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Céline Masclaux-daubresse, Marie-Hélène Valadier, Elisa Carrayol, Michèle Reisdorf-Cren, Bertrand Hirel. Diurnal changes in the expression of glutamate dehydrogenase and nitrate reductase are involved in the C/N balance of tobacco source leaves. *Plant, Cell and Environment*, 2002. hal-04495760

HAL Id: hal-04495760

<https://hal.science/hal-04495760>

Submitted on 19 Mar 2024

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Diurnal changes in the expression of glutamate dehydrogenase and nitrate reductase are involved in the C/N balance of tobacco source leaves

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ABSTRACT

A novel cDNA encoding glutamate dehydrogenase (GDH) from tobacco (*Nicotiana tabacum*), named *gdh1*, was characterized. The *gdh1* mRNA was detected in roots, stems and source/senescent leaves. In order to investigate diurnal regulation of *gdh1* in leaves, the content in *gdh1* mRNA was measured every 3 h over a 48 h period and compared to *nia* and *gs2* mRNA levels, encoding, respectively, nitrate reductase (NR) and chloroplastic glutamine synthetase (GS2). In source leaves, *gdh1* mRNA levels exhibit diurnal fluctuations. A 12 h shift was observed between the day–night rhythms of *gdh1* and *nia* expression. Metabolite contents were also measured and a shift in the day–night fluctuations of both glutamate (GLU) and γ -aminobutyric acid (GABA) was observed between sink and source leaves, whereas the diurnal rhythm of α -ketoglutarate showed no change. A possible role of GDH in the shift of GLU and GABA contents is discussed. Leaf disc experiments showed that *gdh1* expression is enhanced in conditions of continuous darkness. This trend is inhibited by sucrose feeding. The opposite was observed for *nia* expression. An important outcome of this work is the reverse regulation of *gdh1* and *nia* genes. A possible role of sugars and amino acids in the co-regulation of *gdh1* and *nia* genes is suggested.

Key-words: C/N balance; diurnal rhythm; glutamate dehydrogenase; nitrate reductase; senescence; sink/source relationships; sugar regulation.

INTRODUCTION

At first, glutamate dehydrogenase (GDH, EC 1.4.1.2) was considered as the key enzyme for ammonia assimilation in plants (Sims & Folkes 1964). Since the discovery of glutamate synthase (GOGAT), the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway has been shown to be the major process for ammonia assimilation

into amino acids (Mifflin & Lea 1980). Therefore, the role of GDH in plant metabolism is still a matter of debate (Ireland & Lea 1999 for review). Because of its low affinity for ammonia, it has been suggested that GDH was a catabolic enzyme involved in the deamination of glutamate (Pahlich 1996). Moreover, GDH has been identified in mitochondria and a high range of ammonia concentrations has been found in this organelle (5–10 mM) (Ireland & Lea 1999). From this it was proposed that GDH could synthesize glutamate in mitochondria (Givan 1979; Wallsgrove *et al.* 1983). Due to its connection with carbon and nitrogen metabolism, a possible role of GDH as an adaptive enzyme whose action depends on the carbon skeleton or glutamate requirements of the cell, has been proposed (Ireland & Lea 1999; Stitt *et al.* 2002).

GDH can be a homo- or hetero-hexameric protein complex. The genes that encode the different subunits are differentially expressed according to plant organs and tissues, as well as C/N balance (Cammaerts & Jacobs 1985; Ireland & Lea 1999; Morkunas *et al.* 2000). Therefore, the content in GDH isoenzymic forms is largely modulated and this might be one integrative aspect of GDH activity regulation (Cammaerts & Jacobs 1985; Loulakakis & Roubelakis-Angelakis 1996).

To date, the regulation of GDH expression has been described mainly through C/N feeding experiments (Tassi *et al.* 1984; Athwal, Pearson & Laurie 1997). In *Arabidopsis*, GDH activity and *gdh* genes are differentially regulated according to the supply of N or C metabolites (Cammaerts & Jacobs 1985; Melo-Olivera, Olivera & Coruzzi 1996; Turano *et al.* 1997). In several plant species, sugar feeding appeared to be a signal inhibiting *gdh* expression and GDH activity (Laurière & Daussant 1983; Tassi *et al.* 1984; Maestri *et al.* 1991; Melo-Olivera *et al.* 1996) whereas the addition of ammonia stimulated both. Studies of GDH regulation during plant development have indicated an age-related increase in leaf GDH activity (Thomas 1978; Laurière & Daussant 1983; Kar & Feierabend 1984; Ireland & Lea 1999 for a review). The age-related induction of GDH in a range of plant tissues was explained either by an increase in ammonia content or by the depletion of carbo-

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hydrates that are usually observed during senescence (Srivastava & Singh 1987). Indeed, natural senescence can be mimicked by dark adaptation or sugar starvation. Some studies have shown dark-induced *-gdh* expression and *-GDH* activities (Peeters & Van Laere 1992; Melo-Oliveira *et al.* 1996; Turano *et al.* 1997). Therefore, it is likely that in some plants *gdh* behaves as a dark-induced gene (Fujiki *et al.* 2000a)

Recently, we have shown that in tobacco leaves, *gdh* mRNA can be detected exclusively in source leaves where it accumulated during ageing (Masclaux *et al.* 2000). Therefore, source leaves of tobacco appeared to be a good model to study *gdh* age-related regulation. In this study, we observed that, in source leaves, GDH activity increased and *gdh* mRNA accumulated, whereas sucrose content decreased when ammonia accumulated (Masclaux *et al.* 2000). Our interpretation, with respect to the physiological aspects, was coherent with knowledge from previous works. However, our picture was static and relative to events taking place at 1100 h, the time when the plant material was harvested.

Diurnal changes in metabolite contents such as sugars or ammonia are well-known (Scheible, Krapp & Stitt 2000) and many enzymes involved in carbohydrate and nitrogen metabolism also exhibit diurnal rhythms. The influence of metabolites as signals that can modulate the diurnal fluctuation of the expression of certain genes has been largely debated (Cheng *et al.* 1992; Matt *et al.* 1998; Klein *et al.* 2000; Scheible *et al.* 2000; Matt *et al.* 2001; Lillo, Meyer & Ruoff 2001 for a review). If we assume that GDH plays a role in and is influenced by the C/N balance, then potential *gdh* day–night oscillations could be important. In the present study, we report the isolation and the characterization of a novel cDNA encoding GDH from tobacco (*Nicotiana tabacum*) and named *gdh1*. The *gdh1* mRNA was detected in roots, stems and source leaves. In source leaves, *gdh* expression followed a diurnal rhythm. Moreover, mRNA content was enhanced by continuous darkness and inhibited by sucrose feeding. An important outcome of this work is the opposite regulation of *gdh1* and nitrate reductase (*nia*) genes. Results are discussed in relation of nitrogen- and carbon-management that occur during leaf ageing and day–night transitions.

MATERIALS AND METHODS

Plant material and growth

Tobacco (*Nicotiana tabacum*, cv. Xanthi XHFD8; INRA, Versailles, France) was grown on a clay loam soil during 4 months until flowering.

