

# Resolving the Role of Plant Glutamate Dehydrogenase. I. *in vivo* Real Time Nuclear Magnetic Resonance Spectroscopy Experiments

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In higher plants the glutamate dehydrogenase (GDH) enzyme catalyzes the reversible amination of 2-oxoglutarate to form glutamate, using ammonium as a substrate. For a better understanding of the physiological function of GDH either in ammonium assimilation or in the supply of 2-oxoglutarate, we used transgenic tobacco (*Nicotiana tabacum* L.) plants overexpressing the two genes encoding the enzyme. An *in vivo* real time <sup>15</sup>N-nuclear magnetic resonance (NMR) spectroscopy approach allowed the demonstration that, when the two GDH genes were overexpressed individually or simultaneously, the transgenic plant leaves did not synthesize glutamate in the presence of ammonium when glutamine synthetase (GS) was inhibited. In contrast we confirmed that the primary function of GDH is to deaminate Glu. When the two GDH unlabeled substrates ammonium and Glu were provided simultaneously with either [<sup>15</sup>N]Glu or <sup>15</sup>NH<sub>4</sub><sup>+</sup> respectively, we found that the ammonium released from the deamination of Glu was reassimilated by the enzyme GS, suggesting the occurrence of a futile cycle recycling both ammonium and Glu. Taken together, these results strongly suggest that the GDH enzyme, in conjunction with NADH-GOGAT, contributes to the control of leaf Glu homeostasis, an amino acid that

plays a central signaling and metabolic role at the interface of the carbon and nitrogen assimilatory pathways. Thus, *in vivo* NMR spectroscopy appears to be an attractive technique to follow the flux of metabolites in both normal and genetically modified plants.

**Keywords:** Ammonium • Glutamate • Glutamate dehydrogenase • Gene overexpression • Homeostasis • Tobacco.

**Abbreviations:** AlaAT, alanine aminotransferase;  $\alpha$ -Glx,  $\alpha$ -amino groups of Glu and Gln; AOA, aminooxyacetate; GABA,  $\gamma$ -aminobutyrate; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; MSX, methionine sulfoximine; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; WT, wild-type.

## Introduction

Plant physiologists have agonized for more than 30 years as to the role of glutamate dehydrogenase (GDH; EC 1.4.1.2) in higher plants. This is because the enzyme catalyzes the reversible amination of 2-oxoglutarate to form Glu and therefore it is theoretically able to either assimilate or liberate ammonium. Although a considerable amount of

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evidence has built up demonstrating that >95% of the ammonium available to higher plants is assimilated via the glutamine synthetase-glutamate synthase (GS/GOGAT; EC 6.3.1.2/EC 1.4.7.1 and 1.4.1.14) pathway (Lea and Mifflin 1974), proposals that GDH could operate in the direction of ammonium assimilation have been put forward on a regular basis (Yamaya and Oaks 1987, Oaks 1995, Melo-Oliveira et al. 1996), particularly under stress conditions favouring ammonium accumulation (Skopelitis et al. 2006). Others have argued equally strongly that under standard growth conditions, GDH operates in the direction of Glu deamination to form ammonium (Robinson et al. 1992, Fox et al. 1995, Stewart et al. 1995, Glévevec et al. 2004). Finally, the isolation of non-viable photorespiratory mutants lacking the plastidic GS isoenzyme (Leegood et al. 1995), and the fact that the  $K_m$  for ammonium of GDH aminating activity is >100 times higher than that of GS (Stewart et al. 1980), led to the general consensus that GDH plays a negligible role in the assimilation of ammonium when the whole plant is considered.

Recently, this consensus was again questioned following the discovery that GDH is mainly if not exclusively localized in the phloem companion cells (Dubois et al. 2003, Tercé-Laforgue et al. 2004a, Fontaine et al. 2006). In particular, the finding that an increase in GDH activity was induced in both the mitochondria and cytosol of the companion cells when the ammonium concentration increased above a certain threshold, led Tercé-Laforgue et al. (2004a) to hypothesize that the enzyme may act as a sensor to evaluate the C/N status of the plant with respect to ammonium and sugar concentration and/or fluxes through the phloem stream. To further support this hypothesis, a parallel can be made with the flexible function of human GDH in the formation of Glu, which is the prevailing direction for the enzyme reaction in many organs with the exception of astrocytes. This indicates that GDH could serve as a major link between carbohydrate and amino acid metabolism in different organs or cell types (Yoon et al. 2004).

In many studies both the metabolic environment and the tissue localization of GDH were not really taken into account, since the catalytic function of the enzyme in higher plants was studied *in vivo* by providing  $^{15}\text{N}$ -labeled ammonium or  $^{15}\text{N}$ -labeled Glu to cells in suspension culture, isolated mitochondria or leaf discs. Nevertheless,  $^{15}\text{N}$ -labeling experiments have provided strong lines of evidence that GDH is involved in the supply of 2-oxoglutarate when carbon becomes limiting (Robinson et al. 1992, Aubert et al. 2001, Masclaux-Daubresse et al. 2006). As argued by Oaks (1995) and Pahlich (1996), it is evident that the use of such biological systems has some limitations, since the integration of an enzyme in a metabolic chain is largely dependent on both its metabolic and its cellular environment. This is why both of these authors suggested the use of NMR

techniques to unambiguously ascertain the catalytic function of GDH in plants.

The GDH holoenzyme has a hexameric structure consisting of two subunit polypeptides  $\alpha$  and  $\beta$ . The two subunits can be associated as homohexamers of the  $\beta$  and  $\alpha$  subunit or heterohexamers composed of different ratios of  $\alpha$  and  $\beta$  subunits, thus leading to the formation of seven active isoenzymes in both stems and leaves and roots (Loulakakis and Roubelakis-Angelakis 1991, Loulakakis and Roubelakis-Angelakis 1992, Fontaine et al. 2006). Recently, the use of transgenic tobacco plants modified in the expression of the genes encoding the two GDH polypeptides  $\alpha$  and  $\beta$  (Purnell et al. 2005, Skopelitis et al. 2007), has firmly established *in vivo* that under normal growth conditions the GDH isoenzyme 1, a  $\beta$ -subunit homohexamer, solely deaminates Glu (Purnell and Botella 2007), whereas the GDH isoenzyme 7, an  $\alpha$ -subunit homohexamer, exhibits strong deaminating activity and only a very low aminating one (Skopelitis et al. 2007). The finding that the  $\alpha$  and  $\beta$  subunits apparently exhibit two slightly different catalytic functions could explain why two of the three genes encoding NAD(H)-GDH (Jaspard 2006) are highly expressed in most plants examined so far (Melo-Oliveira et al. 1996, Pavesi et al. 2000, Restivo 2004, Purnell et al. 2005, Skopelitis et al. 2007). However, the physiological significance of the organ- and metabolic-dependent variability of the GDH isoenzyme composition and its regulation is still unclear and cannot be explained solely in terms of metabolic function (Skopelitis et al. 2006, Purnell and Botella 2007). As an example, the use of transgenic tobacco plants with modified amounts of the GDH  $\alpha$  or  $\beta$  subunits showed that N metabolism in general and ammonium assimilation in particular, were not markedly modified although an accumulation of Asp was observed when the  $\beta$ -subunit gene was overexpressed (Purnell et al. 2005, Skopelitis et al. 2007).

