

RESEARCH PAPER

# Concerted modulation of alanine and glutamate metabolism in young *Medicago truncatula* seedlings under hypoxic stress

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## Abstract

The modulation of primary nitrogen metabolism by hypoxic stress was studied in young *Medicago truncatula* seedlings. Hypoxic seedlings were characterized by the up-regulation of glutamate dehydrogenase 1 (GDH1) and mitochondrial alanine aminotransferase (mAlaAT), and down-regulation of glutamine synthetase 1b (GS1b), NADH-glutamate synthase (NADH-GOGAT), glutamate dehydrogenase 3 (GDH3), and isocitrate dehydrogenase (ICDH) gene expression. Hypoxic stress severely inhibited GS activity and stimulated NADH-GOGAT activity. GDH activity was lower in hypoxic seedlings than in the control, however, under either normoxia or hypoxia, the *in vivo* activity was directed towards glutamate deamination. <sup>15</sup>NH<sub>4</sub> labelling showed for the first time that the adaptive reaction of the plant to hypoxia consisted of a concerted modulation of nitrogen flux through the pathways of both alanine and glutamate synthesis. In hypoxic seedlings, newly synthesized <sup>15</sup>N-alanine increased and accumulated as the major amino acid, asparagine synthesis was inhibited, while <sup>15</sup>N-glutamate was synthesized at a similar rate to that in the control. A discrepancy between the up-regulation of GDH1 expression and the down-regulation of GDH activity by hypoxic stress highlighted for the first time the complex regulation of this enzyme by hypoxia. Higher rates of glycolysis and ethanol fermentation are known to cause the fast depletion of sugar stores and

carbon stress. It is proposed that the expression of GDH1 was stimulated by hypoxia-induced carbon stress, while the enzyme protein might be involved during post-hypoxic stress contributing to the regeneration of 2-oxoglutarate via the GDH shunt.

Key words: Alanine, alanine aminotransferase, ammonia, glutamate, glutamate dehydrogenase, glutamate synthase, hyperammonia, hypoxia, nitrogen.

## Introduction

Flooding is one of the adverse environmental factors that inhibit germination, seedling establishment, and plant development (Subbaiah and Sachs, 2003). In soils saturated with water, O<sub>2</sub> availability is very low because O<sub>2</sub> diffusion through water is approximately 10 000 times slower than in air and because of competition for O<sub>2</sub> with respiring micro-organisms (Jackson, 1985). In species adapted to survive long-term submergence, developmental changes such as internode and petiole elongation, alteration of root porosity, morphology and depth, root aerenchyma formation, and adventitious root development occur as an adaptive response to flooding. However, the initial adaptive response to decreased oxygen availability in both tolerant and intolerant species is the start of anaerobic metabolism of pyruvate. Several studies support the importance of the role played by ethanol fermentation

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Abbreviations: ADH, alcohol dehydrogenase; AlaAT, alanine aminotransferase; GABA,  $\gamma$ -aminobutyric acid; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MSX, methionine sulphoximine; PDC, pyruvate decarboxylase.

in the tolerance of hypoxia/anoxia. Fermentative pathways catalysed by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) for ethanol production, and lactate dehydrogenase (LDH) for lactate production (Saglio *et al.*, 1999; Sato *et al.*, 2002) are induced in hypoxic/anoxic tissues. In *Arabidopsis thaliana*, overexpression of the anaerobically induced *PDC1* resulted in increased contents of acetaldehyde and ethanol and decreased contents of pyruvate in roots and shoots, which were associated with an increased tolerance of the plants to low oxygen (Roberts *et al.*, 1989; Ismond *et al.*, 2003). Conversely, reduced tolerance to oxygen deficiency was reported in *adh* (alcohol dehydrogenase) null mutants in a number of species (Harberd and Edwards, 1982; Matsumura *et al.*, 1995; Jacobs *et al.*, 1998).

In hypoxic/anoxic tissues, increased glycolysis supported by the induction of glyceraldehyde-3-phosphate dehydrogenase compensates for the lower ATP yield due to the inactivation of oxidative phosphorylation. The accumulation of pyruvate, acetaldehyde (an intermediary component in the ethanol synthetic pathway), ethanol, and lactate allow the regeneration of NAD<sup>+</sup> from NADH. Regeneration of NAD<sup>+</sup> is critical for hypoxia/anoxia tolerance because glycolysis ceases in the absence of NAD<sup>+</sup> (Ismond *et al.*, 2003; Kursteiner *et al.*, 2003).