For diurnal rhythm experiments, each emerging leaf was numbered and tagged from the base of the seedling. Nine plants of uniform development and numbering, were divided into three batches (A, B and C) and transferred to a controlled environment growth chamber (16 h photoperiod, 350/400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, day/night temperature 26/18 °C). Plants were automatically watered with N12

(10 mM NO_3^- and 2 mM NH_4^+) (Coic & Lesaint 1971) for 1 min (flow rate for each plant: 50 mL min^{-1}) every 2 h. When plants were flowering, they had grown up to about 45 leaves which were numbered. Leaf disc tissues (1 cm^2) were randomly collected avoiding the veins, from source leaves (numbered 13, 14 and 15) and sink leaves (numbered 30, 31 and 32) (see Masclaux *et al.* 2000). Leaf discs were pooled on the three-plant batch basis (as described as above) to give three batches of source-leaf tissue and three batches of sink-leaf tissue, respectively. Leaf discs were collected over a 48 h period, at each designated sampling time (i.e. 1200, 1500, 1800, 2200, 2400, 0300, 0600, 0700 and 0900 h). Leaf tissue was frozen in liquid nitrogen and immediately reduced to a homogeneous powder that was stored at -80°C until use in all further experiments.

For sucrose feeding and dark treatment experiments, leaf discs (1 cm^2) were collected, avoiding the veins, from 12 tobacco plants, 3 h after the beginning of the light period. The discs were rinsed in distilled water and floated on 20 mL of buffer (10 mM MES pH 6.5, 40 mM KCl and 10 mM CaCl_2) in Petri dishes. The discs were incubated for 5 h in the growth chamber (23 °C), with or without sucrose (50 mM) in dark or light (150 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) condition. In each floating solution, molality was maintained at 280 mmol kg^{-1} by the addition of polyethylene glycol (PEG, 200 AMW). The leaf discs were collected and frozen in liquid nitrogen before storing at -80°C . The viability of the leaf discs was checked as discussed previously (see Ferrario-Mery *et al.* 2001).

Chlorophyll, phaeophytin and total protein determinations

The chlorophyll and phaeophytin content was estimated on crude leaf extracts (Arnon 1949; Vernon 1960). Soluble protein was determined using a commercially available kit (Coomassie Protein assay reagent, Bio-Rad, Hercules, CA, USA).

Metabolite extraction and analysis

NO_3^- , amino acids and carbohydrates were determined after ethanolic extraction of frozen leaf disc powder (50 mg fresh weight (FW) mL^{-1} of 80% (v/v) ethanol in water for 2 h at 4 °C). The extract was centrifuged at 12 000 $\times g$ for 5 min. The pellet, kept for the determination of starch, was resuspended in water and incubated at 100 °C for 2 h. Starch was determined after release of glucose by incubation with α -amylase and amyloglucosidase (Boehringer Mannheim, Mannheim, Germany) at 50 °C for 3 h in 20 mM acetate buffer (pH 4.6). Ethanol was removed from the supernatant under vacuum and the residue suspended in water to determine the levels of NO_3^- , amino acids and soluble carbohydrates. The NO_3^- was measured according to Cataldo *et al.* (1975). Total amino acids content was assayed by the Rosen colorimetric method using glutamine as a reference (Rosen 1957). Individual amino

acid composition was determined on pooled samples extracted from an equal dry weight of each leaf material by ion-exchange chromatography (Rochat & Boutin 1989). Soluble carbohydrates were determined by an enzymatic method using a commercially available assay kit (Boehringer Mannheim). The α -ketoglutarate was extracted in 1 M HClO₄ (1 mL per 100 mg FW) and determined enzymatically (Bergmeyer 1965; Scheible *et al.* 1997).

Free NH₄⁺ was extracted in 2% solution of 5-sulfosalicylic acid (50 mg FW mL⁻¹) and determined by the phenol hypochlorite colorimetric method (Berthelot reaction) using (NH₄)₂SO₄ as a reference.

Protein extraction, fractionation and enzymatic assays

Enzymes were extracted from frozen leaf material stored at -80 °C in cold extraction buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM MgCl₂, PVP 0.5% (w/v), β -mercaptoethanol 0.1% (v/v) and 4 μ M leupeptin. All extractions were performed at 4 °C. Glutamate dehydrogenase NAD and NADH activities were measured as described by Turano *et al.* (1996).

For GDH fractionation, 2 g FW of old/source leaf tissue were ground in extraction buffer as described above. The extract was filtered and loaded onto a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column as described by Brugière *et al.* (1999). After elution, using a NaCl gradient (0–0.4 M), 48 fractions were collected according to Becker, Carrayol & Hirel 2000, and assayed for GDH activity. Protein fractions were concentrated using micro concentrators (Microcon YM-3; Amicon, Bedford, MA, USA) for Western blot experiments in order to contain the same amount of (NADH) GDH activity.

Gel electrophoresis and Western blot analysis

Proteins (10 μ g) were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). The percentage of polyacrylamide in the running gels was 10%. Denatured proteins were electrophoretically transferred to nitro-cellulose membranes. Polypeptide detection was performed using polyclonal antiserum raised against grape leaf GDH (Loulakakis & Roubelakis-Angelakis 1990).

Statistics

Results are presented as mean values for the three plant-material batches (as described as above) with standard errors ($n = 3$).

Screening of a *Nicotiana tabacum* library

A λ gt10 cDNA library of tobacco was kindly provided by Dr Klaus Palme (Max-Planck Institut für Züchtungsforschung, Köln, Germany). The library was screened with a

random primed ³²P-labelled 443 bp *gdh* probe (Masclaux *et al.* 2000) and cDNA were cloned as described by Dubois *et al.* (1996). The nucleotide sequencing of the *gdh1* cDNA was carried out at the Genopole E.S.G.S, Evry, France.

Southern blot analysis

Genomic DNA was extracted according to Paszkowski *et al.* (1984). Genomic DNA (100 μ g) was digested and resolved by electrophoresis using a 0.8% agarose gel, transferred to a nylon membrane (Genescreen biotechnology systems; NEN Life Sciences Products, Boston, MA, USA) and hybridized with the random primed ³²P-labelled 443 bp *gdh* probe (Masclaux *et al.* 2000). Hybridization was performed at 65 °C according to Church & Gilbert (1984). Filters were washed with 2 \times SET (0.06 M Tris-HCl pH 8, 0.3 M NaCl, 4 mM EDTA) at room temperature for 5 min and at 65 °C for 10 min. Additional washings were performed successively using 1 \times SET and 0.5 \times SET at 65 °C for 20 min before drying and exposure to X-ray film.