To investigate further the function of GDH in plants, we have used an *in vivo* NMR spectroscopy approach to follow under real time conditions the assimilation of  $^{15}\text{N}$ -labeled substrates by the leaves of tobacco plants when the ammonium assimilatory pathway was inhibited. This method is minimally invasive and non-destructive compared with those based on the use of isolated mitochondria (Aubert et al. 2001), the extraction of leaves or roots prior to NMR (Purnell and Botella 2007, Skopelitis et al. 2007) or GCMS analysis (Stewart et al. 1995, Skopelitis et al. 2006, Skopelitis et al. 2007). It thus provides an efficient and reliable way of following the fate of a labeled substrate such as  $^{15}\text{NH}_4^+$  or  $[^{15}\text{N}]\text{Glu}$  through a metabolic pathway in living tissues (Mesnard and Ratcliffe 2005). *In vivo* NMR spectroscopy was therefore employed to determine whether, in the leaves of tobacco plants overexpressing the two GDH subunits individually or simultaneously, the enzyme assimilates ammonium or deaminates Glu.

## Results and discussion

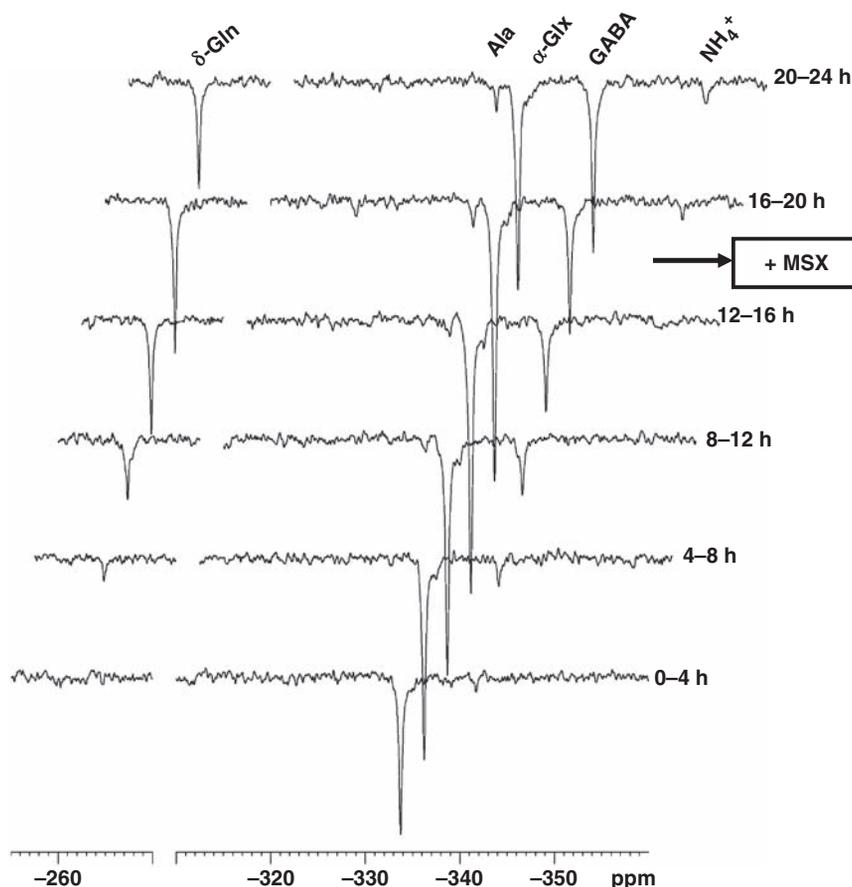
### GDH deaminates glutamate in vivo in untransformed tobacco leaves

Using a real time in vivo NMR approach, a first series of experiments was conducted in order to determine whether GDH in young developing tobacco leaves is able to assimilate  $^{15}\text{NH}_4^+$  or deaminate [ $^{15}\text{N}$ ]Glu. Some spectra from one leaf sample are shown in **Supplementary Fig. 1A** for  $^{15}\text{NH}_4^+$  and in **Fig. 1** for [ $^{15}\text{N}$ ]Glu.

When  $^{15}\text{NH}_4^+$  was provided to leaf fragments in the absence of methionine sulfoximine (MSX), an inhibitor of the GS enzyme (Rhodes et al. 1986), the incorporation of label into both the  $\delta$ -amido group of Gln ( $-263.8$  ppm) and the  $\alpha$ -amino groups of Glu and Gln (referred to as  $\alpha$ -Glx at  $-334.9$  ppm) was visible. The peak located at  $-354.7$  ppm corresponded to  $^{15}\text{NH}_4^+$ . After 20–24 h, the amount of label

incorporated into the  $\delta$ -amido group of Gln was about twice as high as that incorporated into  $\alpha$ -Glx, indicating that the GS/GOGAT cycle was operating, meaning that the leaf tissue samples were in good physiological conditions (**Supplementary Fig. 1A**). When  $^{15}\text{NH}_4^+$  was provided to leaf fragments in the presence of MSX, which does not have an effect on GDH activity (Bechtold et al. 1998), no labeling was observed either in the  $\delta$  position or in the  $\alpha$ -amino group of Glu and Gln ( $\alpha$ -Glx). This result indicates that on one hand GS activity was inhibited and thus unable to provide Gln for the reaction catalyzed by GOGAT, on the other hand GDH was not able to synthesize [ $^{15}\text{N}$ ]Glu, which would have appeared as a peak of labeled  $\alpha$ -Glx in the presence of  $^{15}\text{NH}_4^+$  (**Supplementary Fig. 1A**).

Following the addition of [ $^{15}\text{N}$ ]Glu to the perfusion medium, progressive incorporation of the label into both the  $\delta$ -amido group of Gln ( $-263.8$  ppm) and the  $\alpha$ -amino groups



**Fig. 1** Time series of in vivo  $^{15}\text{N}$ -NMR spectra of young fully developed tobacco leaves from WT plants. At time zero,  $7.0$  mM [ $^{15}\text{N}$ ]Glu was added to the perfusion medium, and after  $17$  h,  $1$  mM MSX, an inhibitor of GS activity, was added. Peak identifications are from left to right:  $\delta$ -Gln =  $\delta$ -amido group of Gln; Ala = alanine;  $\alpha$ -Glx =  $\alpha$ -amino group of Glu and Gln; GABA =  $\gamma$ -aminobutyrate;  $\text{NH}_4^+$  = ammonium. The various spectra of the time course experiment were shifted slightly to the right in order to obtain a better visualization of the fate of  $^{15}\text{N}$  originating from Glu. Similar spectra were obtained with three individual plants.

of Glu and Gln (referred to as  $\alpha$ -Glx at  $-334.9$  ppm) was visible over the first 16 h of the experiment (Fig. 1). These two peaks decreased following the addition of the GS inhibitor MSX. Concomitant with this decrease, a small peak of  $^{15}\text{NH}_4^+$  appeared at  $-354.7$  ppm after the application of MSX. This shows that Glu was deaminated when ammonium assimilation was inhibited, presumably through the reaction catalyzed by GDH.

