrogen from protein is remobilized into asparagine (69). Radioactive nitrogen feeding experiments in peanut indicate that up to 80% of the label of ^{15}N-[N_2] was recovered as asparagine in the sap of nodules (89). Asparagine also acts as the major constituent of nitrogen transported out of nodules in leguminous plants (69, 109). In nonleguminous plants such as Arabidopsis, asparagine is also a major transported amino acid detected in the phloem exudates (62, 107). In the following sections, we discuss AspAT and AS, which are the two major enzymes involved in the downstream metabolism of assimilated nitrogen into aspartate and asparagine.

ASPARTATE AMINOTRANSFERASE

Biochemistry Background of Aspartate Aminotransferase

Biochemical studies show that aspartate aminotransferase (AspAT: E.C.2.6.1.1) can exist as distinct isoenzymes (144). The activities of various AspAT isoenzymes have been found in different tissues and different subcellular locations such as the cytosol, mitochondria, chloroplasts, glyoxysomes, or peroxisomes (for examples, see 108, 125, 140, 144). The subcellular compartmentation of AspAT isoenzymes suggests that the different forms of AspAT might serve distinct roles in plant metabolism. It is also important to note that individual AspAT isoenzymes respond differently to environmental conditions and metabolic status such as light treatment or nitrogen starvation, which suggests that they serve distinct roles (101, 125).

Molecular and Genetic Studies of Aspartate Aminotransferase

Molecular and genetic analyses of AspAT genes have begun to elucidate the in vivo function of each AspAT isoenzyme. cDNA clones encoding AspAT have been isolated in both legumes and nonlegumes such as alfalfa, Arabidopsis, *Panicum*, and soybean (108, 125, 132, 140, 147). The regulation of AspAT in legumes is tightly coupled with the symbiotic process. In alfalfa, the levels of AspAT mRNA are induced during effective nodule development (35, 132).

In the C3 plant Arabidopsis, the entire gene family of AspAT isoenzymes has recently been characterized (108, 147). Five different AspAT cDNA clones

[*ASP1–4* and *ASP5* (formally *AAT1*)] were obtained, including those encoding the mitochrondrial, plastidic, peroxisomal, and cytosolic forms of AspAT. Although two of the five *ASP* genes encode cytosolic forms of AspAT (*ASP2* and *ASP4*), only *ASP2* is expressed at high levels, especially in roots (108). The *ASP1* and *ASP3* genes, which encode a mitochondrial and a peroxisomal form of AspAT respectively, are each expressed at relatively high levels in all organs examined (108). In the C4 plant *Panicum miliaceum*, AspAT genes encoding the cytosolic, mitochrondrial, and plastidic AspAT isoenzymes are all expressed at higher levels in green leaves than in mesocotyls and root tissue (125). It has also been reported that nitrogen availability shows a positive effect on the levels of mRNAs for cytosolic AspAT and mitochondrial AspAT genes but not for plastidic AspAT genes in *Panicum miliaceum* (125).

To help determine the in vivo function(s) of each AspAT isoenzyme, a screen for mutants defective in the predominant forms of AspAT in leaf extracts was performed in Arabidopsis. Crude leaf extracts contain two major AspAT isoenzymes, AAT2 (cytosolic) and AAT3 (chloroplastic), as detected by activity staining of native gels (62, 107). M2 seedlings were screened for alterations in AAT profiles using the native gel screen on crude leaf extracts (107). Four classes of AspAT mutants were obtained from a screen of 8000 EMS-mutagenized Arabidopsis seeds: (*a*) loss of cytosolic AAT2 activity, (*b*) loss of chloroplastic AAT3 activity, (*c*) alteration of cytosolic AAT2 gel mobility, and (*d*) alteration of chloroplastic AAT3 gel mobility (107). By analyzing the effects of these mutations on the growth phenotypes and the balance of free amino acid pools in different classes of mutant plants, the in vivo importance of each isoenzyme can be determined. Preliminary analyses of these AAT mutants show that a mutation in the cytosolic *ASP2* gene results in a retarded growth phenotype and a decrease in the pools of free aspartate (C Schultz & G Coruzzi, unpublished data). Thus, despite the presence of two genes for cytosolic AspAT (*ASP2* and *ASP4*), a mutation in the highly expressed *ASP2* gene causes a growth defect and aspartate-deficient phenotype.