Extraction of total RNA and Northern blot analysis

Total RNA was extracted as described by Verwoerd, Dekker & Hoekema (1989) from plant material stored at -80 °C. Northern blot analysis was performed as described previously (Masclaux *et al.* 2000). The following ³²P-labelled probes were used for mRNA detection: *nia* from *Nicotiana sylvestris* (Vaucheret *et al.* 1989), *gs2* from *N. tabacum* (Becker *et al.* 1992) and a 1121 bp *EcoRI* fragment of the *gdh1* cDNA isolated in this work. Hybridization was performed under high stringency conditions as described for Southern blot analyses. The relative amounts of mRNA were determined by densitometric scanning of Northern blot autoradiograms (Power Look II scanner; UMAX Data systems, Taiwan) and quantified by an advanced quantifier (J-D Match; BioImage System Corp., Ann Arbor, MI, USA).

RESULTS

Cloning and sequence analysis of the tobacco glutamate dehydrogenase *gdh1* cDNA

A DNA fragment of 443 bp containing part of the coding region of a *N. tabacum gdh* gene (Masclaux *et al.* 2000) was used as a probe to screen a tobacco seedling cDNA library (provided by Dr Klaus Palme at the Max-Planck Institut für Züchtungsforschung, Köln, Germany). A single clone containing an open reading frame of 1630 nucleotides and designated *gdh1* was isolated (Accession number AJ420266). The corresponding translation product gave a polypeptide of 411 amino acids with a predicted molecular mass of 44.5 kDa and a pHi of 7.33. The deduced amino acid sequence showed an homology of 86 and 95% with *Nicotiana plumbaginifolia gdhA* and *gdhB*, respectively (Ficarelli, Tassi & Restivo 1999) and 92% with

Legdh1 of *Solanum lycopersicum* (Purnell, Stewart & Botella 1997). The amino acid sequence deduced from *gdh1* showed all of the structural characteristics of known plant *gdh* protein sequences (Turano *et al.* 1997; Purnell *et al.* 1997).

A genomic Southern blot analysis was performed under high stringency conditions using as a probe the ^{32}P -labelled 443 bp fragment originally used to screen the cDNA library. Since this sequence did not contain any *EcoRI*, *HincII* or *SspI* restriction sites, tobacco genomic DNA was digested with these three enzymes. Following autoradiography, a single major band and three minor bands were visible (Fig. 1). Using low stringency hybridization, the four hybridization signals were of similar intensity (data not shown). This result suggests that GDH is encoded by more than one gene in *N. tabacum* and that the *gdh1* sequence corresponds to one of them.

Organ-specific expression of *gdh1* mRNA in *N. tabacum*

Northern blot analysis was performed using the same RNA extracts previously isolated from anthers, pistils, petals, stems, midribs, young leaves and roots (Dubois *et al.* 1996) and the ^{32}P -labelled 1121 bp *gdh1 EcoRI* fragment as a probe. Compared to floral organs and midribs, higher amounts of *gdh1* mRNA were detected in stem and root tissues (Fig. 2). As previously observed (Masclaux *et al.*

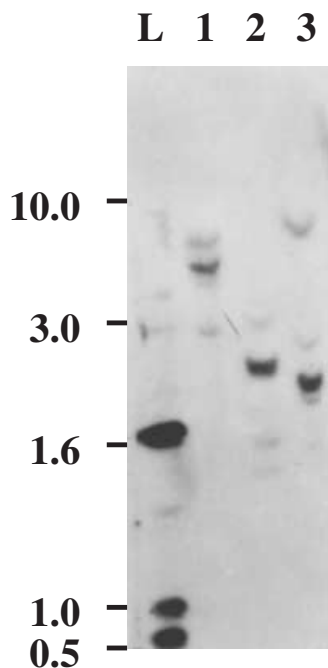


Figure 1. Genomic Southern blot analysis of *gdh1* in *N. tabacum*. Genomic DNA was digested with *EcoRI* (lane 1), *SspI* (lane 2) and *HincII* (lane 3). The size of the DNA markers is indicated in kilobases and the unspecific hybridization of the 1.6, 1.0 and 0.5 kbp fragments of the λ 1 kb ladder (Boehringer Mannheim) is shown in lane L.

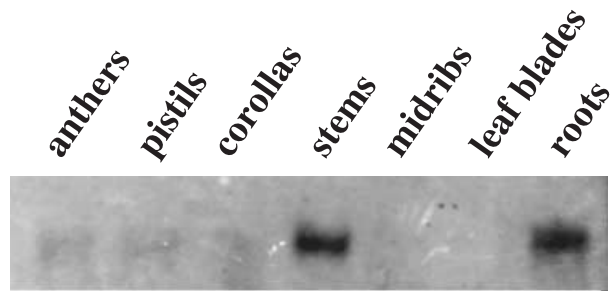


Figure 2. Northern blot analysis of *gdh1* expression in various organs from *N. tabacum*. Total RNA were extracted from anthers, pistils, corollas, stems, midribs, young leaf blades and roots. Ten micrograms of total RNA were loaded in each track. mRNA was detected in stems and roots after hybridization with the ^{32}P -labelled *gdh1* probe.

2000), the gene was not expressed in young leaf mesophyll tissue (Fig. 2).

Characterization of the GDH protein in source leaves

Two main sections along the plant axis of a mature tobacco plant (35 leaves) have been defined previously (Masclaux *et al.* 2000). The upper section contains the youngest leaves (45–15) which behave as sink organs. In the lower section, older/senescent leaves (9–15) behave as source organs and they have been characterized by the expression of specific marker genes and proteins such as *gdh*. Therefore, leaf mesophyll tissues from source leaves (13–15) were used for GDH protein characterization.

Figure 3a shows the elution profile of GDH activity following DEAE-Sephacel ion exchange chromatography of a protein extract prepared from source leaves. A single peak of GDH activity was detected whether the enzyme activity was measured in the aminating or the deaminating direction (Fig. 3a). Protein fractions containing GDH activity were subjected to Western blot analysis using GDH-specific antiserum (Loulakakis & Roubelakis-Angelakis 1990). A single protein exhibiting a molecular mass of about 42 kDa was detected in each fraction, the highest GDH content corresponding to the fractions containing the highest enzyme activity (Fig. 3b).

These results suggest that, in tobacco source leaves at least, GDH activity is associated with a single type of subunit. However, it cannot be excluded that the enzyme contains several polypeptides of identical molecular mass that could differ in charge (Loulakakis & Roubelakis-Angelakis 1990).