We found that, as well as the reactions catalyzed by GS/GOGAT and GDH, other metabolic pathways in which Glu can play an important role needed to be considered with respect to the overall flux of reduced N in intact leaf cells. For example after 4 h, a peak was visible at  $-342.7$  ppm corresponding to  $\gamma$ -aminobutyrate (GABA) that rapidly increased after 8 h. Interestingly, the addition of MSX did not inhibit the incorporation of  $^{15}\text{N}$  into GABA as it did for both  $\delta$ -Gln and  $\alpha$ -Glx (Fig. 1, Supplementary Fig. 2), suggesting that the increase in the amount of  $^{15}\text{N}$ GABA probably originates directly from  $^{15}\text{N}$ Glu decarboxylation (Ratcliffe 1995, Shelp et al. 1999). Moreover, we found in earlier studies that, in the same leaf stage used for the NMR experiments, GABA can represent up to 10% of the total free amino acid content (Tercé-Laforgue et al. 2004b). It could be argued that GABA accumulation is the result of a stress due to the compaction of the leaf tissues in the NMR tube (Wallace et al. 1984, Bown et al. 2006). However, in the absence of MSX, we did not find any accumulation of GABA when  $^{15}\text{NH}_4^+$  was added to the perfusion medium, although substantial amounts of  $\alpha$ -Glu (peak of labeled  $\alpha$ -Glx) were synthesized through the GS/GOGAT pathway, indicating that the leaf tissues were under good physiological conditions even after several hours of incubation inside the NMR tube (Supplementary Fig. 1A).

At  $-332.5$  ppm a small peak of Ala appeared between 8 and 12 h. Ala can be synthesized through the reaction catalyzed by alanine aminotransferase (AlaAT) (Good et al. 2007), using Glu as N donor molecule for transamination (Streeter and Thompson 1972, Stewart and Larher 1980). The activity of this enzyme is strongly enhanced under anoxic conditions (Miyashita et al. 2007, Limami et al. 2008). In our experiment, the amount of Ala produced by the leaf tissues remained very low (Fig. 1, Supplementary Fig. 2), thus indicating that the perfusion medium was sufficiently aerated over the 24 h time course experiment and not subjected to anoxia.

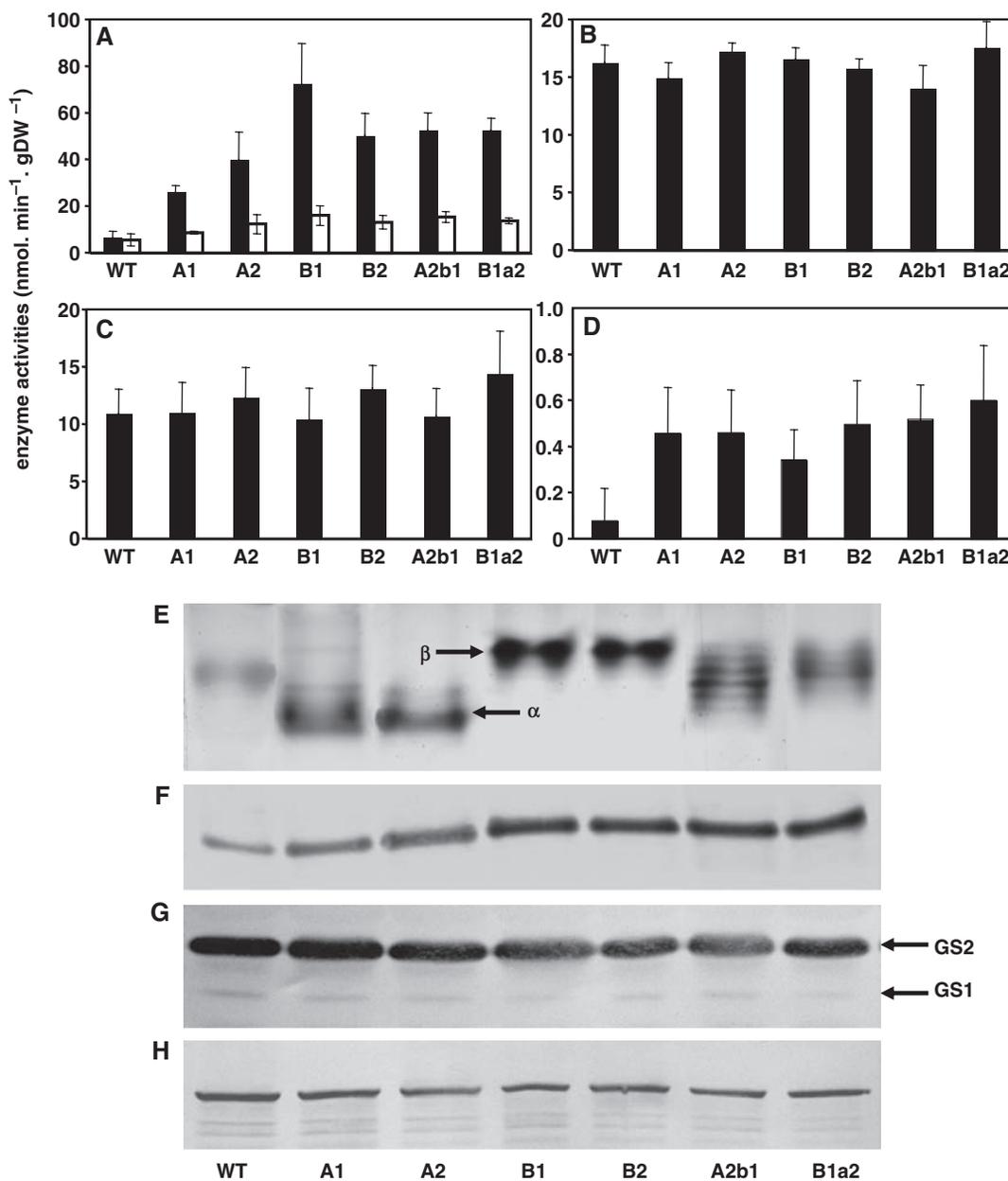
When  $^{15}\text{N}$ -labeled compounds are provided to the plant, Ala and GABA, along with Glu and Gln, are the most enriched amino acids in terms of percentage  $^{15}\text{N}$ , which is consistent with their role as N storage compounds regardless of the cellular oxygen status (Fan et al. 1997). In particular, the importance of Ala in the plant N economy has also already been emphasized using transgenic plants overexpressing AlaAT (Good et al. 2007, Shrawat et al. 2008). Thus, in vivo NMR

appears to be an attractive technique that can be used to investigate further the physiological basis of improved N use efficiency through the measurement of metabolic fluxes in intact tissues, in combination with standard metabolite profiling or metabolomic studies (Wiechert et al. 2007).

### Increased GDH activity enhances Glu deamination but does not induce aminating activity of the enzyme

To produce tobacco plants overexpressing the genes encoding GDH, two *Nicotiana plumbaginifolia* cDNAs encoding distinct NAD(H)-dependent GDH subunits (GDHA and GDHB) were made constitutive by fusing them with the CaMV 35S promoter to obtain, after selection and regeneration on kanamycin, homozygous T3 transgenic plants overexpressing the two proteins. Overexpressors were selected by measuring NAD(H)-dependent GDH activity. For each construct 35S-*NpGDHA* and 35S-*NpGDHB*, two independent transformants (lines A1, A2 and B1, B2, respectively) exhibiting the highest increase in leaf GDH activity, were selected. Two transgenic lines expressing both genes (lines A2b1 and B1a2) were obtained by reciprocal crossing between the *GDHA* and *GDHB* overexpressors (see Materials and Methods).