ASPARAGINE SYNTHETASE

Biochemistry Background of Asparagine Synthetase

Asparagine was the first amino acid discovered and was isolated in asparagus 190 years ago (138). Despite this historical placement, the mechanism of asparagine biosynthesis in plants has been elucidated only recently. The glutamine-dependent asparagine synthetase enzyme (AS: E.C.6.3.5.4) is now generally accepted as the major route for asparagine biosynthesis in plants (70, 100). However, ammonia is also a possible AS substrate, particularly in the case of maize roots (86). In some cases, asparagine is believed to act as an

ammonia detoxification product produced when plants encounter high concentrations of ammonia (39, 113).

The hypothesis that asparagine serves to transport nitrogen in plants is supported by high levels of AS activity detected in nitrogen-fixing root nodules (10, 51, 109) and in cotyledons of germinating seedlings (28, 60, 68). Biochemical studies on partially purified plant AS enzymes have been seriously hampered by the copurification of a heat-stable, dialyzable inhibitor (54, 60), the instability of AS enzyme in vitro (113), and the presence of contaminating asparaginase activity in plant extracts (51). These problems in detection of AS activities have made it difficult to monitor low-level AS activities in certain organs or slight but important changes of AS activity levels resulting from changes in growth conditions.

Molecular Studies of Asparagine Synthetase

The first two cDNA clones encoding plant AS (*AS1* and *AS2*) were obtained from a pea library using a human AS cDNA clone as a heterologous probe (130, 131). Both the pea *AS1* and *AS2* genes are expressed in leaves as well as in roots. Subsequently, studies of AS cDNA clones isolated from Arabidopsis and asparagus have shown that AS genes in these plants are expressed primarily in the leaves or the harvested spears, respectively (24, 63). The AS polypeptides encoded by these cDNA clones each contain a PurF-type glutamine-binding domain (100). This supports the notion that glutamine is the preferred substrate of plant AS. Moreover, studies of these AS cDNA clones, together with the previous biochemical data, have suggested that asparagine metabolism is regulated by the carbon/nitrogen status of a plant (63). The levels of asparagine and AS activities are also controlled by environmental and metabolic signals. Both the asparagine content in phloem exudates and AS activities are induced when light-grown plants are dark adapted (133, 134). Conversely, light and/or sucrose have been shown to result in a decrease in AS activity, as observed in sycamore cell cultures (38) and root tips of corn (12, 120).

The first striking observation of AS gene expression in pea and Arabidopsis was the high level of AS mRNA in dark-grown or dark-adapted plants (63, 130, 131). The light repression of gene expression of *AS1* in pea and *ASN1* in Arabidopsis is at least in part mediated through the action of phytochrome (63, 130). In addition to the direct phytochrome-mediated effects, light appears to exert indirect effects on AS gene expression via associated changes in carbon metabolites. In asparagus spears, it was shown that AS mRNA levels increase in harvested spears, in parallel with the decline of cellular sugar content and independent of light (24). In Arabidopsis, *ASN1* mRNA is high in dark-adapted plants, and treatment with exogenous sucrose represses the steady state level

of *ASN1* transcripts (63). These molecular data are consistent with the biochemical data discussed above. Further information concerning the metabolic control of AS gene expression was obtained by demonstrating that the addition of exogenous amino acids (glutamate, glutamine, asparagine) to the growth medium was able to partially relieve the sucrose repression of the *ASN1* gene of Arabidopsis (63). This finding suggests that the ratio of organic nitrogen to carbon in a plant may be the ultimate factor controlling *ASN1* gene expression. Under conditions where levels of carbon skeletons are low relative to organic nitrogen, asparagine synthesis stores the excess nitrogen as an inert nitrogen reserve. Interestingly, in high-protein maize lines and high-protein rye ecotypes, there seems to be a shift in the composition of transported nitrogen from metabolically active glutamine to inertly stored asparagine (27).

Two new cDNA clones for Arabidopsis AS (*ASN2*, *ASN3*) were recently obtained by functional complementation of a yeast mutant lacking all AS activity (H-M Lam & G Coruzzi, unpublished data). The expression of the *ASN2* and *ASN3* genes seems to be at relatively lower levels compared with the Arabidopsis *ASN1* gene (H-M Lam & G Coruzzi, unpublished data). The mRNA levels of *ASN2* gene are regulated in an opposite manner than the *ASN1* gene. The AS enzymes encoded by these new AS genes may function to provide the required asparagine for other physiological processes such as photorespiration (124).