Day–night fluctuation in the expression of *gdh1* mRNA

All previous studies of *gdh1* expression in tobacco leaves were performed on samples collected in the middle of the light period (Masclaux *et al.* 2000). In order to determine

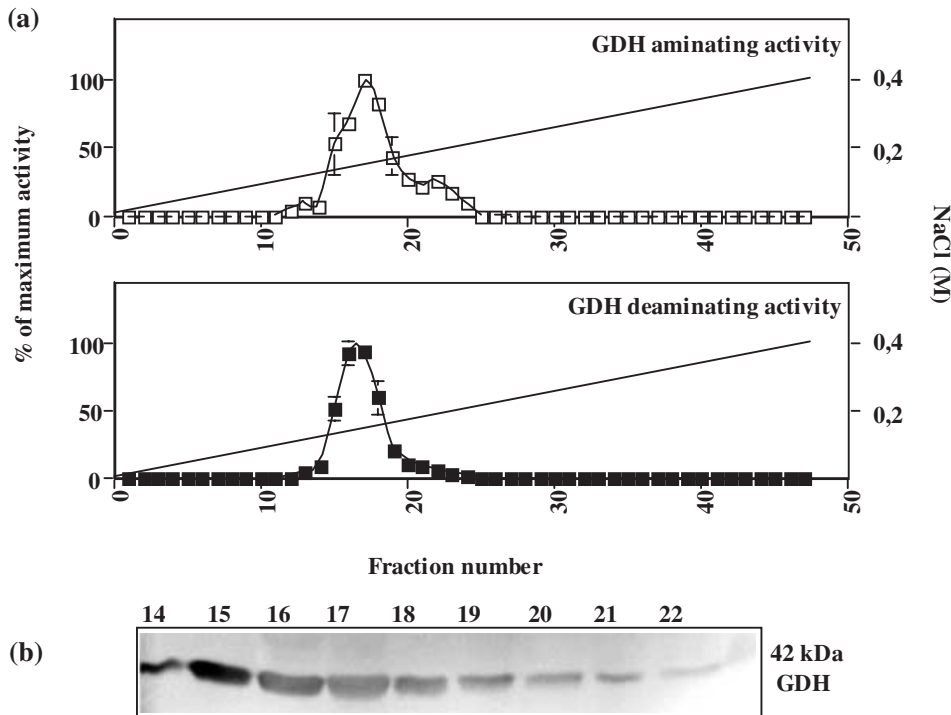


Figure 3. DEAE-Sephacel chromatography of GDH activity in source leaves of *N. tabacum*. (a) Relative GDH aminating and deaminating activities were measured in eluted fractions and expressed as a percentage of the maximum activity. A single major peak for both activities was observed (fractions 14–22). The results are the mean \pm Sn (Sn = standard error with $n = 3$). (b) Western blot analysis of GDH protein content in fractions 14–22 shows a single band.

whether the *gdh1* gene expression was subject to day–night fluctuations, total RNA were extracted from leaf disc samples collected during a 48 h period from both young/sink ($n = 30$ – 32) and old/source ($n = 13$ – 15) leaves. Northern blot analysis was performed using the *EcoRI* fragment of *gdh1* cDNA as a probe.

Because of the trauma caused to the leaves every 3 h due to leaf disc sampling, control samples of unwounded leaves were collected at 36 and at 57 h. Nitrate reductase gene (*nia*) expression, which is known to be subjected to large day–night fluctuations (Galangau *et al.* 1988; Scheible *et al.* 2000), was used as a positive control. Although different results concerning the day–night fluctuations of plastidic GS gene (*gs2*) expression have been reported in the literature (Becker *et al.* 1992; Matt *et al.* 2001), *gs2* mRNA content was used as a negative control in the Northern blot experiments (Fig. 4). A 18S rRNA probe was used as a loading control. A diurnal rhythm for *nia* mRNA was detected whether sink or source leaves were examined (Fig. 4). Although the steady state level of *nia* mRNA was lower in old/source leaves (Masclaux *et al.* 2000) it reached a maximum of expression during the night, increasing between 1800 and 0600 h, and a minimum during the day, decreasing from 0700 to 1500–1800 h (Fig. 4).

In sink leaves, no *gdh1* transcripts were detected whatever the time of extraction (data not shown). In old/source leaves, *gdh1* mRNA levels exhibited diurnal fluctuations, rising between 0700 and 1500–1800 h and then decreasing dramatically from 1800 h to the end of the dark period until 0600 h (Fig. 4). This result shows that, in source leaf mesophyll cells, a shift of 12 h occurs between the day–night

rhythm of *gdh1* and *nia* RNA levels. In contrast, we did not detect any diurnal oscillations in either the aminating or deaminating activities of the GDH enzyme (data not shown).

Metabolite diurnal fluctuations

Levels of carbohydrates and nitrogen metabolites were measured in both young/sink and old/source leaves of tobacco. Regardless of the age of the leaf, characteristic diurnal oscillations were detected for starch and sucrose (Matt *et al.* 1998; Fig. 5A–D). No oscillations were detected for nitrate (Fig. 5E & F). Although the sink leaf free ammonia content tended to accumulate during the day, probably as a result of photorespiratory ammonia accumulation, these minor fluctuations were not observed in source leaves (Fig. 5G & H). No fluctuations were observed in the amino acid content of sink leaves, whereas the total amino acids accumulated in source leaves during the first part of the light period (Fig. 5I & J).

A day–night fluctuation was observed in α -ketoglutarate levels that were similar to those described by Scheible *et al.* (2000) (Fig. 5K & L). It is interesting to note that, in sink leaves, α -ketoglutarate accumulated when ammonia reached its lowest concentration during the day–night cycles.

In both sink and source leaves, diurnal rhythms were detected for glutamine (GLN), glycine (GLY), aspartate (ASP) and asparagine (ASN) (Fig. 6). ASP and ASN accumulated during the first part of the night (Fig. 6E–H) whereas GLN and GLY accumulated during the light