During hypoxia/anoxia, metabolic perturbation jeopardizes cell and plant survival. Perturbation directly linked to the lack of oxygen and related metabolic changes, for example (i) shortage of cellular energy supply (ATP) due to the inhibition of oxidative phosphorylation and (ii) cytoplasm acidification, a major determinant in intolerance to O<sub>2</sub> deficiency (Roberts *et al.*, 1989). Acidification can be caused by several parameters including the release of H<sup>+</sup> accompanying the hydrolysis of pools of Mg<sup>2+</sup> nucleoside triphosphates and sugar phosphates, impaired functioning of the plasma membrane H<sup>+</sup>-pumping ATPase (Gout *et al.*, 2001), accumulation of non-processed acidic intermediates such as glycolytic compounds (Felle, 1996), and poor CO<sub>2</sub> removal (Saglio *et al.*, 1999). Other perturbations are consequences of the onset of lactate and ethanol fermentation that paradoxically also allow the cell to combat hypoxic/anoxic stress by promoting NAD<sup>+</sup> regeneration, for example (i) synthesis of lactate that worsens cytoplasm acidification (Davies *et al.*, 1974); (ii) accumulation of acetaldehyde, a highly reactive chemical affecting cellular damage, by forming acetaldehyde-protein adducts (Jackson, 1985; Braun *et al.*, 1995; Jackson and Armstrong, 1999); and (iii) carbon stress caused by faster depletion of sugar stores due to higher rates of glycolysis and ethanol fermentation. Ethanol is a dead end product that either accumulates or leaks out of the tissue representing, in both cases, a net loss of carbon skeletons. Indeed improved tolerance of hypoxia/anoxia of *Arabidopsis thaliana* overexpressing *PDC1* was effective only in plants growing on a Murashige and Skoog medium

containing 3% (w/v) of sugar (Ismond *et al.*, 2003; Albrecht *et al.*, 2004). Therefore, control of the damaging consequences of lactate and ethanol fermentation appears a determinant issue in plant survival under low oxygen conditions.

It is well established that proline accumulates in plants submitted to drought and salt stress. Asparagine also accumulates in plants submitted to various stress conditions such as drought, salt, mineral deficiencies, toxic metals, and pathogen attack (for a review, see Lea *et al.*, 2007). It is, however, interesting to note that neither asparagine nor proline accumulation was reported in plants subjected to hypoxic stress, while alanine accumulation is generally observed in these plants. It has been shown that almost all the assimilated <sup>15</sup>NH<sub>4</sub><sup>+</sup> accumulates in alanine in the green alga *Selenastrum minutum* under anoxia (Vanlerberghe *et al.*, 1991) and alanine becomes the major accumulated amino acid instead of asparagine in anoxic and hypoxic *Medicago truncatula* (Ricoult *et al.*, 2005, 2006).

The transcriptional induction of alanine aminotransferase (*AlaAT*) genes in response to low-oxygen conditions has been demonstrated in several species, i.e. barley (*Hordeum vulgare*), corn (*Zea mays*), *Panicum miliaceum* (Good and Muench, 1993; Muench and Good, 1994), *Medicago truncatula* (Ricoult *et al.*, 2005), and *Arabidopsis thaliana* (Miyashita *et al.*, 2007). However, in the latter work, the involvement of *AlaAT* in hypoxic alanine production was shown to be more complex than expected. The mutant *alaat1.1* affected in *AlaAT1* that encodes the major *AlaAT* isozyme in *A. thaliana* accumulated even more alanine than the wild type when subjected to hypoxic stress. Upon return to normoxia, the decline in alanine content was delayed in the mutant, highlighting the importance of *AlaAT* activity in the catabolism of alanine during the post-hypoxic period. Very recently, the overexpression of *AlaAT* has been shown to improve the nitrogen use efficiency of canola plants (Good *et al.*, 2007).

With the exception of alanine accumulation and related *AlaAT* gene regulation, nitrogen metabolism under oxygen deficiency conditions has been less well studied in comparison to carbon metabolism, i.e. ethanol and lactate glycolysis. Alanine synthesis occurs at a critical branch point between aerobic and anaerobic metabolism and offers at least two advantages under oxygen-limiting conditions. These are the conservation of carbon skeletons that would otherwise be lost by ethanol fermentation (Good and Muench, 1993; Muench and Good, 1994) and the reduction in pyruvate availability for lactate and acetaldehyde synthesis. However, whether alanine accumulation *per se* contributes and how it may participate in the tolerance of low oxygen stress is still matter of debate. Alanine intrinsically may not play a role in hypoxic stress tolerance, but rather alanine production may be

a mechanism by which the plant stores nitrogen, in preparation for the return to normal oxygen condition as proposed by Miyashita *et al.* (2007). In this context the objective of the present work was to study changes in nitrogen primary metabolism in hypoxic *M. truncatula* seedlings in order to understand whether and how N metabolism in a broader sense than just alanine synthesis, may contribute to short-term adaptive responses to hypoxic stress.