In young developing leaves of the transgenic lines A1, A2, B1, B2, A2b1 and B1a2, NAD(H)-GDH aminating activity was increased by at least 4-fold and up to 7-fold compared with the wild-type (WT) control plants (Fig. 2A). Activity staining following native PAGE was used to detect the GDH isoenzyme composition of the leaves of the various tobacco transgenic lines and WT control plants (Fig. 2E). Following electrophoresis of extracts of the WT tobacco leaves, only a weak and diffuse area of enzyme activity was visible in a zone located at an equal distance from the most cathodic and the most anodic parts of the gel. In the leaves of the two *GDHA* transformants, only the most anodal isoenzyme ( $\alpha$  subunit) was visible. In the leaves of the two *GDHB* transformants, only the most cathodal isoenzyme ( $\beta$  subunit) was visible. In the two double transformant lines A2b1 and B1a2, the GDH isoenzyme pattern was typical of that found in the leaves of *N. plumbaginifolia* (Restivo 2004) indicating that in *N. tabacum* the two subunits  $\alpha$  and  $\beta$  from *N. plumbaginifolia* were assembled into an active heterohexameric enzyme. The increase in enzyme activity measured in vitro (Fig. 2A) was qualitatively similar to that detected by native gel staining (Fig. 2E). Western blot analysis confirmed that the amount of GDH protein was increased in the three types of transformant, approximately matching the enzyme activity measured in vitro in the aminating direction (Fig. 2F). GDH deaminating activity measured in vitro was at least three times lower compared with the aminating activity both in the WT and in the three *GDH* overexpressors. The ratio of aminating NAD(H) activity to deaminating NAD



**Fig. 2** NAD(H)-GDH, GS, Fd- and NADH-GOGAT activities and protein content of tobacco leaves from WT and transgenic plants overexpressing the two genes encoding GDH. (A) NAD(H)-GDH, (B) GS, (C) Fd-GOGAT and (D) NADH-GOGAT activities of WT and transgenic plants. The enzyme activities were measured in leaf samples of WT control plants, *GDHA* overexpressors (transgenic lines A1, A2), *GDHB* overexpressors (transgenic lines B1, B2) and *GDHA/B* overexpressors (transgenic lines A2b1, B1a2). NAD(H)-GDH aminating activity (black bars) and NAD-GDH deaminating activity (white bars), GS, Fd- and NADH-GOGAT activities were measured on three individual plants. Values are the mean  $\pm$  SD. (E) NAD-GDH isoenzyme patterns of WT and transgenic plants. The soluble protein extracts of leaves were subjected to native PAGE followed by NAD-GDH in-gel deaminating activity staining. The amount of protein loaded onto each lane was calculated on a similar DW basis for each leaf sample (0.5 mg). The position of the homohexameric forms of GDH from *N. plumbaginifolia* expressed in the transgenic lines of *N. tabacum* is indicated by the symbols  $\alpha$  and  $\beta$ . (F) GDH, (G) GS and (H) Fd-GOGAT protein contents of WT and transgenic plants. Leaf protein extracts were subjected to protein gel blot analysis using an antiserum raised against GDH from grapevine (Loulakakis and Roubelakis-Angelakis 1990), GS (Becker et al. 1992) and Fd-GOGAT from tobacco (Zehnacker et al. 1992). GDH and Fd-GOGAT proteins are represented by single polypeptides of 43 and 164 kDa respectively, whereas GS is represented by the plastidic GS2 isoenzyme (subunit mol. wt 45 kDa) and the cytosolic GS1 isoenzyme (subunit mol. wt 41 kDa).

activity is usually at least 2- to 3-fold in many plant species (Roubelakis-Angelakis and Kliewer 1992). However, the physiological significance of the ratio needs to be treated with caution, since the presence of other enzymes using NAD as a cofactor can interfere with the measurement of deaminating NAD-GDH activity (Fricke and Pahlich 1992, Loulakakis and Roubelakis-Angelakis 1996, Skopelitis et al. 2007). Total leaf GS (Fig. 2B), Fd-GOGAT (Fig. 2C) activities and protein content (Fig. 2G and H, respectively) were not significantly different between the WT and the three *GDH* overexpressors. GS was mostly represented by the plastidic form of the enzyme (GS2), its cytosolic counterpart (GS1) being also expressed at a similar level both in the *GDH* overexpressors and in the WT. In contrast, we observed a 2- to 3-fold increase in NADH-GOGAT activity (Fig. 2D). We were not able to detect the corresponding protein using antibodies raised against the protein from rice (Hayakawa et al. 1994).

To follow the fate of  $^{15}\text{NH}_4^+$  or  $^{15}\text{N}$ Glu in vivo under real time conditions, NMR spectroscopy experiments were then conducted using leaf fragments from the three types of *GDH* overexpressor, namely *GDHA*, *GDHB* and *GDHA/B*.

When  $^{15}\text{NH}_4^+$  was provided to the leaves of lines A1, A2 (*GDHA* overexpressors), B1, B2 (*GDHB* overexpressors), A2b1 and B1a2 (*GDHA/B* overexpressors) in the presence of MSX, no labeling was observed in the amino group of Glu indicating that, even when GDH activity was increased, the enzyme was not able to aminate 2-oxoglutarate (Supplementary Fig. 1B). In addition, this experiment also showed that neither the  $\alpha$  or  $\beta$  homohexameric nor the heterohexameric (combination of  $\alpha$  and  $\beta$  subunits) forms of the enzyme were able to incorporate ammonium into Glu, which would appear as a peak of labeled  $\alpha$ -Glx. GDH activity was measured in the leaf samples taken from the NMR tube over the  $^{15}\text{N}$ -labeling period. The enzyme activity remained rather stable both in the WT and the *GDH* overexpressors with a slight increase over the first 12 h of the experiment, probably due to a response of GDH activity to mild stress conditions resulting from the compaction of the plant material in the NMR tube, indicating that the leaf tissues were still in rather good physiological condition after 24 h (Supplementary Fig. 3).

When  $^{15}\text{N}$ Glu was added to the perfusion medium, in the three *GDH* overexpressors the time course of the peak integrals derived from a series of spectra (similar to those presented in Supplementary Fig. 1A for the untransformed plants) are shown in Fig. 3A. In the *GDHA* (lines A1, A2), *GDHB* (lines B1, B2) and the double *GDHA/B* (lines A2b1 and B1a2) overexpressors the initial rate of  $\alpha$ -Glx ( $\alpha$ -Glu +  $\alpha$ -Gln) labeling was similar to that of the untransformed WT leaves. However, after 7 h, the accumulation of label in  $\alpha$ - $^{15}\text{N}$ Glx ( $\alpha$ -Glu +  $\alpha$ -Gln) was much lower in the leaves of *GDH* overexpressors with a slight decrease after 12 h indicating that higher amounts of  $\alpha$ -Glu were deaminated compared with

