

Glutamine Synthetase-Glutamate Synthase Pathway and Glutamate Dehydrogenase Play Distinct Roles in the Sink-Source Nitrogen Cycle in Tobacco¹

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Glutamate (Glu) metabolism and amino acid translocation were investigated in the young and old leaves of tobacco (*Nicotiana tabacum* L. cv Xanthi) using [¹⁵N]ammonium and [2-¹⁵N]Glu tracers. Regardless of leaf age, [¹⁵N]ammonium assimilation occurred via glutamine synthetase (GS; EC 6.1.1.3) and Glu synthase (ferredoxin [Fd]-GOGAT; EC 1.4.7.1; NADH-GOGAT; EC 1.4.1.14), both in the light and darkness, and it did not depend on Glu dehydrogenase (GDH; EC 1.4.1.2). The [¹⁵N]ammonium and ammonium accumulation patterns support the role of GDH in the deamination of [2-¹⁵N]Glu to provide 2-oxoglutarate and [¹⁵N]ammonium. In the dark, excess [¹⁵N]ammonium was incorporated into asparagine that served as an additional detoxification molecule. The constant Glu levels in the phloem sap suggested that Glu was continuously synthesized and supplied into the phloem regardless of leaf age. Further study using transgenic tobacco lines, harboring the promoter of the *GLU1* gene (encoding Arabidopsis [*Arabidopsis thaliana*] Fd-GOGAT) fused to a *GUS* reporter gene, revealed that the expression of Fd-GOGAT remained higher in young leaves compared to old leaves, and higher in the veins compared to the mesophyll. Confocal laser-scanning microscopy localized the Fd-GOGAT protein to the phloem companion cells-sieve element complex in the leaf veins. The results are consistent with a role of Fd-GOGAT in supplying Glu for the synthesis and transport of amino acids. Taken together, the data provide evidence that the GS-GOGAT pathway and GDH play distinct roles in the source-sink nitrogen cycle of tobacco leaves.

Plants utilize nitrate, ammonium, and dinitrogen (N₂) molecules as external nitrogen sources. Ammonium is the final form of inorganic nitrogen prior to the synthesis of organic nitrogen compounds. Ammonium is also produced via internal metabolic reactions, including photorespiration, hydrolysis of nitrogen carrying and storage molecules, and amino acid conversion (Ireland and Lea, 1999). In nonleguminous C3 plants, such as tobacco (*Nicotiana tabacum*), the photorespiratory ammonium production by the oxidative decarboxylation of Gly exceeds by about 10-fold the primary nitrate reduction in the vegetative leaves. In the senescing leaves, a large amount of ammonium is produced as a result of protein hydrolysis (Hörteinstainer and Feller, 2002). Therefore, it is essential that toxic ammonium be immediately reassimilated into organic molecules for nitrogen cycling. Ammonium is assimilated into the Gln amide group,

which is then transferred to the position of 2-oxoglutarate, yielding two molecules of Glu by the concerted reaction of Gln synthetase (GS; EC 6.1.1.3) and Glu synthase (ferredoxin [Fd]-GOGAT; EC 1.4.7.1; NADH-GOGAT; EC 1.4.1.14). Nitrogen is then incorporated into Asp, Ala, Asn, and other amides and amino acids. Gln-dependent Asn synthetase (AS; EC 6.3.5.4) provides Asn, which serves as a nitrogen carrier together with Gln and Glu.

Numerous studies have been carried out to define the roles of enzymes in nitrogen assimilation and remobilization, tightly interrelated processes during plant growth and development (Mifflin and Habash, 2002). It was proposed that ammonium might be directly incorporated into Glu by amination of 2-oxoglutarate via mitochondrial Glu dehydrogenase (NADH-GDH; EC 1.4.1.2) and subsequently into Gln by cytosolic GS1 under particular physiological conditions. Studies on source-sink relations have shown that GDH is induced in old leaves when nitrogen remobilization is maximal (Srivastava and Singh, 1987; Masclaux et al., 2000). This led to the proposal that the physiological role of GDH is to synthesize Glu for translocation in senescing leaves (for review, see Mifflin and Habash, 2002). However, there is no evidence to discern a redundant or indispensable role of GDH and GOGAT for Glu synthesis and nitrogen remobilization. In addition, GDH catalyzes the reversible oxidative deamination of Glu to supply 2-oxoglutarate and ammonium (Aubert et al., 2001).

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To better understand the role of GDH and GOGAT in Glu metabolism in the coordinated reaction with GS, we studied the kinetics of *in vivo* turnover of [¹⁵N]Glu fed to leaf discs during aging of tobacco plants. The time course of [¹⁵N]ammonium assimilation into the amino acids was then determined *in vivo* in young and old leaves. To understand the cellular compartmentation of Glu synthesis and amino acid translocation, we investigated the tissue-specific expression and the cellular localization of Fd-GOGAT in tobacco plants transformed by a fusion between the promoter of the Arabidopsis (*Arabidopsis thaliana*) Fd-GOGAT gene (*GLU1*) and a reporter gene.

RESULTS

Respiratory Ammonium Release from Glu

In this experiment, we hypothesized that Glu is deaminated in both young and old leaves. (1) Glu is involved in the photorespiratory nitrogen cycle during the day and provides Gly and 2-oxoglutarate in the peroxisome through the reaction of Glu:glyoxylate

aminotransferase (Fig. 1). Two molecules of Gly are in turn converted to CO₂, ammonia, and Ser via the Gly decarboxylase multienzyme complex and Ser hydroxymethyltransferase. (2) Glu deamination through the anaplerotic pathway, involving GDH, could participate in mitochondrial respiration during the day/night cycle (Masclaux-Daubresse et al., 2002). (3) Ammonium released in mitochondria by photorespiration and oxidative respiration could then be reassimilated by GS (Fig. 1).

To investigate Glu behavior in young and old leaves, leaf discs were incubated with [2-¹⁵N]Glu either in the light or in the dark. Total ammonium levels were higher in young than in old leaves (Fig. 2A). No remarkable difference was detected in ammonium levels between the light and dark conditions. The addition of Met sulfoximine (MSO) dramatically inhibited GS activity, whereas it did not affect the aminating and deaminating activities of GDH (data not shown). Following MSO treatment, ammonium reassimilation through GS was inhibited and the increase in total ammonium content via photorespiration became apparent as the difference between the light and dark treatments both