## Materials and methods

### Germination and seedling growth conditions

Seeds of *Medicago truncatula* Gaertn. (cv. Paraggio) were germinated in darkness at 20 °C. For ammonium ( $^{15}\text{NH}_4^+$ , 99%  $^{15}\text{N}$ , Euriso-top, Saarbrücken, Germany) labelling experiments, seeds of *M. truncatula* were germinated in Petri dishes (diameter 9 cm) on Whatman paper soaked with 3.5 mL sterile deionized water for 19 h until the completion of germination. Two batches of seedlings were constituted, one was maintained for 5 h after completion of germination on sterile deionized water and the second was transferred to aqueous 5 mM of methionine sulphoximine (MSX, an inhibitor of glutamine synthetase) (Sigma, St Louis, MO, USA) before both were transferred for 2 h, 10 h, and 24 h to normoxic or hypoxic labelling media supplemented with 2 mM or 30 mM ( $^{15}\text{NH}_4$ ) $_2\text{SO}_4$ . Normoxic seedlings were maintained in Petri dishes on Whatman paper soaked with labelling solution. Hypoxia treatment was imposed by completely submerging seedlings into 25 ml of labelling medium for the duration of the experiment. Seedlings were collected, cotyledons were discarded and the embryo axes (hypocotyls and roots) were frozen in liquid nitrogen before being stored at -80 °C for amino acid analysis by either high pressure liquid chromatography (HPLC) or gas chromatography coupled to mass spectrometry (GC-MS).

For the determination of gene expression by real-time q-RT-PCR and *in vitro* enzymes activities, seeds and seedlings were treated as described for the  $^{15}\text{NH}_4^+$  labelling experiment, except that they were supplied  $^{14}\text{NH}_4^+$  without MSX.

For glutamate dehydrogenase (GDH) activity staining following native PAGE, seeds were germinated for 19 h and seedlings maintained for 5 h on sterile deionized water before being transferred for 10 h, 24 h or 48 h on 2 mM ( $\text{NH}_4$ ) $_2\text{SO}_4$  (control), excess ammonium (30 mM) or 2 mM ( $\text{NH}_4$ ) $_2\text{SO}_4$  under hypoxia.

For GDH immunoblot analysis, seeds were germinated for 19 h and seedlings maintained for 21 h on sterile deionized water before embryo axes were harvested.

For either GDH immunoblot or native PAGE, leaves were harvested on plants aged 2 months. Plants were grown in a chamber under a day/night cycle of 15 h light at 24 °C (50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 9 h dark at 24 °C.

### Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from radicles were extracted using TRIzol Reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. Reverse transcription, real-time PCR, and SYBR Green detection were performed as previously described (Glévarec *et al.*, 2004). The primer sequences for the amplification of each gene are given in Table 1 of the Supplementary data at JXB online.

### Enzymes, glutamine synthetase (GS), glutamate synthase (GOGAT), and GDH *in vitro* assay

GS and GDH enzymes were extracted and assayed as described in Glévarec *et al.* (2004). Fd- and NADH-GOGAT were determined according to Suzuki *et al.* (2001).

### Immunoblot and native PAGE for GDH subunit analysis

Soluble proteins were extracted from frozen plant material in TRIS-HCl buffer (25 mM, pH 7.6) supplemented with 1 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1  $\mu\text{l ml}^{-1}$  2-mercaptoethanol and polyvinylpyrrolidone (Sigma).

After denaturation, protein samples were separated in SDS-PAGE gel, 11% (w/v) polyacrylamide. The proteins were electrophoretically transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Antiserum raised in rabbits against grapevine GDH was used to detect GDH protein (Loulakakis and Roubelakis-Angelakis, 1990). Protein-antibody complexes were located using peroxidase-conjugated goat anti-rabbit immunoglobulin G.

To identify different GDH isoenzymes, proteins were separated in a native 7% polyacrylamide gel. NAD-GDH activity was revealed on the gel in the presence of 100 mM TRIS-HCl, pH 9, 50 mM L-glutamate, 500  $\mu\text{M}$  NAD, 250  $\mu\text{M}$  nitro blue tetrazolium, 100  $\mu\text{M}$  phenazine methosulphate, for 1 h in the dark at 37 °C.