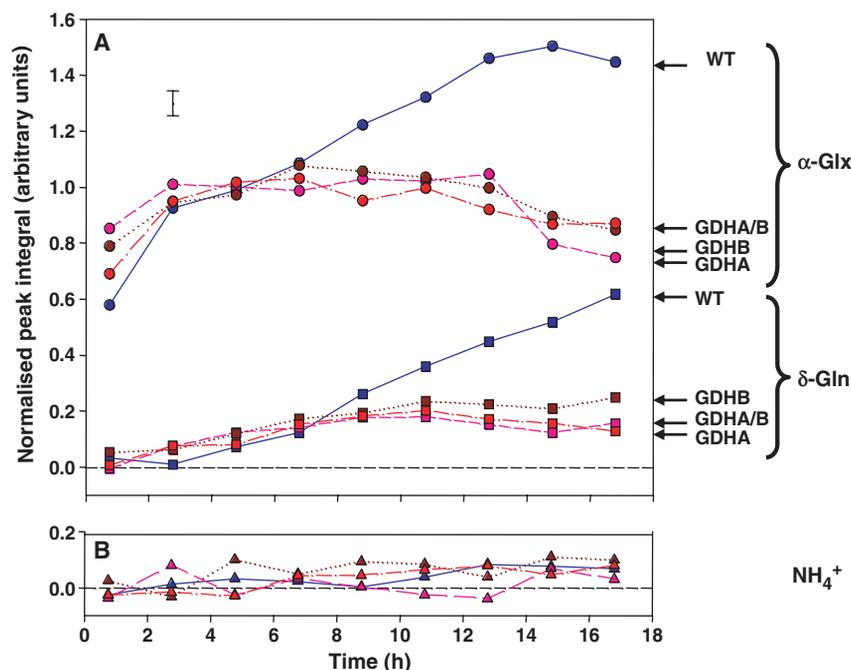
the WT as the result of enhanced enzyme activity. This finding clearly shows that Glu deamination was catalyzed by GDH and that neither other enzymes nor our experimental conditions are causing Glu degradation.

The accumulation profile of  $\delta$ - $^{15}\text{N}$ Gln (representing the  $\delta$ -amido group from Gln) over the time course of the experiment was similar to that of  $\alpha$ - $^{15}\text{N}$ Glx (Fig. 3A). Since  $\alpha$ - $^{15}\text{N}$ Glu deamination was enhanced in the *GDH* overexpressors, less  $\alpha$ - $^{15}\text{N}$ Glu was thus available for the synthesis of  $\delta$ - $^{15}\text{N}$ Gln, explaining that the flux of  $^{15}\text{N}$  going first through the reaction catalyzed by GS and further on through that catalyzed by GOGAT was also reduced. The results presented in Fig. 3D suggest that more Glu is consumed by the leaf cells as the result of increased NADH-GOGAT activity. In contrast, Fd-GOGAT and GS activities remain at a similar level in the *GDH* overexpressors compared with the WT, indicating that both enzymes are probably not involved in the reduction of Glu accumulation. Moreover, in the *GDHA*, *GDHB*, *GDHA/B* overexpressors we observed that in the same leaf samples as those used for the NMR spectroscopy experiment, there was also a decrease (30%) in the free Gln content (Supplementary Fig. 4), thus confirming the results obtained from the NMR spectroscopy experiment. This finding confirms that NMR spectroscopy is a reliable technique even when the leaf samples had been placed in a tube and kept in the dark for several hours (Mesnard and Ratcliffe 2005). Some authors used an optical fibre inserted in the NMR magnet (Ratcliffe et al. 2001, Kikuchi et al. 2004) to study metabolic dynamics in illuminated plant tissues. It may be interesting to perform additional experiments using such an optical fibre, although due to the compaction of the plant tissues in the NMR tube obtaining enough sensitivity and light intensity may limit the feasibility of such experiments.

Both in WT plants and in the *GDH* overexpressors  $^{15}\text{NH}_4^+$  remained at a very low level (Fig. 3B), since it was reassimilated by the GS enzyme that was not in this experiment inhibited by the addition of MSX as in the experiment described in Fig. 1.

By estimating the difference between the accumulated  $\alpha$ - $^{15}\text{N}$ Glx and the  $\delta$ - $^{15}\text{N}$ Gln it is possible to deduce that the level of  $\alpha$ - $^{15}\text{N}$ Glu remains practically constant both in WT and all three types of *GDH* overexpressor throughout the experiment. Although it could be argued that this may be due to the fact that large amounts of labeled Glu are provided from the perfusion medium, the finding that the amount of free Glu in leaf extracts of the three types of *GDH* overexpressor are very similar (Supplementary Fig. 4), suggests that there is both long- and short-term metabolic control of Glu homeostasis.

The originality of our strategy to investigate the function of the different GDH subunits in vivo was to overexpress separately the two *GDHA* and *GDHB* gene products from the



**Fig. 3** Kinetics of Glu incorporation in young leaf of transgenic plants overexpressing the two genes encoding GDH in comparison with WT plants. (A) NMR kinetics of  $^{15}\text{N}$ -label incorporation into the  $\alpha$  position of Glu and Gln ( $\alpha$ -Glx, circles), and into the  $\delta$  position of glutamine ( $\delta$ -Gln, squares). (B) NMR kinetics of  $^{15}\text{N}$  label into ammonium (triangles) after the addition at time zero of 7.0 mM [ $^{15}\text{N}$ ]Glu to the perfusion medium: WT (blue, solid lines) and GDH overexpressors (GDHA: pink, dashed lines; GDHB: dark red, dotted lines; GDHA/B: red, dash-dotted lines). The results presented in (A) and (B) are on the same scale and are presented on separate figures for clarity. All data except for GDHB are the mean of two independent experiments. The mean error is indicated in the upper left corner.

same species (*N. plumbaginifolia*). Each of the resulting overexpressed subunits was capable of forming an active homohexameric enzyme in another species from the same genus (*N. tabacum*). Moreover, we found that when expressed simultaneously, the two GDH subunits were assembled into an active heterohexameric enzyme. This approach allowed us to demonstrate that both the homohexameric enzyme (composed of either of the two subunits) or the heterohexameric enzyme deaminates Glu in vivo at a higher rate when overexpressed in transgenic plants and that the flux of N going through this reaction under real time in vivo is very similar whatever the GDH isoenzyme composition in the three types of GDH overexpressor. However, since the flux of  $^{15}\text{N}$  going through the reaction catalyzed by GDH in the transgenic plants is the same as that of the WT for the first 7 h, it is likely that overexpressing the enzyme does not enhance Glu deamination during this period, probably due to a lag phase corresponding to the incorporation of the  $^{15}\text{N}$ -labeled Glu into the leaf cells. In a previous study (Purnell and Botella 2007), tobacco plants overexpressing only one of the two GDH genes from tomato encoding isoenzyme 1 (corresponding to GDHB in the present study) were produced. In these plants,  $^{15}\text{N}$  labeling was performed on intact roots and  $^{15}\text{N}$ -NMR analysis of the root extracts

indicated that at least in this organ, GDHB deaminates Glu. In another work, Skopelitis et al. (2007) showed that when the GDH gene encoding isoenzyme 7 from *Vitis vinifera* (corresponding to GDHA in the present study) was overexpressed in tobacco leaves, the corresponding enzyme strongly deaminated Glu in vivo. However, these authors also showed that under standard growth conditions, GDHA exhibited very low aminating activity in vivo when compared with deaminating activity. In the present study, this low aminating activity for the GDHA isoenzyme was not detected (Supplementary Fig. 1B), even though the increase in total GDH enzyme activity obtained was similar to that reported by Skopelitis et al. (2007). Whether this is due to the sensitivity of the in vivo NMR technique used in the present study or to the fact that GDH genes originating from different plant species were used for overexpression remains to be elucidated.