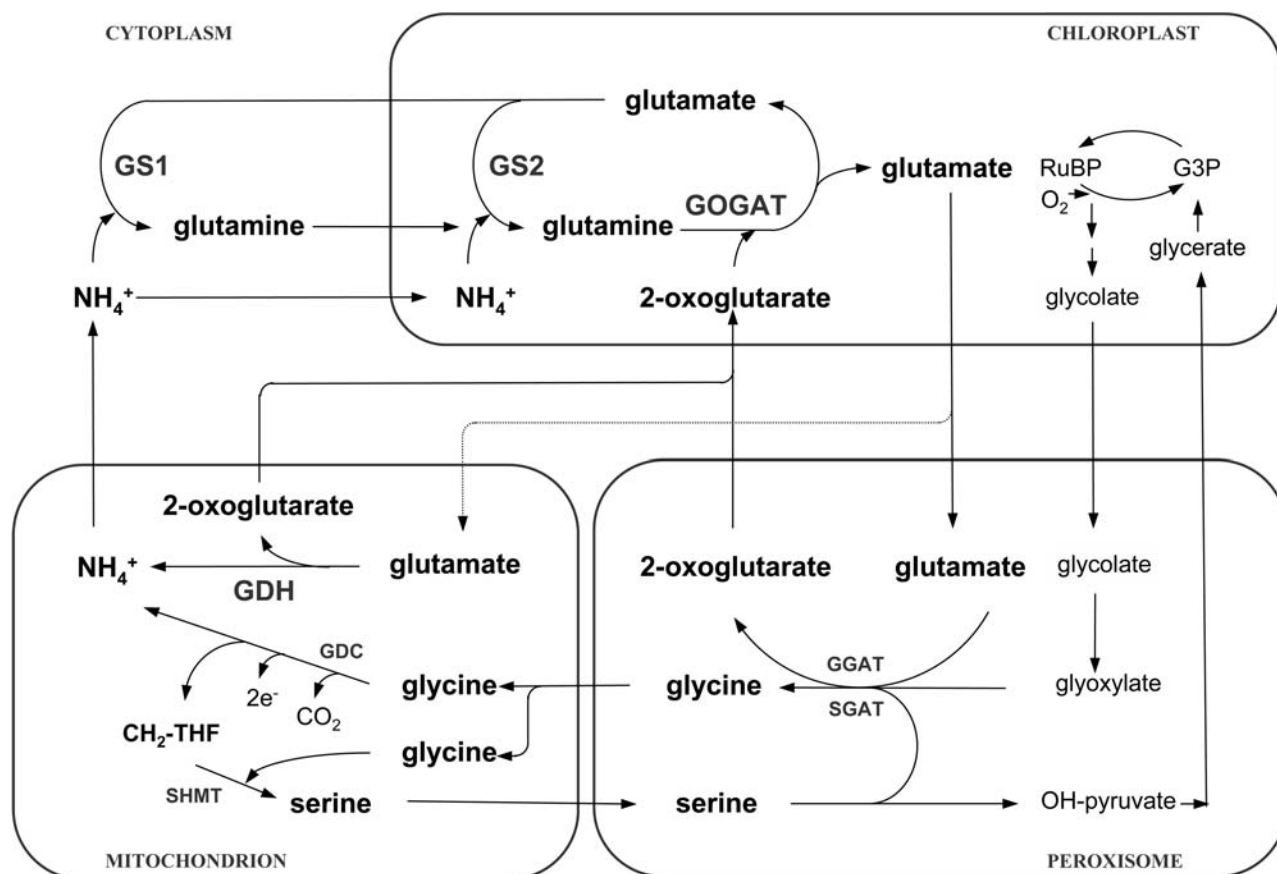


Figure 1. Proposed diagram of the photorespiratory nitrogen cycle involving respiratory Glu metabolism with release of ammonium and 2-oxoglutarate between three subcellular compartments. Ammonium can be produced during photorespiration and oxidative deamination of Glu by GDH and assimilated by the action of the GS-COGAT pathway. The stoichiometry of the cycle is not included. GDC, Gly decarboxylase multienzyme complex (EC 1.4.4.2/2.1.2.10); GGAT, Glu:glyoxylate aminotransferase (EC 2.6.1.4); SHMT, Ser hydroxymethyltransferase (EC 2.1.2.1); SGAT, Ser:glyoxylate aminotransferase (EC 2.6.1.45); CH₂-THF, N⁵,N¹⁰-methylene tetrahydrofolate; G3P, glyceralate-3-P; OH-pyruvate, hydroxypyruvate; RuBP, ribulose 1,5-bisphosphate.

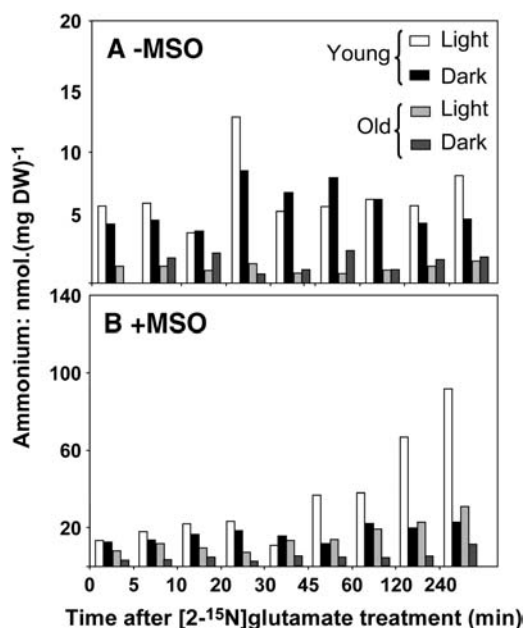


Figure 2. Changes in the levels of total ammonium in young and old leaves either in the light or in the dark. Young and old leaf discs from 10-week-old tobacco plants were floated on incubation buffer for 1 h in the dark. Leaf discs were dipped into the solutions without (A) or with (B) 1 mM MSO, quickly removed, and washed with water (time 0). Leaf discs were further incubated in the light or in the dark and harvested at 5, 10, 20, 30, 45, 60, 120, and 240 min. Ammonium content was expressed as nmol (mg DW)^{-1} . Values represent the means of analysis on leaf discs from five independent plants. DW, Dry weight.

in old and young leaves, except at 30 min in young leaves (Fig. 2B).

Release of ^{15}N ammonium from $[2\text{-}^{15}\text{N}]\text{Glu}$ was higher in the light compared to the dark both in young and old leaves (Fig. 3, A and B). In the light, the addition of MSO led to similar rates of ^{15}N ammonium release from $[2\text{-}^{15}\text{N}]\text{Glu}$ in old and young leaves (Fig. 3A). However, the young leaves accumulated higher amounts of total ammonium than the old leaves in the light (Fig. 2, A and B). In the dark, the inhibition of ammonium assimilation by MSO resulted in higher ^{15}N ammonium accumulation in old than in young leaves after 120 min (Fig. 3B). The results suggest that an extra ^{15}N ammonium was released from $[2\text{-}^{15}\text{N}]\text{Glu}$ deamination in the dark by the reaction independent per se of photorespiration in the old leaves.

Kinetics of Ammonium Assimilation into Amide and Amino Nitrogen

The kinetics of ^{15}N ammonium assimilation were determined in young and old leaves in the light (Fig. 4) and in the dark (Fig. 5). In the light, the high ^{15}N labeling was first detected as $[5\text{-}^{15}\text{N}]\text{Gln}$ as early as 5 min (Fig. 4, A and B), then as $[2\text{-}^{15}\text{N}]\text{Glu}$, $[2\text{-}^{15}\text{N}]\text{Gln}$, and other amino acids, such as Ala, Gly, and Ser, after 10 min (Fig. 4, A–D). These labeling kinetics correlate with a high efficiency of the GS activity that can use Glu as soon as it is synthesized by GOGAT (Fig. 6). The

delay of $[2,5\text{-}^{15}\text{N}]\text{Gln}$ labeling relative to $[2\text{-}^{15}\text{N}]\text{Gln}$ can be explained by (1) a differential availability of chloroplastic $[2\text{-}^{15}\text{N}]\text{Glu}$ pool as a substrate for the cytosolic GS1 and chloroplastic GS2; (2) a higher availability of NH_4^+ than $^{15}\text{NH}_4^+$; or (3) a preference of GS toward nonisotopic ammonium as a substrate. The ^{15}N enrichment patterns of these amino acids were similar between the young and old leaves, despite a slightly lower enrichment in the young leaves (Fig. 4, A–D). The addition of MSO resulted in the complete inhibition of the labeling of $[5\text{-}^{15}\text{N}]\text{Gln}$, $[2\text{-}^{15}\text{N}]\text{Glu}$, $[2\text{-}^{15}\text{N}]\text{Gln}$, and $[2,5\text{-}^{15}\text{N}]\text{Gln}$ (data not shown), suggesting that GS catalyzes the sole efficient entry of ammonium in our conditions.

When young and old leaf discs were treated with azaserine (AZA), the $[5\text{-}^{15}\text{N}]\text{Gln}$ labeling was unchanged (Fig. 4, E and F). In contrast, ^{15}N enrichment in $[2\text{-}^{15}\text{N}]\text{Glu}$, $[2,5\text{-}^{15}\text{N}]\text{Gln}$, and $[2\text{-}^{15}\text{N}]\text{Gln}$ were substantially decreased, while the remaining label of these amino acids was slightly higher in young than in old leaves (Fig. 4, E and F). These results suggest that AZA did not completely inhibit the total GOGAT activity and that the difference was due to the higher GOGAT activity in young than in old leaves. Both MSO and AZA did not inhibit the GDH aminating and deaminating activities (data not shown). As the GDH aminating activity was induced in old leaves (Masclaux et al., 2000), it was expected that GDH assimilates ammonium in the old leaves. However, the ^{15}N -labeling patterns in the old leaves did not show significant differences from those in the young leaves (compare Fig. 4, A and B). Moreover,

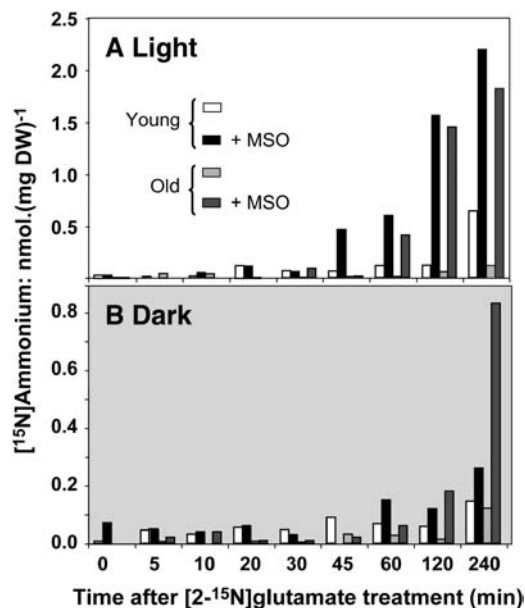


Figure 3. Changes in the levels of ^{15}N ammonium in young and old leaves either in the light (A) or in the dark (B). Experiments were carried out under identical conditions to those described in the legend of Figure 2. Leaf discs were harvested at 5, 10, 20, 30, 45, 60, 120, and 240 min. ^{15}N Ammonium levels were expressed as nmol (mg DW)^{-1} . Values represent means of analysis on leaf discs from five independent plants. DW, Dry weight.