LIGHT AND METABOLIC CONTROL OF NITROGEN ASSIMILATION

Evidence shows that the process of nitrogen assimilation into amino acids is subject to light and metabolic control at the molecular level. Light exerts a positive effect on the expression of genes involved in ammonia assimilation into glutamine/glutamate such as on GS2 and Fd-GOGAT (30, 90, 105, 127, 149; K Coschigano & G Coruzzi, unpublished data). Conversely, light has been shown to repress genes encoding AS and GDH (42, 63, 131; R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data). The involvement of phytochrome in these light effects has been reported in some experiments (30, 63, 127, 130, 131). Further genetic experiments using the available phytochrome-deficient mutants available in Arabidopsis (99, 146) should provide more clues about which phytochrome regulates nitrogen assimilation. Although phytochrome is known to be the primary light receptor (96), the downstream signal transduction cascade is not understood. Thus, a direct linkage between the expression of genes involved in nitrogen assimilation and the light signal pathway is still lacking. The identification of light-responsive elements in plant promoters of genes encoding enzymes such as GS2 and AS in pea

(129; N Ngai & G Coruzzi, unpublished data) may be important in finding the missing link for light regulation of these nitrogen assimilatory genes.

In Arabidopsis, the reciprocal control of *GLN2* vs *ASN1* by light at the mRNA level has been shown to reflect similar light-induced changes in the levels of glutamine and asparagine. Glutamine levels are higher in light-grown plants, whereas asparagine levels are highest in dark-adapted plants (62, 107). This was also found previously in pea (133, 134). Under light conditions, nitrogen is assimilated into metabolically active glutamine and glutamate and transported as such for use in anabolic reactions in plants. Under dark-growth conditions (low carbon concentration relative to organic nitrogen), the plants direct the assimilated nitrogen into inert asparagine for long-distance transport or long-term storage.

Recently, there has been some discussion about the possible cross-talk between light control of gene expression and metabolic regulation by sugars (53). It is interesting to note that sucrose can mimic the effects of light on the expression of genes related to nitrogen metabolism such as nitrate reductase, nitrite reductase, GS2, Fd-GOGAT, GDH, and AS (18, 63; R Melo-Oliveira, I Oliveira & G Coruzzi, unpublished data; K Coschigano & G Coruzzi, unpublished data). Regulation of nitrogen assimilatory genes by the cellular carbon status reflects the interrelationship between carbon and nitrogen metabolism in plants.

Several lines of studies have focused on the metabolic control by sugars on genes related to photosynthesis and carbon metabolism (53, 111, 112). Hexose kinase is proposed to be the switching enzyme that can sense carbon availability inside the cell (53). On the basis of studies in microorganisms, a plant homologue of the yeast catabolic repression *trans*-acting factor SNF1 has been identified in rye (1). Subsequently, SNF1-related genes were isolated from Arabidopsis and barley (46, 71a). In barley, two SNF1-related protein kinases show differential expression patterns in different tissues (46). It will be important to see whether a SNF1 mutant might alter the balance of carbon and nitrogen metabolism.

In addition to the control by carbon status in the cell, it has been proposed that the relative abundance of nitrogen pools also plays a significant role in regulating nitrogen assimilation. In fact, some reports claim that the ratio of cellular carbon to nitrogen is a major player in the metabolic control of nitrogen assimilation. A homologue of a yeast general nitrogen regulatory protein NIT2 was obtained in tobacco (23). Cross-talk between the regulation of two amino acid pathways has also been reported in plants in which a blockage of histidine biosynthesis leads to a decrease in the mRNA levels of most amino acid biosynthetic enzymes, which suggests that general control of amino acid biosynthesis occurs in plants (44).

CONCLUSION

Molecular and genetic analyses have provided important tools to extend our knowledge of nitrogen assimilation based on biochemical studies. The mechanisms by which light and/or metabolic status regulate nitrogen assimilation are beginning to be dissected using cloned genes. For example, some potential regulatory genes have already been identified. In addition, specific screens for mutants in this process can be conducted in a genetically tractable system such as Arabidopsis. A combined molecular and genetic study on the regulatory network by which a gene responds to the metabolic status will lead to a better understanding of the interaction of genes controlling different carbon and nitrogen metabolic pathways. Basic research studies in these areas of nitrogen metabolism may also make significant contributions to the improvement of nitrogen usage efficiency and crop yield.

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