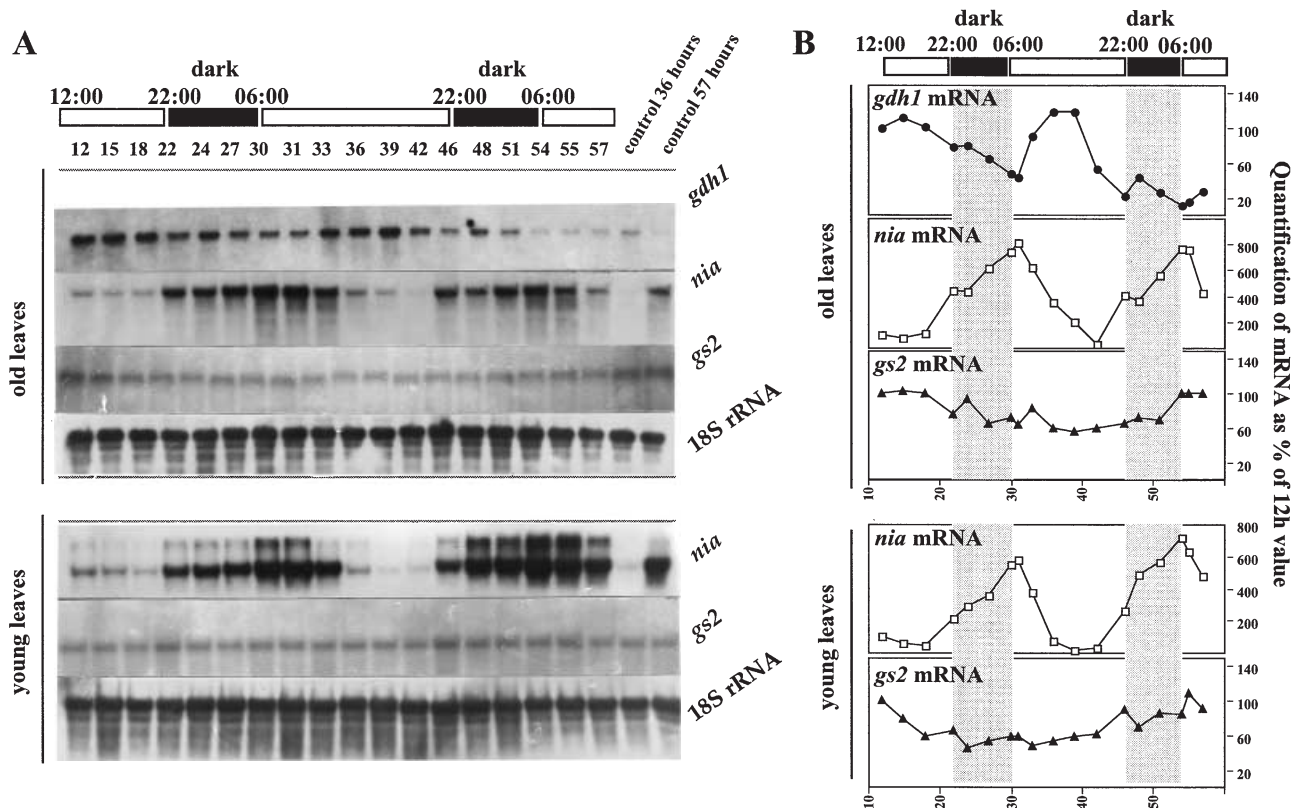


Figure 4. Diurnal changes in *gdh1* and *nia* mRNA accumulation during 48 h of a 18 h light : 6 h dark photoperiod. The dark and light periods are indicated by closed and open bars, respectively. Old/source and young/sink leaf sampling began at 1200 h and samples were numbered from 12 to 57, corresponding to the time in hours after the beginning of the experiment. Total RNA was extracted from each sample and *gdh1*, *nia* and *gs2* mRNA contents were analysed by Northern blotting and specific ^{32}P -labelled probe hybridization. A 18S ribosomal probe was used as a loading control. (A) The result of the Northern blot analysis. (B) Quantification of the accumulation of the different transcripts by the values of the densitometric scanning of the bands corresponding to each transcript. Each value was corrected according to the one found for 18S control.

period (Fig. 6A–D). Amounts of GLY were higher in source leaves in comparison with sink leaves suggesting that glycine decarboxylase could be the main limiting factor in the photorespiratory pathway in ageing leaves.

Serine (SER), glutamate (GLU) and γ -aminobutyric acid (GABA) also showed a day–night fluctuation but the rhythm patterns were different between sink and source leaves (Fig. 6I–N). In sink leaves, GLU and GABA accumulation was observed at the beginning of the night and in the middle of the day, respectively (Fig. 6K & M). In source leaves, the accumulation of both of these amino acids was shifted by about 6 h in comparison with sink leaves (see Fig. 6L & N and 6K & M, for comparison). Moreover, in both types of leaf, a 5–6 h delay was observed between the accumulation of GABA preceding that of GLU (see Fig. 6K & L and 6M & N, for comparison).

Accumulation of proline (PRO) occurred only in sink leaves at the end of the light period–beginning of the night period (Fig. 6O & P) just as GLN. Thus, GLN and PRO might be the two main nitrogen storage compounds at the end of the light period in sink leaves. PRO accumulation was attenuated after a 24 h day–night; this could be as a

consequence of leaf disc sampling. Such a hypothesis was reinforced by measuring the PRO content of samples collected from unwounded controls after 36 h. The 36 h unwounded leaf tissues contained the same amount of PRO ($814 \text{ nmol (mg Pheo)}^{-1}$) as the early-collected leaf discs (see Fig. 6O).

Influence of sucrose and light on *gdh1*, *nia* and *gs2* expressions

To better understand the putative regulatory control mechanisms controlling the day–night fluctuation of *gdh1* expression in tobacco source leaves, we have investigated both the effect of a dark treatment and sucrose feeding on *gdh1* transcript accumulation using leaf discs (Ferrario-Mery *et al.* 2001). The expression of both *nia* and *gs2* mRNA was monitored in parallel and used as controls.

In leaf discs isolated from source leaves and placed under light, *gdh1* transcripts were barely detectable (Fig. 7, lane 2). Sucrose feeding resulted in a decrease of *gdh1* transcript content (Fig. 7, lane 1). In contrast, when leaf discs were

incubated in the dark, the *gdh1* mRNA content was strongly enhanced (Fig. 7, lane 4). The dark-induced accumulation of *gdh1* transcripts was inhibited when sucrose was added to the incubation medium (Fig. 7, lane 3).

On the other hand, in dark-treated leaf discs, *nia* gene expression was strongly induced by sucrose (Fig. 7 lane 3) in comparison with samples placed in the light with or without sucrose (Fig. 7, lanes 1 and 2). In light-treated leaf discs, *nia* transcript accumulation under sucrose feeding (Fig. 7, lane 1) was lower than in dark-treated discs (Fig. 7, lane 3), suggesting that dark-treated tissues were sugar starved and then more sensitive to sucrose feeding.