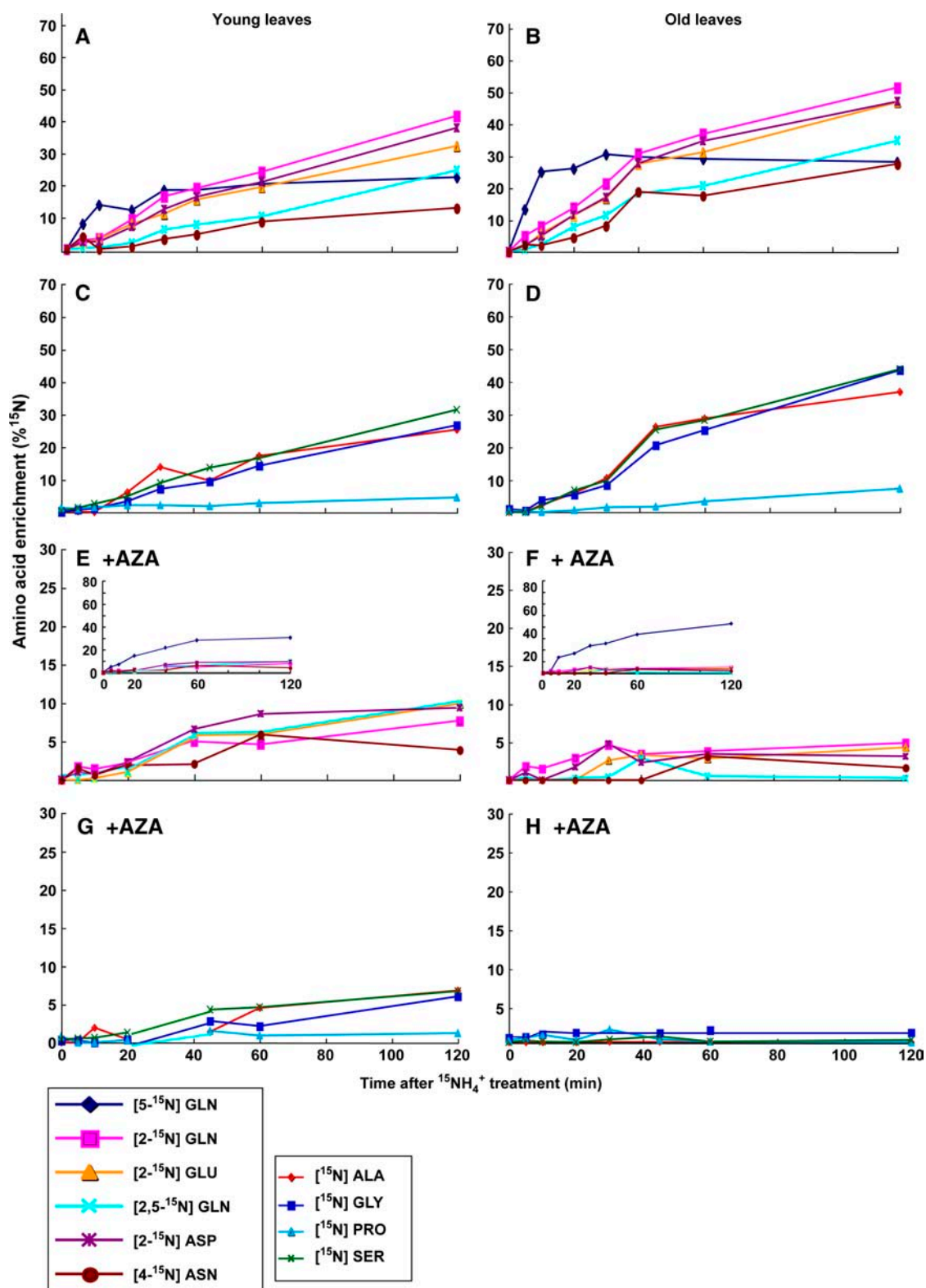


Figure 4. Kinetic analysis of [¹⁵N]ammonium incorporation into amides and amino acids in the light. Young and old leaf discs from 10-week-old tobacco plants were floated on incubation buffer for 1 h in the dark. After addition of [¹⁵N]ammonium (time 0), samples were incubated in the light and harvested at 5, 10, 20, 30, 45, 60, and 120 min from the solution without (A–D) or with (E–H) AZA (GOGAT inhibitor). ¹⁵N labeling in amide and amino nitrogen was determined by a GC-MS analyzer. Values represent the means of analysis on leaf discs from five independent plants.