Evidence that GDH can assimilate ammonium at a very low rate has been presented in a limited number of studies either using isolated mitochondria (Yamaya and Oaks 1987, Yamaya et al. 1987) or intact tissues (Skopelitis et al. 2007). This may have been due to the fact that as  $\text{NH}_4^+$  was provided in excess, the equilibrium of the enzymatic reaction would have been shifted towards the synthesis of

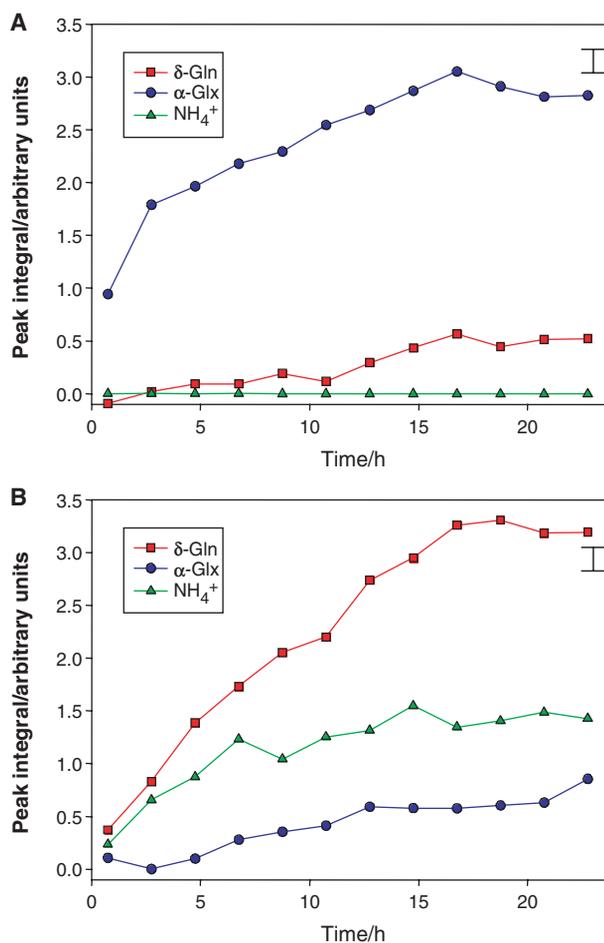
Glu (Pahlich 1996). The same situation occurs when measuring the *in vitro* aminating activity of GDH using 50 mM  $\text{NH}_4^+$  (Skopelitis et al. 2007). It could be argued that the amount of  $\text{NH}_4^+$  released in the mitochondria during photorespiration would provide a concentration sufficient to allow the synthesis of Glu in the organelle; however, Keys et al. (1977) clearly demonstrated that GDH was unable to operate in this direction at least in photosynthetically active cells.

### Occurrence of a futile cycle GDH–GS/GOGAT

To investigate further the physiological function of GDH, taking into account (i) that the enzyme does not assimilate ammonium even when it is overexpressed, and (ii) that the level of Glu remains constant even when Glu deamination is increased in the *GDH* overexpressors, additional experimentation was conducted on untransformed WT tobacco leaves.

In a first experiment, [ $^{15}\text{N}$ ]Glu and non-labeled  $\text{NH}_4^+$  were simultaneously applied to the perfusion medium in the presence of aminooxyacetate (AOA) a specific inhibitor of transaminase reactions (Brunk and Rhodes 1988). This inhibitor was used to prevent Glu utilization for the synthesis of derived amino acids and thus only measure the accumulation of  $^{15}\text{N}$ -labeled metabolites going through the GS/GOGAT cycle and the catabolism of Glu catalyzed by GDH. We observed over a time course experiment that there was no accumulation of  $^{15}\text{NH}_4^+$ . Following deamination of [ $^{15}\text{N}$ ]Glu accumulation of  $^{15}\text{N}$  in the  $\delta$ -amido group of Gln originating from [ $^{15}\text{N}$ ]Glu was visible (Fig. 4A). Therefore, this experiment confirms that [ $^{15}\text{N}$ ]Glu is deaminated through the reaction catalyzed by GDH releasing  $^{15}\text{NH}_4^+$ . The resulting  $^{15}\text{NH}_4^+$  was entirely reassimilated by the GS enzyme to form Gln, even if the flux of  $^{15}\text{N}$ -labeled  $\text{NH}_4^+$  released from Glu deamination was diluted by the addition of unlabeled  $\text{NH}_4^+$ .

In a second experiment, we applied  $^{15}\text{NH}_4^+$  together with unlabeled Glu in the presence of AOA. Fig. 4B shows that the  $^{15}\text{NH}_4^+$  is rapidly incorporated first into the  $\delta$ -amido group of Gln and then into the  $\alpha$ -amino groups of  $\alpha$ -Glx through the GS/GOGAT cycle, even when unlabeled Glu was added to the perfusion medium. If we consider the original slope of the curves, one can see that the flux of reduced N going through the reaction catalyzed by GS ( $\delta$ -Gln accumulation of around 3 in 12 h) is about six times higher compared with that going through the reaction catalyzed by GOGAT ( $\alpha$ -Glx accumulation of around 0.5 in 12 h). This result indicates that there is a lower flux of reduced N passing through the reaction catalyzed by GOGAT when Glu and  $\text{NH}_4^+$  are present in the perfusion medium in a range of concentrations close to that circulating in the apoplast for ammonium or measured in leaf cells for Glu (Winter et al. 1994, Husted and Schjoerring 1995). Since leaf tissues were maintained for several hours in the dark, it probably explains why GOGAT activity was lower compared with that of



**Fig. 4** Occurrence of a futile cycle GDH–GS/GOGAT.  $^{15}\text{N}$ -NMR kinetics of young tobacco leaves from WT plants perfused with (A) 7.0 mM [ $^{15}\text{N}$ ]Glu and 3.5 mM  $\text{NH}_4\text{Cl}$  and (B) 7.0 mM Glu and 3.5 mM  $^{15}\text{NH}_4\text{Cl}$ . The inhibitor of transaminase AOA was added to the perfusion medium at a concentration of 1 mM. All data are the mean of two independent experiments in which three individual plants were used. The mean error is indicated in the upper right corner.

GS (Masclaux-Daubresse et al. 2006). Despite this, one can observe that unlike [ $^{15}\text{N}$ ]Gln, both  $^{15}\text{NH}_4^+$  and [ $^{15}\text{N}$ ]Glx remain at a fairly constant level after 12 h. This observation indicates that the rate of Glu catabolism is very similar to its rate of synthesis, the former being controlled by GDH and the latter by GOGAT. At the same time the flux of reduced N is mainly going through the reaction catalyzed by GS to synthesize Gln thus avoiding a build-up of ammonium.