### Amino acid extraction and analysis by GC-MS and HPLC

The total amino acids were extracted from embryo axes in 96% ethanol for 1 h at 4 °C. After centrifugation, the ethanol fraction was removed and the same process was then repeated with deionized water. The ethanol and water fractions were combined and stored at -20 °C. After evaporation of the extract under vacuum, organic residues were dissolved in deionized water and

**Table 1.** Primer sequences for real-time quantitative PCR amplification

Primers for specific amplification of GS1a and GS1b, Fd and NADH-GOGAT, ICDH, GDH1 and GDH3, and mAlaAT target specific sequences in the 3' or 5'-NTR.

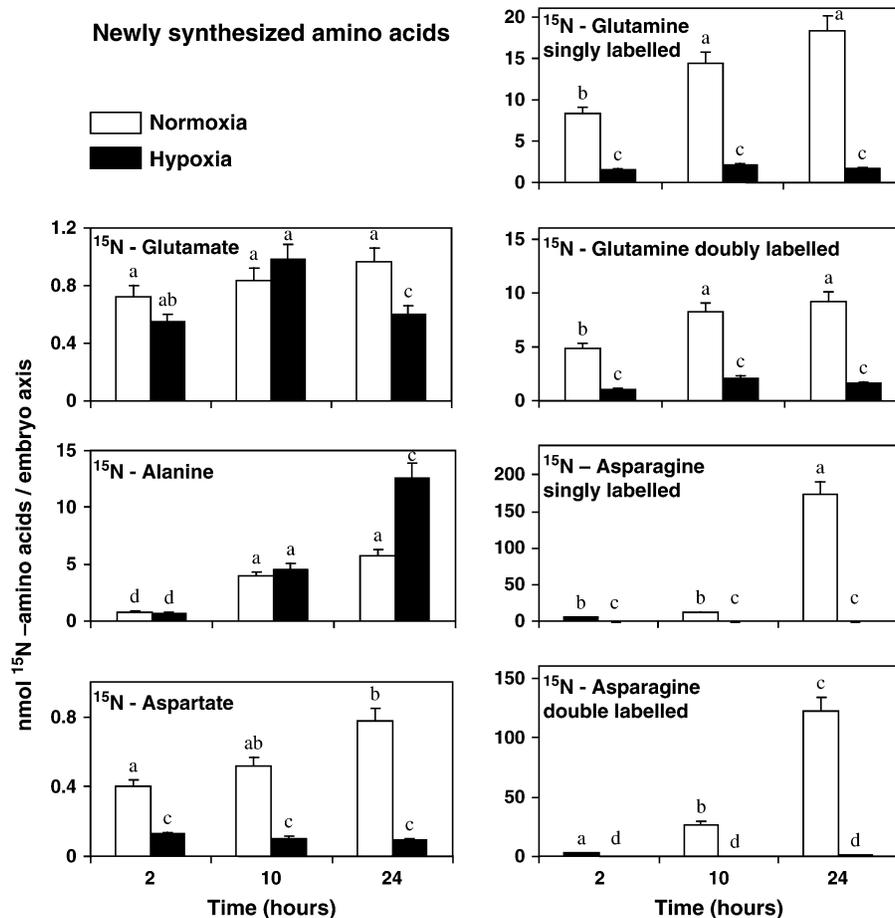
Gene	Forward primer	Reverse primer
<i>GS1a</i>	5'-GTCCAAGTGGTCTTTAAGCACAAA	5'-GAGTAAAATCAAGAAGCAACCATGTTA
<i>GS1b</i>	5'-ACCACCATTCTCTGGAAACCAT	5'-ACAATGCATGTGTGTGTTTTATAGCA
<i>Fd-GOGAT</i>	5'-GCACTGTTGCATCCGGG	5'-TCCTGCATGTTTGTATGGAACCTT
<i>NADH-GOGAT</i>	5'-CAGGTGACTCTCGGCGTG	5'-TCTTTGGTGAGGTAGCTGTCAACT
<i>ICDH</i>	5'-GTGGAGAAACCAGCACAAACAG	5'-TGGGCTAGACCTCTTTGCCAA
<i>GDH1</i>	5'-CAGTGACACTGCAAGATCTATTTTCC	5'-AACCAGAAGAACCTAACACAATATTCAA
<i>GDH3</i>	5'-CATAAGAGTAATATGAGCAACCATTCTTG	5'-GAAATAGACGTATCCACATTAAGAAATGA
<i>mAlaAT</i>	5'-CCGCACCTGATGcCTTCTAT	5'-AAAATGCCATGTGCCAGGAA