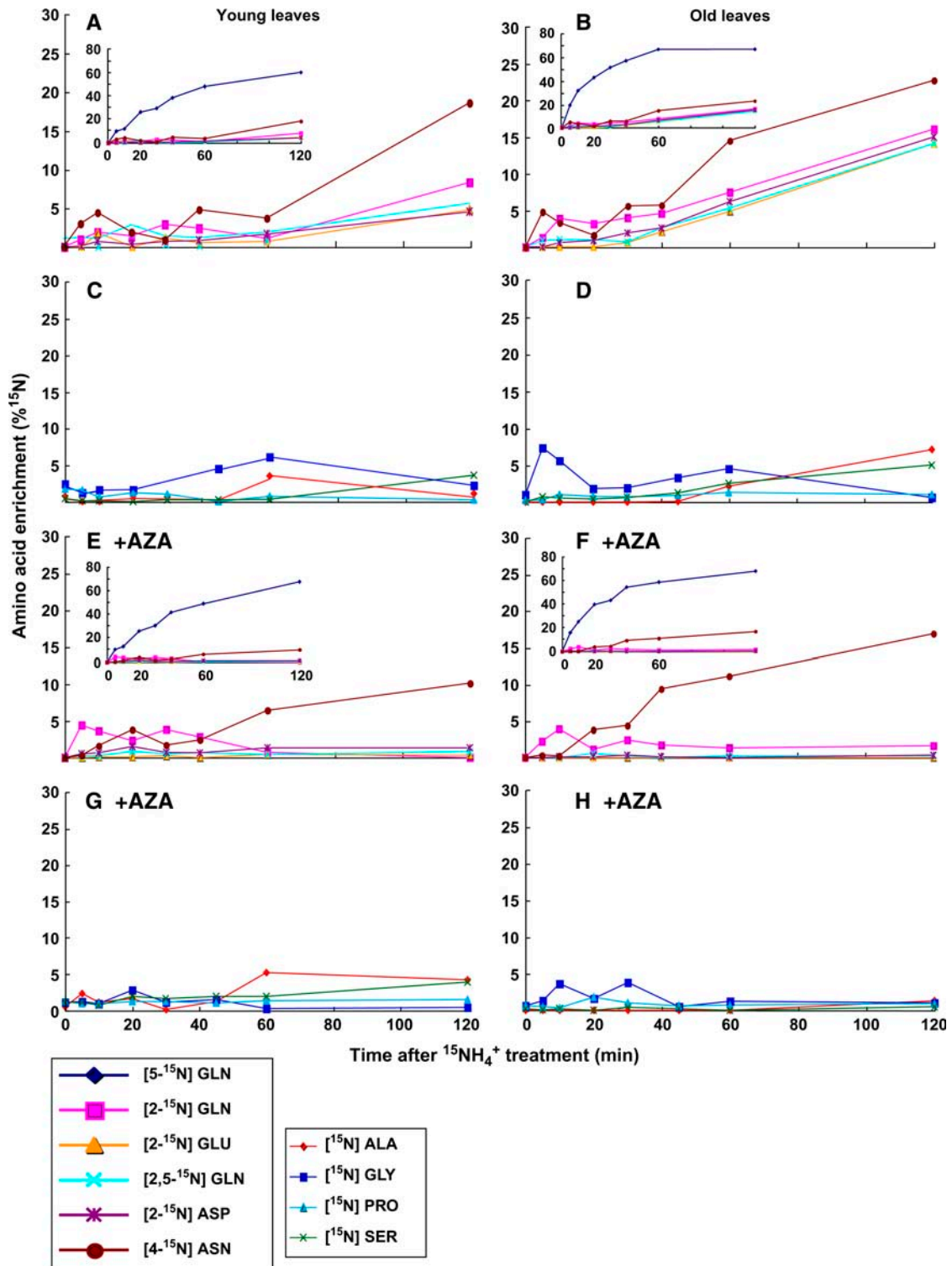


Figure 5. Kinetic analysis of [¹⁵N]ammonium incorporation into amides and amino acids in the dark. After addition of [¹⁵N]ammonium (time 0), samples were incubated in the dark and harvested at 5, 10, 20, 30, 45, 60, and 120 min from the solution without (A–D) or with (E–H) AZA. Values represent the means of analysis on leaf discs from five independent plants from discs.

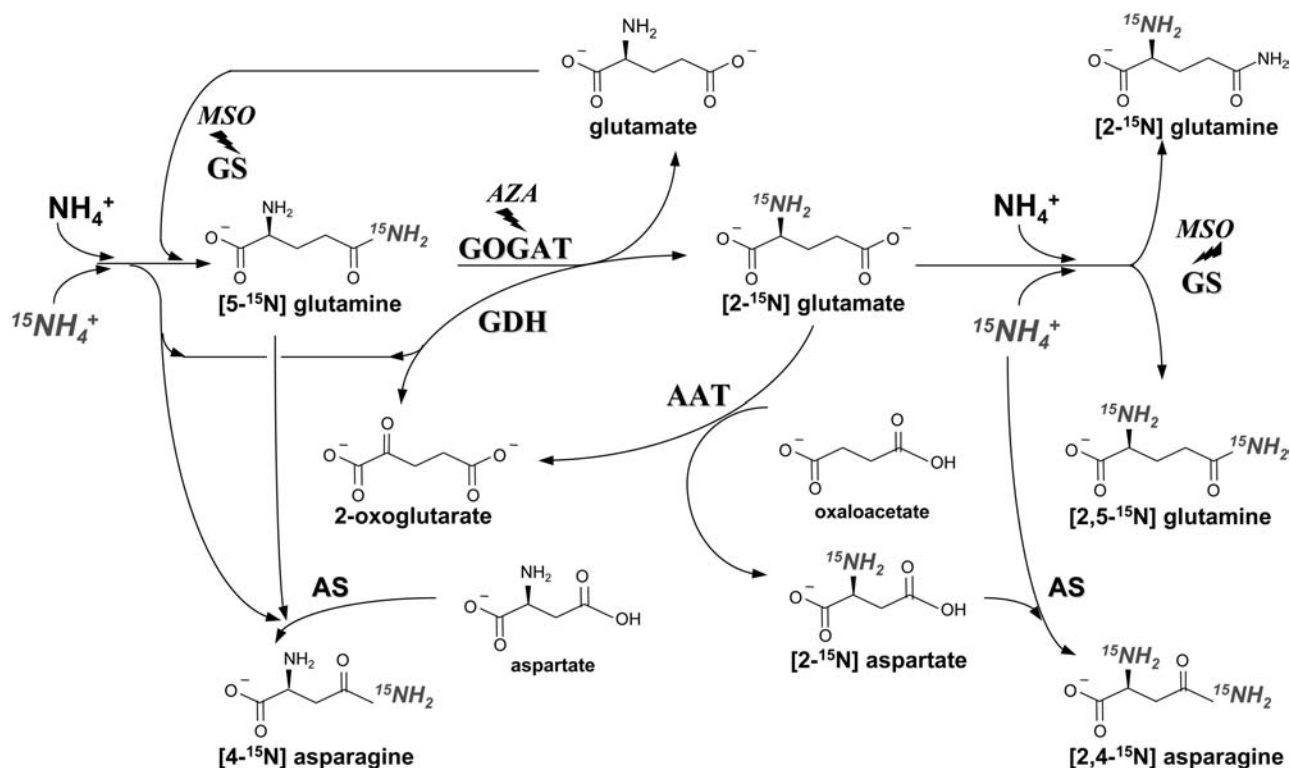


Figure 6. Scheme of ammonium assimilation into amides and amino acids. The first step of [^{15}N]ammonium entry is into the Gln-amide group by GS. Then GOGAT transfers the ^{15}N amide group of Gln to the 2-oxoglutarate position yielding one [$2\text{-}^{15}\text{N}$]Glu and one Glu. AS catalyzes Asn formation from Asp using either the Gln-amide group or ammonium. AAT, Asp aminotransferase (EC 2.6.1.1); GOGAT, Glu:gluoxylate aminotransferase; AZA, GOGAT inhibitor; MSO, GS inhibitor.

AZA inhibited the labeling of [$2\text{-}^{15}\text{N}$]Glu, [$2,5\text{-}^{15}\text{N}$]Gln, [^{15}N]Ser, [^{15}N]Ala, and [^{15}N]Gly in the young leaves and to a higher extent in the old leaves (Fig. 4, E–H). The labeling patterns correlate with the operation of the GS-GOGAT cycle.

The feeding of [^{15}N]ammonium resulted in the recovery of label in Gly, Ser, and Ala in young and old leaves with a constant increase during 120 min (Fig. 4, C and D). The labeling rates of these amino acids were almost as high as those of Glu and Asp (Fig. 4, A–D). In contrast, the labeling of Pro was very low. The AZA treatment substantially inhibited the transfer of label to these amino acids (Fig. 4, G and H).

In the dark, [^{15}N]ammonium was assimilated into [$5\text{-}^{15}\text{N}$]Gln by GS at 5 min after the [^{15}N]ammonium addition in both young and old leaves (Fig. 5, A and B). Compared to the light conditions, [$5\text{-}^{15}\text{N}$]Gln enrichment was higher in the dark (60%–70% versus 30%–40%). In contrast, the labeling of [$2\text{-}^{15}\text{N}$]Glu, [$2\text{-}^{15}\text{N}$]Gln, [$2,5\text{-}^{15}\text{N}$]Gln, and [$2\text{-}^{15}\text{N}$]Asp was delayed and lower than 5% during the first 30 min, presumably because of a lower Fd-GOGAT activity in the dark. It can be noted that, after 30 min, the labeling rates of [$2\text{-}^{15}\text{N}$]Glu, [$2\text{-}^{15}\text{N}$]Gln, [$2,5\text{-}^{15}\text{N}$]Gln, [$2\text{-}^{15}\text{N}$]Asp, and [$4\text{-}^{15}\text{N}$]Asn were increased more in old than in young leaves (compare Fig. 5, A and B), suggesting that old leaves might contain metabolic pathways that increase Glu labeling in the dark. The dark treatment caused a substantial reduction of labeling in [^{15}N]Gly, [^{15}N]Ser, and [^{15}N]Ala,

probably as a consequence of the low labeling of [$2\text{-}^{15}\text{N}$]Glu (compare Fig. 5, C and D, and Fig. 4, C and D, respectively).

The AZA treatment in the dark completely inhibited ^{15}N enrichment in [$2\text{-}^{15}\text{N}$]Glu, [$2\text{-}^{15}\text{N}$]Gln, [$2,5\text{-}^{15}\text{N}$]Gln, and [$2\text{-}^{15}\text{N}$]Asp in the old leaves, whereas residual labeling remained in the young leaves (compare Fig. 5, B and F, and A and E, respectively). These results suggest that if old leaves contain particular metabolic pathways that increase the levels of Glu in the dark, such pathways are AZA sensitive. In addition, the MSO treatment completely inhibited label transfer into [$2\text{-}^{15}\text{N}$]Glu, [$2,5\text{-}^{15}\text{N}$]Gln, and [$2\text{-}^{15}\text{N}$]Gln in the old leaves (data not shown), ruling out a role for GDH. It is interesting to note that the dark treatment favored [$4\text{-}^{15}\text{N}$]Asn formation in young and old leaves, even in the presence of AZA (Fig. 5, A, B, E, and F). Because [$2\text{-}^{15}\text{N}$]Asp labeling was absent in the AZA-treated leaves in the dark, $^{15}\text{NH}_4^+$ and [$5\text{-}^{15}\text{N}$]Gln served as the potential amino donor to [$4\text{-}^{15}\text{N}$]Asn in the dark (Fig. 6). The labeling of Gly, Ser, Ala, and Pro was inhibited by the addition of AZA (compare Fig. 5, C and G, and D and H, respectively).