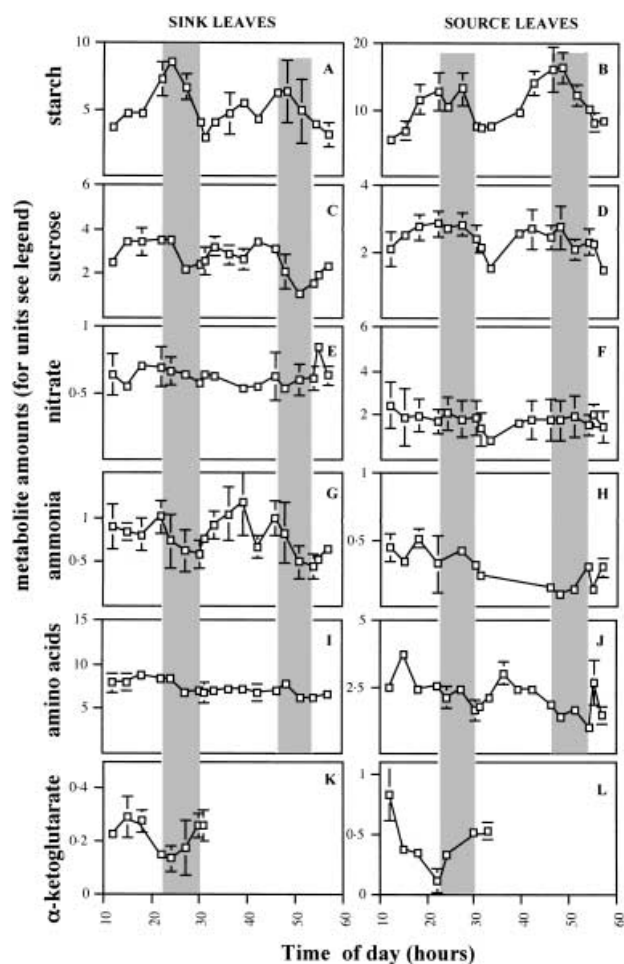


Figure 5. Diurnal changes of starch (A; B); sucrose (C; D); nitrate (E; F); ammonium (G; H), amino acids (I; J) and α -ketoglutarate (K; L) contents in both young/sink (A; C; E; G; I; K) and old/source (B; D; F; H; J; L) tobacco leaf mesophyll tissues. The shaded blocks represent the dark period. The numbers show the time in hours after the beginning of the experiment. Phaeophytin (Pheo) or chlorophyll (Chl) contents in young and old leaves were, respectively, $2.2 (\pm 0.2)$ and $0.9 (\pm 0.1) \mu\text{g} (\text{mg FW})^{-1}$. Quantities of starch, sucrose and amino acids were expressed in $\mu\text{mol} (\text{mg Chl})^{-1}$. Quantities of ammonium and α -ketoglutarate were expressed in $\mu\text{mol} (\text{mg Pheo})^{-1}$. Quantities of nitrate were expressed in $\text{mmol} (\text{mg Chl})^{-1}$. The results are the mean \pm Sn (Sn = standard error with $n = 3$).

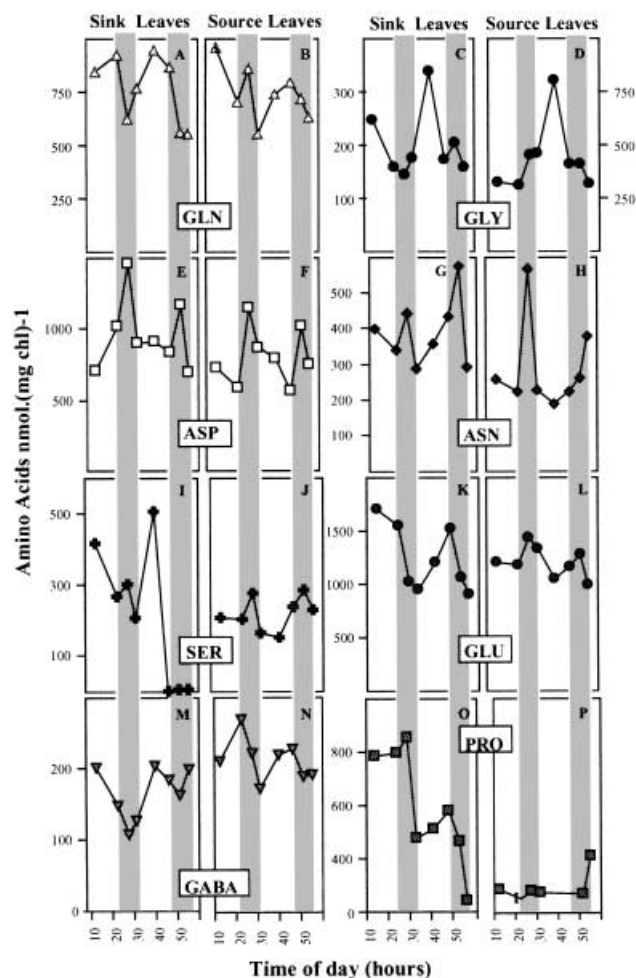


Figure 6. Diurnal changes of glutamine (A; B); glycine (C; D); aspartate (E; F); asparagine (G; H); serine (I; J); glutamate (K; L); GABA (M; N) and proline (O; P) contents in both sink (A; C; E; G; I; K; M; O) and source (B; D; F; H; J; L; N; P) tobacco leaf mesophyll tissues. The shaded lanes represent the dark period. The numbers show the time in hours after the beginning of the experiment. Standard errors were not shown because of sampling (see Materials and methods). Note that scales are different for GLY amount in source and sink leaves.

In light-treated leaf discs, the accumulation of *gs2* mRNA was higher than in dark-treated leaf discs (Fig. 7, lanes 1 and 2 compared with lanes 3 and 4). The addition of sucrose to leaf discs incubated either in dark or light condition, led to a decrease in the amount of *gs2* transcript. Sucrose did not then reverse the negative effect of darkness (Fig. 7, lanes 1 and 3 compared with lanes 2 and 4), suggesting that *gs2* behaves like other genes encoding enzymes involved in photosynthesis (Krapp, Quick & Stitt 1991; Winger *et al.* 1998).

We did not observe any modifications in either GDH protein content or GDH activity using the same leaf disc samples incubated either in the light or in the dark, treated or not with sucrose (data not shown).

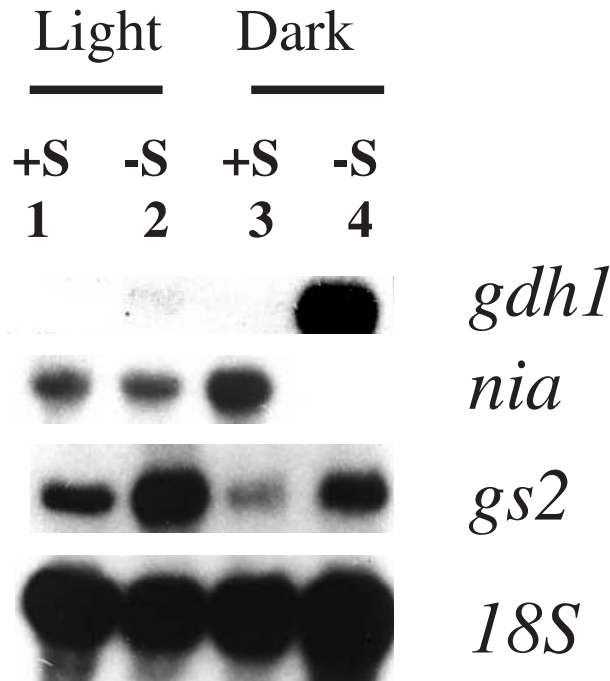


Figure 7. Effect of sucrose feeding and dark treatment on mRNA accumulation. Northern blot experiments, using *gdh1*, *nia* or *gs2* ^{32}P -labelled probes and 10 μg of total RNA extracted from leaf discs, incubated in the presence of light with sucrose (lane 1, + S) or without sucrose (lane 2, -S), in the presence of dark with sucrose (lane 3, + S) or without sucrose (lane 4, -S). A 18S ribosomal probe was used as a loading control.

DISCUSSION

In a previous work, we have shown that ageing induced gradual changes in metabolite and enzymatic contents in leaves (Masclaux *et al.* 2000). Two main sections along the tobacco main axis could be roughly defined and characterized in terms of importance of the occurring metabolic pathways (Masclaux *et al.* 2000). Upper leaves are defined as a sink section mainly involved in primary carbon and nitrogen assimilation. The lower leaves were part of a source section involved in nitrogen remobilization and characterized by the induction of GDH and cytosolic glutamine synthetase (GS1). However, the comparison of sink and source leaves metabolism was based on data from leaf samples taken at 1100 h. Since numerous enzymes involved in C and N metabolism and either their substrate or products are known to exhibit day–night fluctuations (Scheible *et al.* 2000), *gdh1* gene expression was monitored in both source and sink leaves along a 48 h day–night period.