extracted with the same volume of chloroform. After centrifugation, an aqueous phase containing amino acids was vacuum dried. The amino acids were then redissolved in deionized water and passed through a 2 ml column of Dowex 50 W×8 200–400 mesh, H<sup>+</sup>-form resin (Fluka (Sigma)). Columns were washed three times with 1 ml of water and amino acids were eluted with 5 ml of 6 M NH<sub>4</sub>OH. Samples were divided into two parts and were vacuum dried. One part was derivatized by *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (Alltech, Templemars, France) for 30 min at 90 °C. The BDMS derivatives obtained were analysed by GC-MS using a Fisons MD800 quadrupole GC-MS system in order to determine the incorporation of <sup>15</sup>N into amino acids as described by Glévarac *et al.* (2004). The other part was analysed by HPLC. The amino acids were determined by the Waters (MA, USA) AccQTag method. The AccQTag method uses Waters AccQFluor Reagent (patent pending) to derivatize the amino acids. The reagent is a highly reactive compound, 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC), which forms stable derivatives with primary and secondary amino acids. Derivatives are separated by reversed-phase (C18 column) HPLC and quantified by fluorescence detection.

## Results

### *The fate of ammonium in M. truncatula seedlings under hypoxia stress analysed by <sup>15</sup>NH<sub>4</sub><sup>+</sup> labelling*

*Medicago truncatula* seedlings were maintained on sterile water for 5 h after the completion of germination before being transferred for 2, 10, and 24 h to two different labelling media supplemented with 2 mM (<sup>15</sup>NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> under control (normoxia) and hypoxia conditions. Labelled ammonium was supplied at 99% atom excess. The percentage of <sup>15</sup>N atom excess in each amino acid (% <sup>15</sup>N) was determined by GC-MS (data not shown) and amounts of newly synthesized amino acids during the labelling period (nmol <sup>15</sup>N-amino acids) were calculated (Fig. 1). Under control conditions with 2 mM (<sup>15</sup>NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> calculations of newly synthesized amino acids throughout the 24 h labelling period showed that the main accumulated amino acid in the embryo axes was <sup>15</sup>N-asparagine



**Fig. 1.** <sup>15</sup>NH<sub>4</sub><sup>+</sup> labelling experiment for the determination of the fate of ammonium in *M. truncatula* seedlings under normoxia and hypoxic stress. Young seedlings (24 h) were fed with 2 mM (<sup>15</sup>NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (99% <sup>15</sup>N atom excess) for 2, 10, and 24 h. Results are the mean of three replicates, corresponding to <sup>15</sup>N atom excess in glutamate, alanine, aspartate, glutamine (single and double labelled), and asparagine (single and double labelled) (% <sup>15</sup>N-amino acids) and the amounts of newly synthesized amino acids during the labelling period, expressed as nmol <sup>15</sup>N-amino acids per embryo axis. Values followed by different letters, are significantly different according to ANOVA analysis and Newman-Keuls test ( $\alpha = 5\%$ ).

either single or double labelled. The amounts of  $^{15}\text{N}$ -glutamine (single and double) and  $^{15}\text{N}$ -alanine increased at least 2 times and 5 times, respectively, between 2 h and 24 h of labelling.  $^{15}\text{N}$ -Aspartate showed a slight increase, while  $^{15}\text{N}$ -glutamate was relatively constant (Fig. 1).

Under hypoxia stress, calculations of newly synthesized amino acids throughout the 24 h labelling period showed that the main accumulated amino acid in the embryo axes was  $^{15}\text{N}$ -alanine. Twice as much alanine was synthesized during the labelling period than in the control, and alanine became the major amino acid in the hypoxic embryo axes. The amounts of  $^{15}\text{N}$ -aspartate and  $^{15}\text{N}$ -glutamine were significantly lower in the hypoxic embryo axes compared to the control, while the amounts of newly synthesized  $^{15}\text{N}$ -glutamate were similar in both treatments, showing very little difference over the experimental time-course (Fig. 1).