Amino Acid Transport in the Phloem

Amino acids were analyzed in phloem sap from six leaf ranges, collected along the tobacco main axis, from leaf 9 (old leaf) to leaf 30 (young leaf), according to

Masclaux et al. (2000). The total amino acid content in the phloem exudates was 1.6-fold higher from old leaves (10th and 11th leaves from the bottom) than from young leaves (22nd and 23rd leaves; Table I). These data suggest that older leaves exported more amino acids in their phloem sap than younger ones, and that they then behave as source leaves. Gln was the most abundant amino acid in the phloem, and it remained constant during leaf development (Table I). In contrast, the most important amino acid in leaf blades was Glu (Masclaux et al., 2000); thus, Gln can be considered the major nitrogen-transporting form in the phloem. In the same way, Asn proportions were higher in the phloem sap than in the leaf blade, suggesting that this amino acid is also dedicated for export. Its level stayed nearly constant over leaf development (Table I).

Pro continuously decreased in the phloem with developmental age, and a similar reduction was observed in the blades (Table I). The phloem sap also transported high levels of Ser, especially in the mature leaves found at the intermediate position between young and old leaves. In the leaf blades, Ser remained almost constant (8%) along the main axis (Masclaux et al., 2000). It can be noted that the most abundant amino acids found in phloem (Gln, Asn, Ser, and Pro) are all derived from Glu, the most abundant amino acid present in the leaf blade. Gly, Ala, Glu, and Asp levels remained constant in exudates from the leaves collected along the main axis (Table I).

Changes in the Levels of Nitrogen Assimilatory Enzymes and Expression of Fd-GOGAT in Transgenic Tobacco Plants Harboring a *GLU1* Promoter Fused to *GUS*

Two transgenic tobacco lines (*GLU1::GUS*1* and *GLU1::GUS*2*) harboring the *GLU1* promoter of the *Arabidopsis* Fd-GOGAT fused to a *GUS* reporter gene (*GLU1::GUS*) were subjected to a comparative study of

the enzymes of nitrogen metabolism in young and old leaves. Nitrate reductase activity in the old leaf discs was as low as 25% to 30% of the activity in the young leaf discs (Table II). A similar reduction was observed for GS (21%–25%). In contrast, the NADH-dependent aminating and NAD-dependent deaminating activities increased about 1.5-fold and 2.4-fold, respectively, in the old leaves. GOGAT activity decreased to about one-third in the old leaves: 27% to 28% for Fd-GOGAT and 32% to 35% for NADH-GOGAT.

Concomitant with the enzyme activity, the level of Fd-GOGAT protein in the mesophyll decreased to 30% to 42% of that in the young leaf mesophyll (Fig. 7A). In contrast, the higher levels of Fd-GOGAT protein were recovered in the veins (72%–75%) and in the petiole (1.1–1.8-fold) from the old leaves (Fig. 7A).

To understand the function of the GS-GOGAT cycle that was active in NH_4^+ reassimilation regardless of leaf age, the tissue-specific expression of GOGAT was determined in the young and old leaves. The activity of the Fd-GOGAT *GLU1* promoter decreased to a higher extent in the mesophyll than in the veins during aging (Fig. 7B). *GLU1* promoter expression was quantified by in vitro GUS activity in the mesophyll, veins, and petioles of the young and old leaves and also in the roots of two transgenic tobacco lines (Table III). *GLU1* promoter activity in the mesophyll decreased by 42% to 44% in the old leaves compared to the activity in the young leaves, whereas its decrease was only by 7% to 12% in the vascular tissues of veins and petioles from the old leaves. GUS activity remained relatively constant in the roots (Table III).

Steady-state levels of Fd-GOGAT mRNA were measured by relative quantitative reverse transcription (RT)-PCR in mesophyll and veins from young and old leaves of both *GLU1::GUS*1* and *GLU1::GUS*2* transgenic plants. Equal total amounts of 18S ribosomal RNA were used as the internal standard for RT (data

Table I. Amino acid determination (% and total content) in phloem sap collected from tobacco leaves of different ages

Amino acids were pooled prior to analysis, which was carried out according to Chaffei et al. (2004). Numbers in parentheses correspond to the amino acid determination (% and total content) in leaf blades, determined as described by Masclaux et al. (2000). Boldface type shows amino acids with higher values as indicated in the text. Values represent the means of analysis on phloem exudates from five independent plants.

Amino Acids	Amino Acids in Phloem Saps				
	Leaf No. 9	Leaf No. 10	Leaf No. 13	Leaf No. 20	Leaf No. 30
Asp %	10.0	9.7	7.6	8.4	8.1
Asn %	10.3 (5.2)	15.4 (3.7)	8.9 (1.8)	10.2 (1.2)	9.6 (1.8)
Ser %	13.9	10.9	17.3	16.2	7.2
Glu %	8.7 (25.5)	7.6 (27.7)	6.2 (25.3)	6.5 (28.8)	5.4 (15.3)
Gln %	27.9 (17.0)	22.1 (14.9)	30.3 (12.4)	21.9 (10.6)	21.8 (14.0)
Pro %	7.6 (1.3)	9.7 (2.3)	12.9 (1.3)	21.1 (2.9)	34.6 (14.1)
Gly %	4.9	6.9	2.0	1.9	2.5
Ala %	4.2	4.9	4.0	3.8	5.6
Total nmol/mg dry weight	109.0 (55.0)	65 (41.4)	59.0 (43.1)	46.8 (29.0)	21.5 (51.0)

Table II. Effects of leaf aging on the activity of the enzymes of nitrogen metabolism in transgenic tobacco plants, which harbor the *GLU1* promoter of the *Arabidopsis* Fd-Glu synthase fused to a *GUS* reporter gene (*GLU1::GUS*)

Enzyme assays were carried out in two transgenic lines: *GLU1::GUS*1* and *GLU1::GUS*2*.

Nitrogen Metabolism Enzymes	Young Leaves		Old Leaves	
	<i>GLU1::GUS*1</i>	<i>GLU1::GUS*2</i>	<i>GLU1::GUS*1</i>	<i>GLU1::GUS*2</i>
	Enzyme activity: nmol mg ⁻¹ protein min ⁻¹			
NR	15.50 ± 1.40	18.76 ± 2.06	4.70 ± 0.40	4.67 ± 0.42
GS	90.50 ± 8.21	102.27 ± 11.25	22.67 ± 2.01	34.00 ± 3.10
Fd-GOGAT	16.17 ± 1.48	21.51 ± 2.11	4.50 ± 0.38	5.81 ± 0.55
NADH-GOGAT	0.33 ± 0.03	0.41 ± 0.04	0.11 ± 0.01	0.14 ± 0.02
NADH-GDH				
Aminating	33.77 ± 3.14	31.07 ± 3.01	78.50 ± 7.21	76.12 ± 8.37
Deaminating	14.17 ± 1.28	13.89 ± 1.81	21.33 ± 1.98	19.51 ± 2.15

not shown). The level of Fd-GOGAT mRNA in the old mesophyll decreased between 76% and 85% of the mRNA levels in the young mesophyll (Fig. 7C). In the veins, the Fd-GOGAT mRNA level remained higher, ranging between 91% and 110% in the old leaves (Fig. 7C). The different extent of decrease in the mRNA (Fig. 7C) and protein levels of Fd-GOGAT (Fig. 7A) suggests an involvement of posttranscriptional regulation.