Northern blots revealed a rhythm for *gdh1* mRNA accumulation in source leaves whereas no *gdh1* transcripts could be detected in young leaves. The *gdh1* mRNA oscillations showed a minimum at the beginning of the light period (0600 h) and a peak in the afternoon (1800 h). The *gdh1* mRNA level oscillated in an opposite way compared with that of *nia* transcripts along the day–night period. This

could reflect the fact that the expression of the gene encoding NR is turned on when that of the gene encoding GDH is turned off and reciprocally.

The NR oscillating system is well documented. The diurnal rhythm of NR is not likely to be governed by a clock receiving input through phytochrome receptors but rather dependent on photosynthetically active light absorbed by chlorophylls (Becker *et al.* 1992; Lillo 1994). It is based on a transcription–translation feedback loop in which the positive component that increases *nia* gene transcription is related to light and the component exerting a negative effect is a product of nitrogen assimilation. GLN was suggested to be a good candidate to exert the negative feedback on *nia* day–night fluctuation (Klein *et al.* 2000; Lillo *et al.* 2001 for review), even though some reports have shown that *nia* expression and glutamine accumulation are not so clearly related (Ferrario-Mery *et al.* 2001). Contrary to *nia* regulation, both ammonia and amino acids (GLN for example) are able to induce *gdh* gene expression (Melo-Olivera *et al.* 1996; Turano *et al.* 1997; Masclaux, unpublished; Fig. 8). Moreover, in the present work, we show that glutamine oscillations in source leaves are in phase with *gdh1* mRNA fluctuations (Figs 4, 6E & F). Therefore, it is attractive to suggest that, as for *nia* gene regulation, GLN is a possible effector for the diurnal modulation of *gdh1* (Fig. 8). However, if the biological clock oscillator models are true (Lillo *et al.* 2001), a negative regulation should also be linked to a feedback from a product supplied from the *in vivo* GDH activity (Fig. 8). In the present study, we show that *gdh1* expression is negatively regulated by sucrose, a C metabolite. However a role for sugar as an effector in the feedback regulation of *gdh1* is not obvious, since there is no evidence that GDH activity can modulate sugar content. Another possibility is that the sugar-signalling pathway controlling *gdh1* expression is mediated *via* α -KG replen-

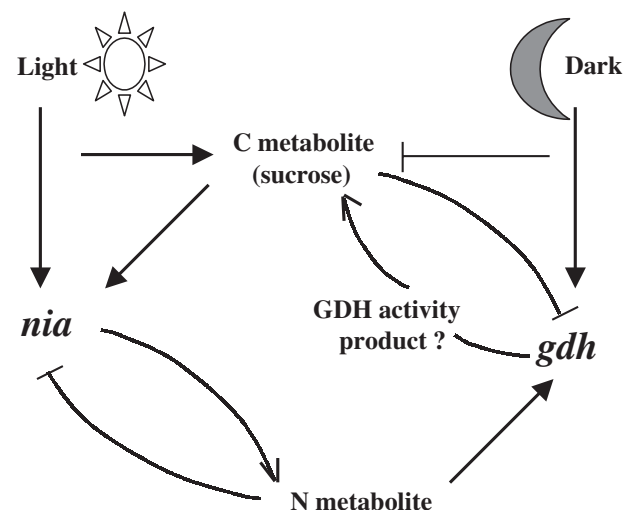


Figure 8. Schematic representation of the antiparallel regulation of the *gdh* and *nia* genes. Arrows indicate positive effects and bars negative effects on gene regulation. *nia*: nitrate reductase gene; *gdh* glutamate dehydrogenase gene, GLN: glutamine.

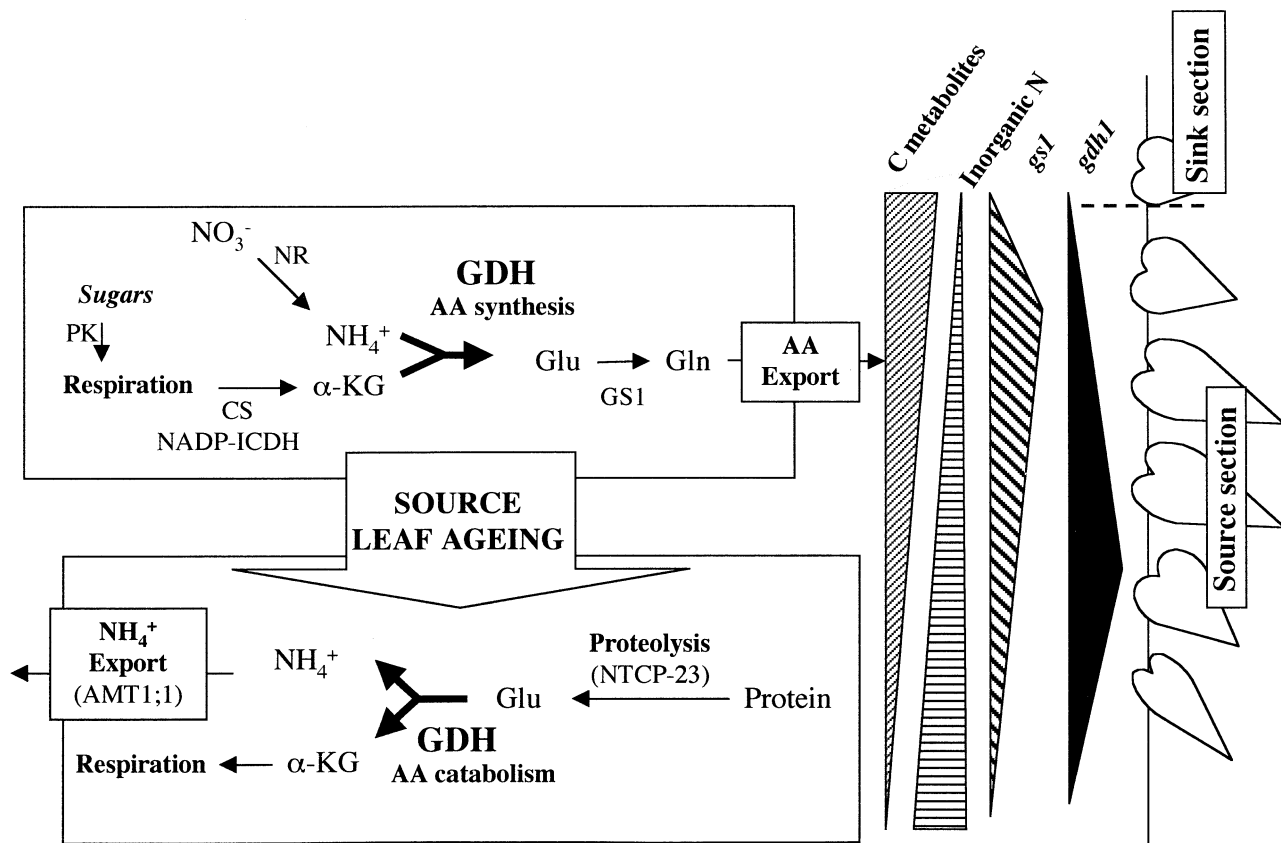


Figure 9. Schematic representation of the hypothetical evolution of the role of GDH in the C/N balance during source leaf ageing. PK: pyruvate kinase (Scheible *et al.* 2000); CS: citrate synthase (Scheible *et al.* 2000); NADP-ICDH: NADP-isocitrate dehydrogenase (Scheible *et al.* 2000); NR: nitrate reductase; GS1: cytosolic glutamine synthetase; AMT1;1: ammonium transporter (Gazzarrini *et al.* 1999); NTCP-23: cysteine protease (Ueda *et al.* 2000); AA: amino acids; α -KG: α -ketoglutarate.