#### *Changes in expression of genes encoding enzymes of nitrogen and carbon metabolism (GS, GOGAT, GDH, AlaAT, and isocitrate dehydrogenase (ICDH)) under hypoxia*

The expression of genes encoding enzymes that have been shown to be involved in ammonium and glutamate metabolism in *M. truncatula* (Glévarec *et al.*, 2004; Ricoult *et al.*, 2006) was determined by q-RT-PCR in embryo axes under hypoxia stress. The genes included *GS1a* and *GS1b*, *Fd-* and *NADH-GOGAT*, mitochondrial *AlaAT* (*mAlaAT*), and cytosolic *ICDH*. Screening the Dana Farber Cancer Institute (DFCI) *Medicago* Gene Index (<http://compbio.dfci.harvard.edu/tgi/>) identified two complete sequences of genes encoding two GDH isoforms, similar to *A. thaliana* *GDH1* and *GDH3*. The expression of the two genes was studied. The latter two genes corresponded to *M. truncatula*, TC94704, similar to *A. thaliana* *GDH1* (Q43314) and *M. truncatula*, TC94778, similar to *Arabidopsis thaliana* *GDH3* (Q9S7A0). Until now, no similar *A. thaliana* *GDH2* (Q38946) has been found in *M. truncatula*. For this reason, TC94704 and TC94778 were named, respectively, *GDH1* and *GDH3* in the present article.

The expression of GS, GOGAT, and GDH were accounted for by analysing the isoforms *GS1b*, *NADH-GOGAT*, and *GDH1*. As expected (Glévarec *et al.*, 2004), expression of the isoforms *GS1a* and *Fd-GOGAT* were very low in the control and not stimulated by hypoxic stress (data not shown). With the exception of *GDH3*, all genes studied were up-regulated during the post-germination phase of development showing at least a 2–3-fold increase between 0 h (24 h of imbibition) and 2 h (Fig. 2). This pattern of expression is consistent with our former study (Glévarec *et al.*, 2004).

*mAlaAT* expression was 4-fold higher in the hypoxic embryo axes than in the control after 2 h under stress, indicating that the adaptive response to hypoxia took

place early (Fig. 2). The expression of the genes *GS1b*, *NADH-GOGAT*, and *ICDH* was down-regulated by at least 50% as early as 2 h or 5 h after hypoxic stress. The expression of *GDH3* remained at the same low level as in the control for up to 12 h of hypoxic stress before decreasing considerably at 24 h. Unlike the latter genes, *GDH1* was up-regulated in hypoxic embryo axes. The expression of *GDH1* was 3–4 times higher than in the control, as early as 2 h after the seedlings were transferred to hypoxia (Fig. 2).

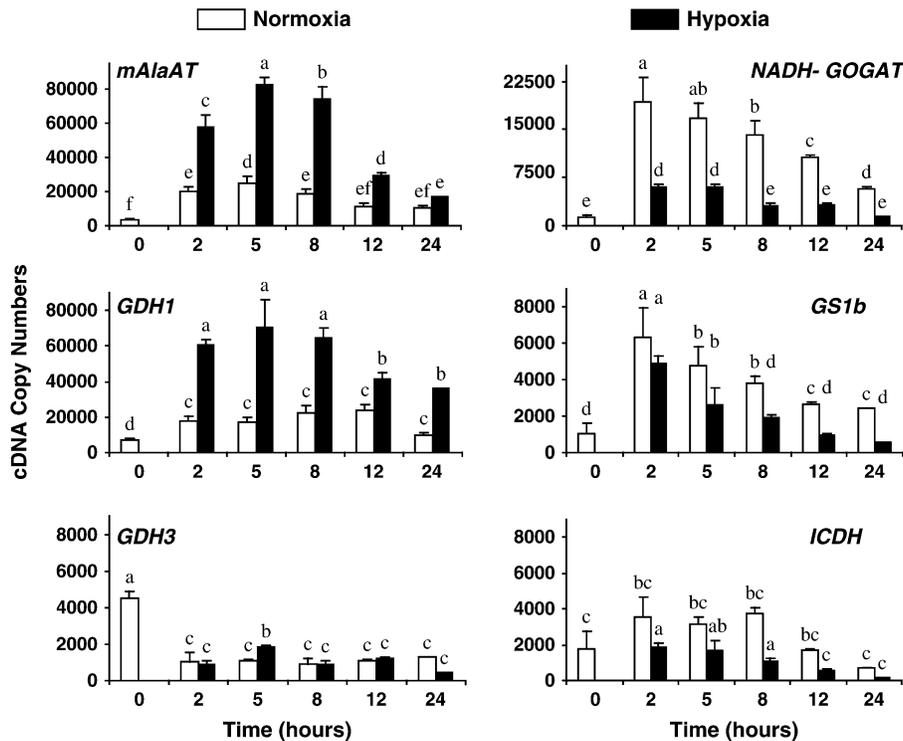
#### *Changes in the activities of GS, NADH-GOGAT and aminating and deaminating GDH in vitro, under hypoxic stress*