Immunolocalization of Fd-GOGAT

Using confocal laser-scanning microscopy, the cellular and subcellular localization of Fd-GOGAT protein was determined in leaf sections of tobacco by the indirect immunofluorescence method. The specific labeling of fluorochrome was detected in the meso-

phyll cells (Fig. 8A) compared to the control treated with only the first antibody (Fig. 8B). The fluorescence was localized to the chloroplasts of palisade and spongy parenchyma. The intensity of specific labeling and the number of labeled chloroplasts increased in the absence of bovine serum albumin (data not shown). Moreover, the Fd-GOGAT protein was found to be located in the vascular system of minor veins (Fig. 8C). Minor veins revealed a collateral organization with only one layer of phloem below a small number of xylem elements. Higher magnification resolution of the vascular bundles showed that the Fd-GOGAT protein was localized in the phloem companion cells (CC) next to the sieve element (SE). Phloem CCs, which appeared within the focal plane, were found to correlate with the cells that were detected by transmission microscopy (Fig. 8D).

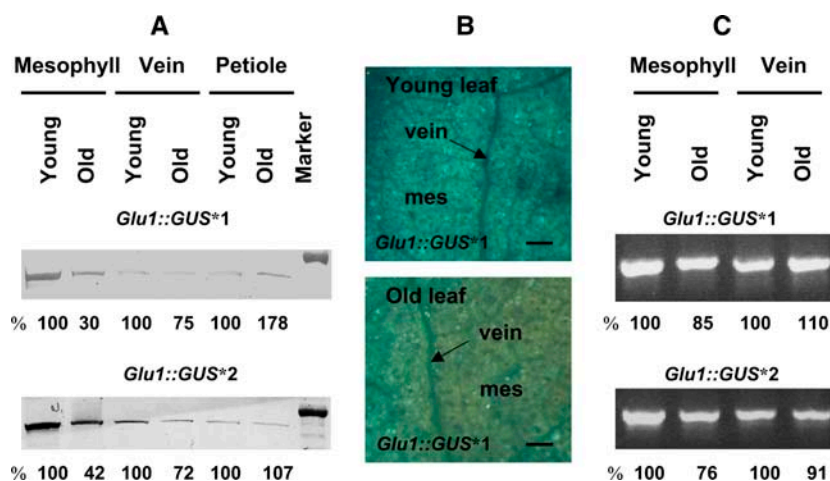


Figure 7. Changes in the level of Fd-Glu synthase in young and old tissues from two 10-week-old transgenic tobacco lines, harboring the promoter of *GLU1* encoding *Arabidopsis* Fd-GOGAT, fused to the *GUS* reporter gene (*GLU1::GUS*1* and *GLU1::GUS*2*). A, Western-blot analysis of Fd-GOGAT. The enzyme protein amounts were compared in the mesophyll, vein, and petiole, and expressed as percent relative to the values in the young leaves. The Fd-GOGAT protein of 165 kD was shown with the prestained markers including a myosin (233 kD). B, In vivo activity of the promoter of *GLU1* (*Arabidopsis* Fd-GOGAT) fused to the *GUS* reporter gene. *GUS* activity was assayed in the young and old leaves of the transgenic line *GLU1::GUS*1*. Bar = 50 μ m. mes, Mesophyll. C, Relative quantitative RT-PCR analysis of the Fd-GOGAT mRNA levels. The mRNA levels were compared in the mesophyll and vein and expressed as percent relative to the values in the young leaves.

Table III. Effects of leaf aging on the activity of the *Fd-Glu* synthase promoter in transgenic tobacco plants, which harbor the *GLU1* promoter of the *Arabidopsis* *Fd-GOGAT* fused to a *GUS* reporter gene (*GLU1::GUS*)

In vitro *GUS* activity was determined in two transgenic lines: *GLU1::GUS*1* and *GLU1::GUS*2*.

Tissues	Young Leaves and Roots		Old Leaves and Roots	
	<i>GLU1::GUS*1</i>	<i>GLU1::GUS*2</i>	<i>GLU1::GUS*1</i>	<i>GLU1::GUS*2</i>
	<i>GUS</i> activity: nmol 4-MU mg ⁻¹ protein min ⁻¹			
Leaves				
Mesophyll	58.41 ± 4.90	14.10 ± 1.27	34.05 ± 3.2	7.86 ± 1.08
Vein	5.02 ± 0.42	4.55 ± 0.41	4.67 ± 0.42	4.01 ± 0.36
Petiole	2.93 ± 0.25	2.17 ± 0.20	2.71 ± 0.23	1.95 ± 0.18
Roots	1.22 ± 0.09	0.45 ± 0.05	1.16 ± 0.11	0.41 ± 0.04

DISCUSSION

Nitrogen metabolism in old source leaves is characterized by a progressive hydrolysis of stromal proteins and degradation of chloroplasts (Jiang et al., 1993; Masclaux et al., 2000; Hörteinstainer and Feller, 2002). Since the main metabolic process in leaf senescence consists of nutrient remobilization, toxic free ammonium should be rapidly refixed into the amino acids to avoid deteriorating effects and provide nitrogenous forms suitable for source-sink transport. Our ¹⁵N-labeling study provides evidence that significant [¹⁵N]ammonium was released from [¹⁵N]Glu deamination by GDH in the dark to higher extents in old than in young leaves. This is consistent with the higher GDH activity in response to natural senescence (Masclaux et al., 2000; Masclaux-Daubresse et al., 2002). It is estimated that up to one-third of the Glu-dependent respiratory rates in isolated mitochondria can be attributed to the GDH deamination reaction (Aubert et al., 2001). The carbon flow from Glu oxidation becomes important under conditions of carbon limitation in darkness (Scheible et al., 2000) and carbohydrate starvation (Robinson et al., 1992; Turano, 1998). Also, the transition of young leaves to old leaves is characterized in part by a decrease in Suc (Masclaux et al., 2000). Therefore, it is conceivable that GDH supplies 2-oxoglutarate by Glu oxidation for the nitrogen and carbon cycle in old leaves.

Label of [¹⁵N]ammonium was rapidly incorporated into [5-¹⁵N]Gln by GS in the light and in the dark regardless of leaf age. Kinetics of [¹⁵N]ammonium assimilation clearly showed that *Fd-GOGAT* and/or *NADH-GOGAT* transferred [5-¹⁵N] of Gln to [2-¹⁵N]-Glu as soon as 10 min after [¹⁵N]ammonium feeding in the light, while the [2-¹⁵N]Glu labeling by *GOGAT* was slightly delayed in darkness. Despite the induction of cytosolic GS1 and a partial degradation of chloroplasts in old leaves, the chloroplastic GS2 protein remains predominant over the cytosolic GS1 protein in tobacco leaves (ratio of 75%:25% in the old leaves and 95%:5% in the young leaves; Masclaux et al., 2000). This implies that both the chloroplastic GS2 and cytosolic GS1 are involved in ammonium assimilation. Our in vivo ¹⁵N-labeling data clearly contrast with the proposal that GDH and cytosolic GS1 play the major role for the synthesis and reallocation of amino acids in

senescing leaves (for review, see Habash, 2002). Indeed, Glu synthesis from ammonium and 2-oxoglutarate in isolated plant mitochondria has been reported (Yamaya et al., 1986), but the rate of Glu formation was as low as 0.2% of photorespiratory [¹⁵N]ammonium release (Yamaya et al., 1986) or 1.2% of the oxidative deamination rate of Glu (Aubert et al., 2001).

GOGAT occupies the central position of photorespiratory nitrogen metabolism by providing Glu that serves as the amino donor via transaminase reactions. The ¹⁵N-labeling patterns of Glu, Gly, Ser, Ala, and Pro in this study correlate with the open flux of nitrogen by import and export of amino acids into and from the photorespiratory nitrogen cycle, respectively (Betsche, 1983). The synthesis of [2-¹⁵N]Glu and the [2-¹⁵N]Glu-derived amino acids was inhibited by AZA in young