From these two sets of experiments, one can deduce that as expected a larger part of the ammonium is channeled through the reaction catalyzed by GS, whether it is released when Glu is deaminated by GDH ([ $^{15}\text{N}$ ]Glu deamination experiment) or coming from an external source (mimicked by the application of  $^{15}\text{NH}_4^+$ ). In turn, one can hypothesize

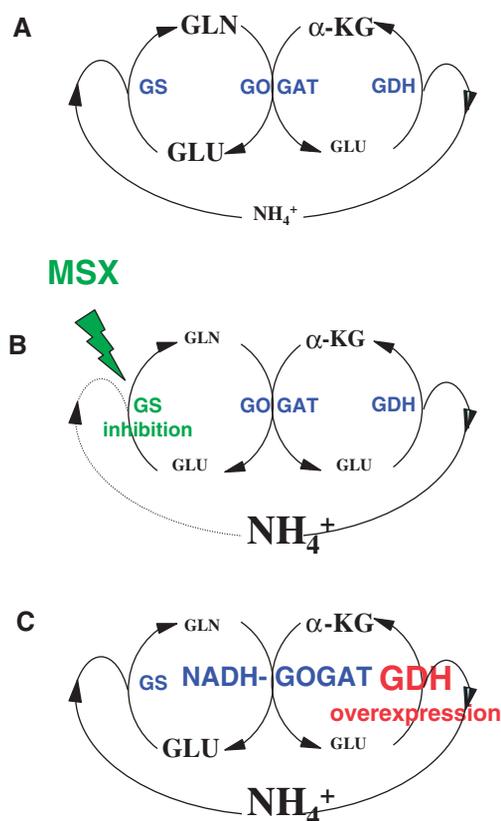
that the concerted action of the GS/GOGAT cycle and of GDH through a futile cycle recycling both ammonium and Glu, controls the steady state level of both molecules thus maintaining at the same time the homeostasis of Glu and avoiding the accumulation of ammonium. In this context, a putative metabolic pathway showing the recycling of  $\text{NH}_4^+$  between the GS/GOGAT cycle and the deamination of Glu catalyzed by GDH under standard physiological conditions is presented in **Fig. 5A**. When this metabolic pathway is perturbed, for example, in the presence of MSX, which inhibits GS activity thus lowering Glu synthesis, a build-up of ammonium occurs following the deamination of Glu by GDH, which cannot be reassimilated by GS. Concomitantly a decrease in the N flux going through Glu and Gln is observed as a result of the shortage in Gln and the deamination of Glu by GDH (**Fig. 5B**). If we artificially increase Glu deamination by overexpressing GDH, we observed that the

pool of Glu remains practically constant (**Fig. 5C**). The GS enzyme should normally reassimilate the  $\text{NH}_4^+$  released by the deamination of Glu. However, the decrease in the flux of reduced N going through Gln suggests that a feedback regulatory mechanism presumably operating through the regulation of GOGAT activity controls N recycling to maintain a steady level of Glu. Our results suggest that this regulation occurs through the increase in NADH-GOGAT activity in the GDH overexpressors and not via an increase in Fd-GOGAT, which remains at a similar level compared with the WT. There are several arguments in the literature in favour of the presence of mechanisms controlling the homeostasis of Glu. It has been observed in several cases that, whatever the plant developmental stage or the growth conditions, the variations in Glu content are relatively minor in comparison with most of the other amino acids (Forde and Lea 2007). Due to its cellular localization restricted to the phloem, GDH may well be one of the key checkpoints controlling the C/N status of the plant, as suggested in one of our previous studies (Tercé-Laforgue et al. 2004a), Glu being one of the key metabolites involved in this control.

### Conclusion

Some authors (Mesnard and Ratcliffe 2005) recommended performing *in vivo* NMR experiments using intact tissues to avoid possible artefacts due to the use of extraction procedures that may lead to misinterpretation of the  $^{15}\text{N}$ -labeling data. Moreover, NMR studies provide a time-dependent dynamic picture of a metabolic pathway. These *in vivo* NMR experiments have been carried out in the present study, thus demonstrating that the major role of the GDH enzyme is to deaminate Glu *in vivo*. It could be argued that performing experiments with leaf samples placed in an NMR tube for several hours in the dark could lead to a misinterpretation of results. It is, however, unlikely, since we showed that the GS/GOGAT cycle was active under our experimental conditions and that the metabolic NMR profile did not reveal any particular stress conditions in terms of either metabolic fluxes or accumulation of molecules such as GABA and Ala. Although it could be argued that NMR experiments only provide relative values for metabolite concentrations compared with standard analytical techniques, they give a dynamic picture of metabolic fluxes occurring within intact tissues and even within different compartments of a living cell (Kikuchi et al. 2004, Mesnard and Ratcliffe 2005).

Moreover, the fact that GDH is confined to the mitochondria of the phloem companion cells, which are not photosynthetically active needs to be considered for a proper interpretation of the physiological role of the enzyme (Tercé-Laforgue et al. 2004b), taking into account that the GS/GOGAT cycle also operates in the phloem and thus does not directly require light for functioning (Masclaux-Daubresse et al. 2006). The finding that



**Fig. 5** Scheme of ammonium recycling in tobacco leaves showing the occurrence of a futile cycle implying the GS/GOGAT cycle and the deamination of Glu by GDH. (A) Under standard physiological conditions in leaves of WT plants. (B) In the presence of the inhibitor of GS, MSX in leaves of WT plants. (C) Under standard physiological conditions in leaves of GDH overexpressors. The font size of the characters for an enzyme activity or a metabolite is proportional to its activity or its concentration, respectively.

NADH-GOGAT, another enzyme localized in the vascular bundles (Hayakawa et al. 1994), is enhanced in response to *GDH* overexpression also strengthens the hypothesis that the phloem cells are directly involved in the control of Glu and Gln synthesis and trafficking.

Our hypothesis is that *GDH* (in conjunction with NADH-GOGAT) is involved in controlling the homeostasis of Glu, which is a central molecule for N transport throughout the plant (Glu represents up to 25% of the total amino acids in young tobacco leaves and up to 10% in the phloem sap, see Tercé-Laforgue et al. 2004b) and a signaling molecule in a variety of metabolic and developmental processes (Forde and Lea 2007). This hypothesis is further supported by previous findings from studies performed to better understand the N economy of plants using tobacco as a model. (i) When plants were grown on a medium containing  $\text{NH}_4^+$  as sole N source, the concentration of Glu remained similar to that detected in plants grown on  $\text{NO}_3^-$ , whereas the level of Gln was dramatically increased (Tercé-Laforgue et al. 2004b). At the same time strong induction of *GDH* was observed (Loulakakis and Roubelakis-Angelakis 1992, Tercé-Laforgue et al. 2004b, Skopelitis et al. 2006), probably to maintain a steady state level of Glu originating from the *GS/GOGAT* cycle through the *GDH* deaminating reaction. The ammonium released from Glu deamination is reassimilated by *GS* and contributes to the build-up of Gln, which is used as an N storage compound and as an amino donor for the synthesis of other amino acids such as Asn (Lea et al. 2007). (ii) Using transgenic plants impaired in *GS* activity in the phloem, we found using  $^{15}\text{N}$  labeling that one of the main providers of amino groups for Pro synthesis is Gln and not Glu, which stays relatively constant, thus indicating that the rate of Glu metabolism is very similar to its rate of synthesis (Brugière et al. 1999). This finding further supports the hypothesis that *GDH*, acting in concert with *GS* and *GOGAT*, two enzymes that are also present in the phloem (Masclaux-Daubresse et al. 2006), is directly involved in the control of Glu homeostasis at least in the vascular tissue.