GS activity was dramatically reduced in hypoxic embryo axes, reaching almost zero after 72 h exposure to hypoxia (Fig. 3). GDH seemed more affected in the deaminating reaction, which decreased dramatically after 72 h under hypoxia, whilst the aminating reactions remained almost constant. However, GDH activity in the control showed a 2–3-fold increase in both reactions, during the time-course of the experiment (Fig. 3). *NADH-GOGAT* activity in the hypoxic embryo axes exhibited a 2-fold increase under hypoxia, while it remained relatively constant in the control (Fig. 3).

#### *Determination of GDH activity in vivo in M. truncatula seedlings under hypoxic stress by $^{15}\text{NH}_4^+$ labelling*

The up-regulation of *GDH1* expression in hypoxic embryo axes as well as the maintenance of GDH aminating *in vitro* activity at a constant level under hypoxia stimulated an investigation into the possible role of GDH in hypoxia stress tolerance, through the synthesis of glutamate and regeneration of  $\text{NAD}^+$ .

As *in vitro* GDH activity does not reflect the true *in vivo* activity of the enzyme (Glévarec *et al.*, 2004),  $^{15}\text{NH}_4^+$  labelling (in the presence of the glutamine synthetase inhibitor: MSX) was used in combination with GC-MS amino acids analyses. In these experiments, *M. truncatula* seedlings were maintained on sterile water for 5 h after completion of germination and then transferred for 2, 10, and 24 h to labelling medium that contained 5 mM MSX and 2 mM  $(^{15}\text{NH}_4)_2\text{SO}_4$  under either normoxia or hypoxia. MSX treatment resulted in a 94% inhibition of GS activity, while GDH and GOGAT activities were not affected (data not shown). In the absence of GS activity, incorporation of  $^{15}\text{NH}_4^+$  into amino acids was too low to be determined accurately in agreement with the fact that GS/GOGAT is the only assimilatory pathway of inorganic nitrogen in plants (Mifflin and Lea, 1976). However, to our knowledge in all the experiments in which GDH has been investigated for a non-redundant role in inorganic nitrogen assimilation, in particular under stress conditions, higher exogenous concentrations of ammonium (20–30 mM