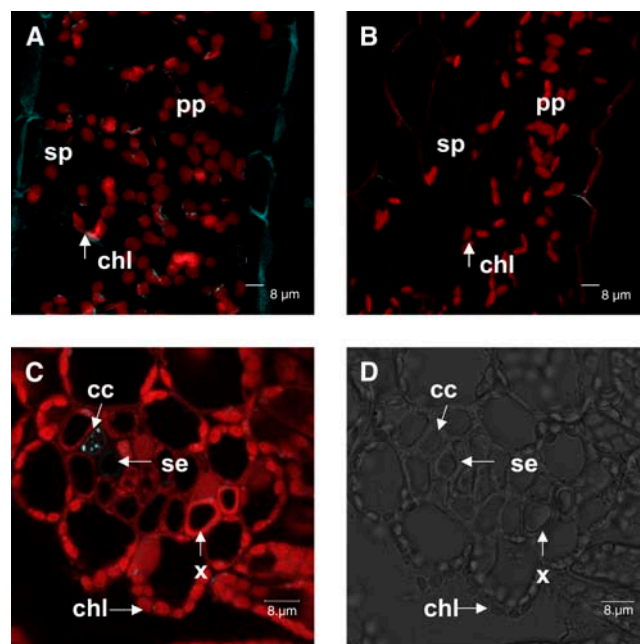


Figure 8. Immunocytochemical localization of *Fd-Glu* synthase in tobacco leaves. A, Leaf mesophyll section. B, Control leaf mesophyll section treated with nonimmune serum as the primary antibody. C, Leaf vascular section. D, Transmission of the leaf vascular section corresponding to C. chl, Chloroplast; pp, palisade parenchyma; sp, spongy parenchyma; x, xylem.

leaves and to higher extents in old leaves, where higher GDH activity was recovered. Moreover, MSO completely blocked [^{15}N] transfer from [^{15}N]ammonium to the amide and amino groups of Gln and Glu in young and old leaves. It can be noted that Fd-GOGAT supplies a sole source of Glu as the amino donor because mutants defective in Fd-GOGAT show reversible lethal phenotypes (Somerville and Ogren, 1980; Blackwell et al., 1988; Ferrario-Méry et al., 2002). These data provide strong evidence that the GS-GOGAT cycle is the primary route of ammonium assimilation in both old and young leaves and that GDH plays a minor role.

Interestingly, a low [^{15}N]Glu labeling by the GS-GOGAT cycle was detected in old leaves after 30 min of [^{15}N]ammonium feeding in the dark. This indicates that the nitrogen cycle takes place in the dark, albeit at lower rates. Given that Ala partially replaces Glu in the transaminase reaction with higher efficiency in the dark, [^{15}N]Glu would become apparent. Consistently, peroxisomal Glu:glyoxylate aminotransferase can use both Glu and Ala as the amino donor at equal rates (Igarashi et al., 2003), and Ser and Ala can equally serve as the amino donor for Ser:glyoxylate aminotransferase (Liepman and Olsen, 2001). Glutamate thus formed by GOGAT could in turn be used for biosynthetic reactions and export from old leaves to maintain the nitrogen cycle. This implies that alternative nitrogen pathways take place to balance the nitrogen when disequilibrium occurs. In fact, the inhibition of Glu synthesis by AZA led to a significant labeling of [$4\text{-}^{15}\text{N}$]Asn in the dark by direct incorporation of [^{15}N]ammonium as the substrate. Consistently, a dendritic group of Asn synthetase isoenzymes have enhanced ammonia-dependent activity (Lam et al., 1998), and AS gene expression is down-regulated by light (Ngai et al., 1997). Therefore, tobacco plants can provide inert Asn as the transient ammonium detoxification molecule in the dark as well as a long-distance nitrogen carrier. It is estimated that as much as 43% of the amino acids in young leaves (youngest leaf 7) would be supplied by nitrogen partitioning via upflow of amino acids through the phloem (Jeschke and Pate, 1991).

The spatial distribution of the enzymes of amino acid synthesis correlates with the pathway of intra- and intercellular transport of amino acids. Fd-GOGAT found in the CC-SE complex corresponds to the expression of the *GLU1* promoter-*GUS* transgene within phloem cells (Feraud et al., 2005). Fd-GOGAT in the interconnected CC-SE complexes, adjacent to the phloem parenchyma cells (Tourgeon, 1996), can allow a channeling of amino acids from the mesophyll chloroplast/cytosol to phloem SEs in minor veins. Moreover, the close location of the CC-SE complexes to a small number of xylem elements appears to be a prerequisite for a direct xylem-phloem transfer of amino acids translocated from roots (Atkins, 2000). The enzymes of ammonium metabolism, including cytosolic GS1 (Carvalho et al., 1992; Kamachi et al., 1992; Pereira et al., 1992),

Asn synthetase (Nakano et al., 2000), and GDH (Tercé-Laforgue et al., 2004), are colocalized in the companion cells as well. Although there is no molecular evidence showing that amino acid trafficking takes place by broad affinity amino acid transporters in minor veins (Fischer et al., 1998; van Bel, 2003), phloem loading of Gln, Asn, and Glu from CC-SE complexes likely regulates nitrogen translocation in the sieve streams where these amino acids are maintained at the constant levels. The presence of Fd-GOGAT in the vascular cells of old leaves reinforces the notion that the enzyme plays a role in amino acid transport to young tissues.

The role of amino acid transport was first assigned for NADH-GOGAT in rice (*Oryza sativa*) because it was found in the vascular tissues, particularly in the metaphloem, metaxylem-parenchyma, and mestome sheath cells of the vascular bundles, whereas it was not detected in the mesophyll, CCs, and SEs (Tobin and Yamaya, 2001). In concert with the GS1 located in the CC-SE complex, Fd-GOGAT presumably plays a complementary role to NADH-GOGAT during development because NADH-GOGAT occurred at higher levels in young and nonexpanded leaves and decreased with leaf age (this study; Yamaya et al., 1992). Consistently, the low level of Fd-GOGAT (15% of wild-type activity) supplies Glu for the normal growth of the transgenic antisense Fd-GOGAT tobacco lines in which NADH-GOGAT is not detected in leaves and roots (Ferrario-Méry et al., 2000; Feraud et al., 2005). As the CC-SE complexes contain a heterogeneous mixture of photosynthetic chloroplasts and nonphotosynthetic plastids at different sites of the same tissue (Thomson and Whitley, 1980; DeWitt and Sussman, 1995), Fd-linked Glu formation in the vascular tissue requires electron donor systems other than chloroplastic PSI. NADPH serves as the primary reductant for Glu formation via the reversed reaction of photosystem-dependent Fd:NADP⁺ oxidoreductase (EC 1.18.1.2; Fig. 5; Hanke et al., 2004; Feraud et al., 2005). Taken together, the data support the notion that the GS-GOGAT cycle takes place in vascular cells for the biosynthesis of Glu and Gln prior to the cycle of amino acids, and that GDH does not play a role in Glu supply even in old leaves of tobacco where GDH is induced.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tobacco (*Nicotiana tabacum* cv Xanthi XHFD8; Institut National de la Recherche Agronomique, Versailles, France) plants were grown on a clay loam soil in a greenhouse under natural lighting (temperature ranging between 20°C and 30°C) until flowering. Twelve plants were watered every 2 d with a 10 mM nitrate and 2 mM ammonium nutrient solution (Coic and Lesaint, 1971). Ten-week-old plants had approximately 30 leaves. Fully expanded leaves, ranging between the 10th and 11th leaf stage from the bottom, were chosen in order to select leaves where GDH and GS1 were expressed, thus allowing us to consider them as old leaves. The 22nd and 23rd leaves were considered as young leaves (Masclaux et al., 2000; Masclaux-Daubresse et al., 2005).

Transgenic tobacco plants harboring the promoter of the Fd-GOGAT gene (*GLU1*) of *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia) were prepared by transforming tobacco cv Xanthi (Ziegler et al., 2003). Wild-type and transformed plants were grown on soil for 10 weeks and then transferred

and grown in a chamber under a regime of 16 h light (photosynthetic photon flux density, 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 23°C/8 h dark, 18°C) using a nutrient solution (Coïc and Lesaint, 1971).

¹⁵N-Labeling Experiments, Amino Acid Analysis, and Gas Chromatography-Mass Spectrometry Measurement

¹⁵N-labeling experiments were performed using leaves of 10-week-old wild-type tobacco plants. Discs of 1 cm in diameter were prepared from leaves numbered 10 to 11 (old) and 22 to 23 (young) from bottom to top. Discs were floated on 10 mM MES buffer, pH 6.5, containing 10 mM CaCl₂, 40 mM KCl, and 2% (w/v) polyethylene glycol, either in the light (photosynthetic photon flux density, 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or in the dark, with or without 1 mM MSO or 1 mM AZA for 1 h. Afterward, [¹⁵N]ammonium (99% enrichment) or [2-¹⁵N]Glu (99% enrichment; Euriso-top S.A.) was added to the medium. Leaf discs were dipped into the labeling solutions, quickly transferred, and rinsed with a large volume of water before freezing in liquid nitrogen (time 0). Leaf discs were further incubated in the light or in the dark and collected at 5, 10, 20, 30, 45, 60, 120, and 240 min. Samples were frozen in liquid nitrogen prior to analysis.