## Materials and Methods

### Plant material and growth

Tobacco (*N. tabacum*, cv. Xanthi XHFD8; INRA, Versailles, France) was grown on coarse (diameter 1–2.5 mm) sand (Bellanger-Sopromat, Courbevoie, France) throughout plant development. From the bottom of the seedlings, each emerging leaf was numbered and tagged. From a batch of 6-week-old plants, 12 plants of uniform development and numbering seven leaves were selected. Plants were grown in a controlled environment growth chamber (16 h light, 350–400  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ , 26°C; 8 h dark, 18°C) and watered with a complete solution (10 mM  $\text{NO}_3^-$  and 2 mM  $\text{NH}_4^+$ ; Coïc and Lesaint 1971). Plants were automatically

watered for 1 min (flow rate for each plant: 50 ml.min<sup>-1</sup>) every 2 h. Three plants were used for each experiment. Four weeks later, leaves were numbered 8, 9, 11, 13, 15, 20, 30 (from bottom to top) as previously described by Tercé-Laforgue et al. (2004b). Leaf 20, corresponding to a fully expanded photosynthetically active leaf (photosynthetic activity of 5.8 mmol.m<sup>-2</sup>.s<sup>-1</sup>), was used for both physiological and NMR analysis. From this leaf, 1 cm×3 cm fragments including major and minor veins were randomly collected and pooled in two groups. One was used to perform NMR analysis and the other weighed, frozen and used to perform amino acid analysis. All the harvesting of fresh material was carried out between 10 am and 2 pm.

### Production of tobacco plants overexpressing *GDHA* and *GDHB*

DNA fragments containing the *GDHA* (*NpGDHA*) cDNA and the *GDHB* (*NpGDHB*) cDNA from *N. plumbaginifolia* (Restivo 2004) were subcloned in the sense orientation into the binary vector pBI121 to obtain *35S-NpGDHA* and *35S-NpGDHB* construct using the procedure previously described by Fontaine et al. (2006). Seeds from treated plants were collected and screened for kanamycin resistance and transgenic plants identified in this generation were classified as T1 plants. Homozygous T3 progeny was then selected for further biochemical and NMR studies following two rounds of self-pollination and selection on kanamycin. For each construct two independent transformants exhibiting the highest *GDH* activity were selected. They were named *GDHA* overexpressors (line A1 and line A2), and *GDHB* overexpressors (line B1 and line B2), respectively. Double transformants were obtained by crossing a *GDHA* overexpressing plant with a *GDHB* overexpressing plant using each transgenic line either as a female receptor (line A2 and line B1) or as a male for pollen donor (line a2 and line b1) to obtain two independent lines of double transformants called *GDHA/B* overexpressors (line A2b1 and line B1a2).

### Protein extraction, enzymatic assays and protein gel blot analysis

Protein was extracted from frozen leaf material stored at –80°C. All extractions were performed at 4°C. NAD(H)-dependent *GDH* was measured in both directions as described by Turano et al. (1996) except that the extraction buffer was that used by Tercé-Laforgue et al. (2004b). *GS* activity was measured according to the method of O'Neal and Joy (1973). Fd- and NADH-GOGAT were assayed by measuring Glu formation by HPLC as described in Suzuki et al. (2001). Soluble protein was determined using a commercially available kit (Coomassie Protein assay reagent; Bio-Rad, München, Germany) using bovine serum albumin as a standard. Visualization of NAD-dependent *GDH* activity following native PAGE was performed as described by

Restivo (2004). For gel blot analysis of the GDH proteins, frozen leaf material was extracted in cold buffer containing 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM MgCl<sub>2</sub>, PVP 0.5% (w/v), 2-mercaptoethanol 0.1% (v/v) and leupeptine 4 μM, and soluble proteins separated by SDS–PAGE (Laemmli 1970). The amount of protein loaded in each track was calculated on a similar DW basis for each leaf tissue sample. Polypeptide detection was carried out using polyclonal anti-serum raised against grapevine leaf GDH (Loulakakis and Roubelakis-Angelakis 1990), tobacco leaf GS (Becker et al. 1992) and Fd-GOGAT (Zehnacker et al. 1992).

### Amino acid extraction and analyses

Frozen leaf material at –80°C was used for metabolite extraction. Free amino acids were extracted with 2% (w/v) 5-sulfosalicylic acid (1 mL per 10 mg DW) as described by Ferrario-Méry et al. (1998) and their concentration determined by the Rosen colorimetric method (Rosen 1957). The composition of individual amino acids was determined by ion-exchange chromatography on replicates of the plant samples extracted from an equal DW (Rochat and Boutin 1989) using an Amino Tac JLC-500/V amino acid analyzer (JEOL, Croissy sur Seine, France).

### NMR spectroscopy experiments

For the NMR experiments, a fully expanded leaf from individual WT or *GDH* overexpressing lines (leaf 20, see section Plant material and growth) was excised between 10 am and 2 pm, carefully washed with distilled water and cut into 0.5 cm × 0.5 cm squares to obtain approximately 2 g FW. Leaf fragments were then packed as densely as possible in an 10 mm screw cap NMR tube and fitted with a perfusion system (Ratcliffe et al. 2001) derived from those described by Labboun and Roscher (2005) and Troufflard et al. (2007). First the leaf tissue was rinsed with approximately 50 mL of perfusion medium consisting of 0.1 mM CaSO<sub>4</sub>, 50 mM sucrose, 10 mM MES adjusted to pH 6.5 with KOH. For the NMR spectroscopy experiment, 60 mL of perfusion medium (bubbled with pre-humidified oxygen in an outside reservoir maintained at room temperature of around 21°C) was recirculated through the tissue in the NMR tube with a peristaltic pump at a flow rate of 10 mL·min<sup>-1</sup> to maintain the tissues under good physiological conditions (Roby et al. 1987; Ratcliffe and Shachar-Hill 2001). The nitrogen source (glutamic acid and/or NH<sub>4</sub>Cl) was added two hours after the start of perfusion to allow for tissue equilibration and recording of reference spectra. Inhibitors of enzyme activities such as MSX and AOA were added directly into the medium reservoir at times indicated in the figures or the figure legends to obtain a final concentration of 1 mM. NMR spectra were recorded with a Bruker Avance 300 (Bruker BioSpin SA, Wissembourg, France) wide bore spectrometer using an 10 mm broadband probe. The magnet was stable enough to

be used in unlocked mode, and shimming was performed on the <sup>1</sup>H water resonance. <sup>15</sup>N-spectra (30.41 MHz) were recorded in 30 min blocks and summed later. Spectral width was 501 ppm, and each free induction decay (FID) contained 32k data points. Spectra were <sup>1</sup>H decoupled with the WALTZ16 sequence. Two acquisition conditions were used alternately: first, a fast acquisition mode with a 90° pulse (22.0 μs) every 2.0 s and low power <sup>1</sup>H decoupling during the relaxation delay (power-gated) using 900 scans for a 30 min spectrum; secondly, a slower acquisition mode optimized for the detection of <sup>15</sup>NH<sub>4</sub> (Fox et al 1992) consisting of a 70° pulse every 10 s without <sup>1</sup>H decoupling during the relaxation delay (inverse-gated) using 180 scans for a 30 min spectrum.

For data treatment, spectra were summed independently for the two acquisition modes and Fourier-transformed into 64k data points with 5 Hz line broadening. Spectra were referenced to the NH<sub>4</sub> peak at –354.70 ppm or the α-Glx peak at –334.75 ppm. The signals were integrated manually after baseline correction always using the same integration limits.

### Statistics

For measurement of enzyme activities and metabolite analysis, results are presented as mean values for three plants for each WT and transgenic line analyzed with standard errors (SE = SD/√*n*–1, where SD is the standard deviation and *n* the number of replicates).

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