ishment, i.e. via the product of the GDH deaminating activity (Morcuende *et al.* 1998; Koch *et al.* 2000). Interestingly, Ferrario-Mery *et al.* (2001) have shown recently that α -KG feeding can promote the accumulation of *nia* transcripts in tobacco leaves. From this, it will be useful to test whether α -KG can induce *gdh1*.

Opposite changes in *gdh1* and *nia* expression observed through reversed day–night oscillations are also illustrated by sucrose feeding and dark-induction experiments. The transcript level of *gdh1* was increased by a dark treatment and decreased by the addition of sucrose. Sucrose feeding was able to abolish the dark induction of *gdh1* as well as the dark inhibition of *nia*. Moreover, leaf developmental changes such as ageing emphasized the opposition between *nia* and *gdh1* regulation. Since sugars produced via photosynthesis became limiting in old/source leaves (Masclaux *et al.* 2000), sugar availability could once more be a potential signal in age-related *gdh1* and *nia* expression.

Nevertheless, the role of diurnal changes in *gdh1* transcript levels remains to be addressed, since we did not detect any day–night fluctuations in the *in vitro* aminating and deaminating activities of the enzyme. However, it has been shown in a number of studies that it is difficult to measure a reliable enzyme activity *in vitro* that reflects the

actual *in vivo* enzyme activity. Indeed, GDH aminating activity measured *in vitro* is always higher in comparison with its deaminating activity. Moreover, *in vivo*, deaminating activity can be revealed accurately only using ^{15}N labelling techniques as described previously (Berger *et al.* 1985; Robinson *et al.* 1991; Stewart *et al.* 1995). Therefore, we cannot exclude that there is a day–night fluctuation for GDH activity *in vivo*.

Interestingly, we observed that following the induction of *gdh1* mRNA, a rhythm for GLU, different to that of sink leaves, appeared in source leaves. Indeed, in sink leaves, GLU accumulated at the end of the day as a result of GOGAT activity and was used during the night. In source leaves, GLU accumulated in the first half of the night and was probably used in the second half of the night and in the morning. The behaviour of GLU in source leaves can then be questioned. As no change in GLN day–night oscillation was observed between sink and source leaves and since GABA oscillations were shifted by 6 h just like GLU, we hypothesize that GABA is buffering GLU production (Satya Narayan & Nair 1990; Shelp *et al.* 1995). Moreover, we observe here that the expression of *gdh1* in source leaves oscillated in an opposite way compared with glutamate, while α -KG rhythm remained unchanged

between source and sink leaves. A role for *gdh1* in GLU oscillation shift can therefore be considered.

Interestingly, it has been shown recently that, in tobacco source leaves, the levels of the transcripts and activities of organic acid-producing enzymes (pyruvate kinase (PK), citrate synthase (CS), NADP-isocitrate dehydrogenase (NADP-ICDH)) also show diurnal changes (Scheible *et al.* 2000). As for *gdh1* mRNA, the transcript oscillations of these genes, were out of phase compared with that of *nia* mRNA. We observed in source leaves the same fluctuation patterns as the one described by Scheible *et al.* (2000) for individual amino acids, sugars and α -KG. Since GDH activity can work in both the aminating and deaminating directions (as shown only *in vitro*), two hypotheses can be considered. GDH deaminating activity could be used to produce α -KG just as PK, CS and NADP-ICDH activities (Scheible *et al.* 2000) in order to supply carbon skeletons usable as an acceptor for the reduced nitrogen products of NR activity. However, because deaminating GDH activity would also release mineral nitrogen, such a hypothesis lacks coherence. In such a context, an aminating pathway that is able to compensate the age-related decrease in Fd-GOGAT activity for GLU synthesis appeared to be more logical as long as α -KG is available and NR active (Fig. 9). Nevertheless, this hypothesis is based on *gdh1* mRNA accumulation and not on GDH activity.

In any case, we should bear in mind that *gdh1* and *nia* expressions behave in opposition with regard to senescence. Moreover, we have shown previously (Masclaux *et al.* 2000) that *gs1* transcripts accumulated with ageing before those of *gdh* (Fig. 9). It is assumed that GS1 participates in nitrogen mobilization and compensates for a deficit in the GS2 isoform due to age-related chloroplast collapse. The delay between *gdh* and *gs1* age-related accumulation suggests that the roles of GDH and GS1 are not co-operative. Interestingly, the senescence-induced tobacco cysteine protease gene (*NTCP-23*) presents a diurnally modulated expression that is very similar to that observed for *gdh1* (Ueda *et al.* 2000). Moreover, the ammonium transporter (*AtAMT1;1*) which is induced in source compared to sink leaves (Gazzarrini *et al.* 1999), exhibits in roots an almost identical diurnal rhythm to that of *gdh1* and *NTCP-23* in leaves. Rhythmic changes in *AtAMT1;1* expression was unfortunately not investigated in leaves, but its homologous *LeAMT1;1* was seen to be induced in tomato leaves at the end of the day and during the dark period (von Wieren *et al.* 2000).

We have shown previously that the process of leaf senescence is accompanied by an increase in ammonium content, proteolysis and *gdh1* expression and a decrease in sugar contents and NR activity (Masclaux *et al.* 2000). These observations indicate that proteins and amino acids were certainly catabolized to provide ammonia (Fujiki *et al.* 2000b). As some ammonium transporters are senescence-induced in source leaves (von Wieren *et al.* 2000), we can suppose that a part of the ammonium is mobilized from old senescing mesophyll cells to vascular tissues and further to sink tissues (Fig. 9). In such a context, GDH appeared a

good candidate for glutamate catabolism. GDH could then participate in protein burning, supplying α -KG in order to sustain respiration and cell viability for nitrogen mobilization efficiency (Fig. 9; Laurière 1983; Srivastava & Singh 1987).

ACKNOWLEDGMENTS

The authors thank Dr K. A. Roubelakis-Angelakis (University of Crete, Heraklion, Greece) for providing antibodies raised against the grape GDH protein. We are also grateful to Dr J. P. Boutin and K. Pageau for their help in the amino acid analysis, to Dr J. F. Morot-Gaudry for helpful discussions and to Dr M. Hodges and Dr H. Vanacker for critically reading the manuscript.

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Received 13 March 2002; received in revised form 22 May 2002; accepted for publication 23 May 2002