Total amino acids and ammonia were extracted with 2% (w/v) sulfosalicylic acid. Extracts were centrifuged at 17,500g for 20 min to eliminate cellular debris. Amino acids were applied to a column (AG 50W-X8 resin, 100–200 mesh, H⁺-form, 5 × 0.5 cm; Bio-Rad Laboratories), washed with 4 mL water, and eluted with 2.5 mL of 6 M NH₄OH, then with 1 mL water. Total amino acids were determined by the method of Rosen (1957) and ammonium content by the Berthelot reaction. One-half of the supernatant was adjusted to pH 2.1 with LiOH, and amino acids were separated and quantified by ion-exchange chromatography on a Biotronic LC5001 analyzer using a standard amino acid mixture (Benson standard PANB) by Perkin-Elmer Nelson 2100 software (Rochat and Boutin, 1989). From the remaining supernatant, amino acids were derivatized with either *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide in acetonitrile at 75°C for 30 min (Chaves Das Neves and Vasconcelos, 1987) or *N,N*-dimethylformamide at 125°C for 1 h (Williams and Wolf, 1994). The atom percentage of amide and amino ¹⁵N was determined by gas chromatography (GC)-mass spectrometry (MS) analysis (model MD800; Fisons).

[¹⁵N]Ammonium released from [2-¹⁵N]Glu was determined after purification and derivatization steps as described by Fujihara et al. (1986) and Ek et al. (1990). The [¹⁵N]ammonium extract (100 μL) was mixed with 1 mL of 5% NaHCO₃ to adjust pH to 8 before adding 4 μL of pentafluorobenzoyl chloride (PFB-Cl; Sigma). After centrifuging twice for 1 min, the mixture was incubated at room temperature for 30 min. Pentafluorobenzamine (PBFA), a product from the reaction of ammonia on PFB-Cl, was extracted with 1 mL acetate. The organic phase was separated by centrifugation at 2,000g for 5 min and mixed with 250 μL of 6% H₃PO₄ to eliminate NaHCO₃ and PFB-Cl. Water was eliminated using CuSO₄. Then PBFA was derivatized by *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide and analyzed as described for amino acid analysis.

Phloem Sap Collection

Phloem exudates were collected from 10-week-old wild-type tobacco plants in the light period as described previously (Chaffee et al., 2004). Leaf petioles were excised and recut under water and rapidly immersed in 1 mL of collection buffer consisting of 10 mM HEPES, pH 7.5, and 1 mM EDTA. Phloem exudates were collected in darkness for 8 h in a growth chamber with 95% relative humidity. Samples were concentrated 10-fold by speed vac, adjusted to pH 2.1, and stored at –70°C prior to amino acid analysis.

Protein Extraction and Enzyme Assays

Nitrate reductase was extracted, and activity and activation state, expressed as the ratio of 10 mM MgCl₂-dependent activity to 5 mM EDTA-dependent activity, were measured as described by Ferrario-Méry et al. (2000). GS activity was measured by the method reported by O'Neal and Joy (1973). Fd-GOGAT and NADH-GOGAT activities were assayed by determining Glu (Suzuki et al., 2001). GDH aminating and deaminating activities were assayed as described by Masclaux et al. (2000). Total soluble proteins were measured according to Bradford (1976).

Western Immunoblotting

Protein samples (20–50 μg) were subjected to SDS-PAGE electrophoresis (Laemmli, 1970) and transferred onto a nitrocellulose membrane according to Towbin et al. (1979). The membrane was probed with rabbit IgG directed

against tobacco Fd-GOGAT as the primary antibody (Suzuki et al., 1996) and with goat secondary antibodies against rabbit IgG conjugated with peroxidase. Peroxidase activity was developed with 3.4 mM 4-chloro-1-naphthol and 0.01% (v/v) H₂O₂. Relative amounts of the enzyme protein were estimated by scanning samples with a FLA-5000 imaging system (FujiFilm France S.A.S.).

Relative Quantitative RT-PCR

Total RNA was extracted from the *GLU1::GUS* transgenic tobacco plants using a kit according to the manufacturer's instruction (Qiagen). To carry out relative quantitative PCR, ribosomal RNA was used as an endogenous standard, and first-strand cDNA was synthesized from 2 μg RNA using an Omniscript RT kit (Qiagen). An abundance of initial cDNA strands between samples was corrected using Quantum RNA 18S internal standards (Ambion). PCR was performed on a LightCycler instrument (Roche). The following specific primer sets were used. *GLU1* was used for Fd-GOGAT: forward primer, 5'-GGACCTGAAAATGCATCTCA-3'; reverse primer, 5'-GCCAAATGCTGCTGATAAAC-3'. The reaction was carried out using 1:20 and 1:40 dilutions of cDNA. PCR was hot started at 95°C and carried out for 32 cycles composed of 94°C for 30 s, 46°C for 90 s, and 72°C for 90 s. PCR products were visualized by ethidium bromide in agarose gel and bands were quantified by scanning of samples with a FLA-5000 imaging system (FujiFilm France S.A.S.).

Histochemical and Quantitative GUS Assays

Leaf sections were incubated in 50 mM sodium phosphate, pH 7.0, 0.1 mM K₃[Fe(CN)₆], 0.1 mM K₄[Fe(CN)₆], and 1.9 mM 5-bromo-4-chloro-3-indolyl- β -D-GlcUA (X-Gluc) at 37°C for 2 to 18 h (Jefferson et al., 1987). Chlorophyll was removed by incubation in 70% (v/v) ethanol. Leaf sections were cut by hand and mounted on slides. GUS staining was observed using a microscope (model BX51; Olympus), and photographs were taken with a digital camera system (model DP50; Olympus). Quantitative GUS assays were carried out in vitro using sample extracts in 50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (v/v) *N*-lauroylsarcosine, and 10 mM β -mercaptoethanol. GUS activity was determined by measuring the kinetics of 4-methylumbelliferyl- β -D-glucuronide (MUG) conversion to 4-methylumbelliferone (4-MU) as described by Jefferson et al. (1987) using a Fluoroscanner II (365-nm excitation and 455-nm emission; Labsystems). GUS activity was expressed as nanomoles of 4-MU mg⁻¹ protein min⁻¹.

Indirect Immunofluorescence Analysis

Leaf sections were hand cut using razor blades. Sections were fixed in 3.7% (w/v) formaldehyde for 1 h, in 50 mM PIPES buffer, pH 6.9, 5 mM MgSO₄, and 5 mM EGTA (MTSB) for 30 min, then in 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3, 14 mM NaCl, and KCl (PBS) for 15 min. Tissues were dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, and 97% [v/v] in PBS). Samples were incubated in a mixture of 100% wax and 97% ethanol (1:1, v/v) at 40°C overnight, and embedded in 100% wax at 40°C for 2 h. Sections (10 μm) were prepared using a microtome and air dried on polylysine-coated glass slides. Samples on slides were dewaxed and rehydrated through a degraded ethanol series (97%, 90%, and 50% [v/v] in PBS). Samples were blocked with 1% (w/v) bovine serum albumin in PBS (blocking solution), and incubated with rabbit antibody against tobacco Fd-GOGAT diluted in blocking solution. After three washes of 5 min each in PBS, slides were incubated with goat anti-rabbit IgG labeled with Alexa 405 (Molecular Probes) in blocking solution. After washing, slides were sealed with PBS. As controls, sections were incubated with preimmune sera as the primary antibodies or without the primary antibodies, and with Alexa 405-labeled secondary antibodies. Immunofluorescence was observed using a spectral confocal laser-scanning microscope (Leica TCS SP2 AOBs). Microscopic observations were carried out with a laser diode (25 mW, 405 nm), and generated using a Leica HC PL APO 63 × /1.20 Water Corr./0.17 Lbd.BL objective. Low-speed scan (200 lines/s) images (512 × 512 pixels) were generated. Alexa 405 fluorescence was collected with a specific bandwidth (407–427 nm) after a spectral adjustment to eliminate the background noise. The red autofluorescence of tissues was observed between 509 and 628 nm. In some experiments, another photomultiplier was used to collect simultaneously the transmitted light with the same configuration as above.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: for *GLU1*, Y09667 and U39287; and for *GLU2*, U39288.

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