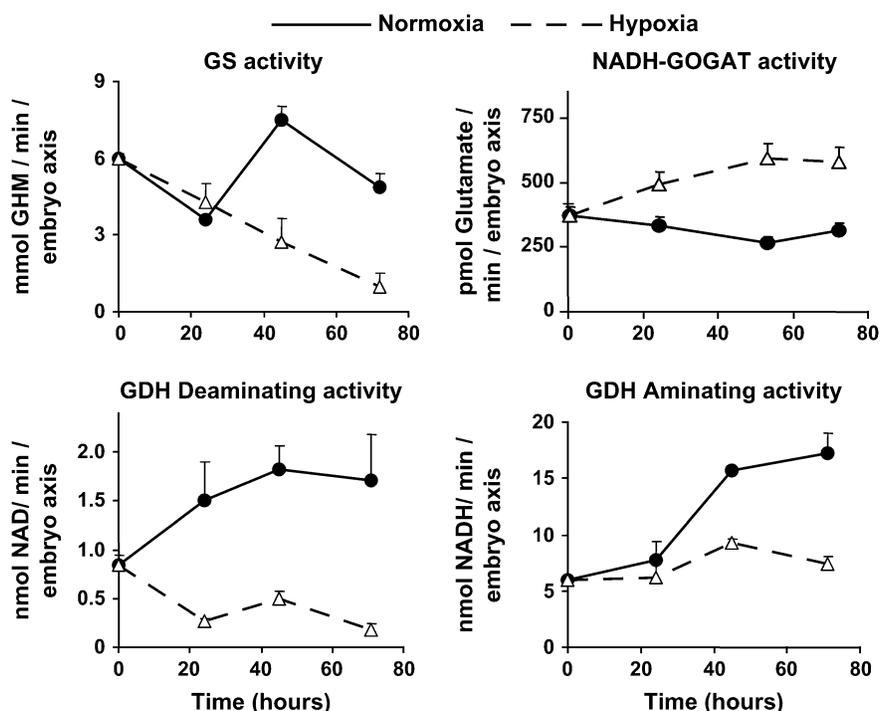


**Fig. 2.** Expression of the genes encoding *mAlaAT*, *GDH1*, *GDH3*, *NADH-GOGAT*, *GS1b*, and *ICDH* in *M. truncatula* seedlings under normoxia and hypoxic stress conditions. Young seedlings (24 h) were sampled at various times throughout post-germination growth and gene expression measured in the embryo axes. Amplified fragments of each gene were cloned into a pGEM T Easy vector (Promega). The plasmids were diluted several times to generate templates ranging from  $10^5$  to  $10^3$  copies, which were used to produce standard curves for the estimation of copy numbers. The results are expressed in copy number of cDNA corresponding to each gene studied in 5  $\mu$ l of 1:2 (v/v) dilution of retrotranscribed total RNAs (Glévec *et al.*, 2004). Results are the mean of three replicates, corresponding to the copy number of cDNA of each gene. Values followed by different letters, are significantly different according to ANOVA analysis and Newman-Keuls test ( $\alpha = 5\%$ ).

ammonium) were used, i.e. *A. thaliana* (Melo-Oliveira *et al.*, 1996), *N. tabacum* and *Vitis vinifera* (Skopelitis *et al.*, 2006). For this reason, the same experiment was also carried out with 30 mM  $(^{15}\text{NH}_4)_2\text{SO}_4$  added or not with 5 mM MSX under normoxia or hypoxia (Fig. 4). In the absence of MSX, under either normoxia or hypoxia, the results were similar to that with 2 mM  $(^{15}\text{NH}_4)_2\text{SO}_4$  (data not shown). In MSX-treated seedlings, under either normoxia or hypoxia, although at much lower levels than in the control (absence of MSX),  $^{15}\text{NH}_4^+$  was significantly incorporated into three amino acids, glutamate, alanine, and aspartate. However, the label did not go beyond these three amino acids. Incorporation of  $^{15}\text{NH}_4^+$  was accompanied by a slight increase in amounts of  $^{15}\text{N}$ -amino acids in normoxia+MSX treatment, in particular  $^{15}\text{N}$ -alanine increased five times between 2 h (0.28 nmol per embryo axis) and 24 h (1.34 nmol per embryo axis). Although lower than that of  $^{15}\text{N}$ -alanine,  $^{15}\text{N}$ -aspartate and  $^{15}\text{N}$ -glutamate showed a 2-fold increase between 2 h and 24 h of the labelling period (Fig. 4). Under hypoxia and in the presence of MSX, the net synthesis of amino acids was negligible, in particular, alanine, the amino acid marker of hypoxia did not accumulate (Fig. 4).

#### *The effect of ammonium concentrations on GDH isoforms in M. truncatula embryo axes under normoxia or hypoxic stress, revealed by activity staining on native PAGE and immunoblotting*

GDH isoforms were revealed by GDH activity staining following native polyacrylamide gel electrophoresis of extracts of embryo axes supplied with 2 mM  $(\text{NH}_4)_2\text{SO}_4$  (control), 30 mM  $(\text{NH}_4)_2\text{SO}_4$  or 2 mM  $(\text{NH}_4)_2\text{SO}_4$  under hypoxic stress (Fig. 5a). In all three treatments, GDH activity increased significantly throughout the 24 h period of the experiment. In agreement with the GDH activity determined *in vitro*, gel activity staining also showed a lower GDH activity in hypoxic embryo axes compared with the control. However, irrespective of the treatment, only a maximum of four GDH activity bands appeared, corresponding to the cationic homohexamer and two/three heterohexamers in which the cationic isoform predominated over the anionic. This result indicates that the cationic isoform of GDH was the major isoform in embryo axes. Consistent with this result, immunoblot analysis of the GDH protein in the leaves of mature plants and embryo axes showed two bands corresponding to two GDH polypeptides  $\alpha$  and  $\beta$  in the leaves, while embryo axes exhibited only one band corresponding to the  $\alpha$



**Fig. 3.** Changes in *in vitro* activities of GS, NADH-GOGAT, and aminating and deaminating GDH (NADH-GDH and NAD-GDH, respectively) in *M. truncatula* seedlings under normoxia and hypoxic stress conditions. Young seedlings (24 h) were sampled at various times throughout post-germination growth and the enzyme activities measured in embryo axes. Among these activities, GS activity was expressed as nmol/ $\gamma$ -glutamyl hydroxamate (GHM)/min/embryo axis (Glévarec *et al.*, 2004). Results are the mean  $\pm$ SE of three replicates.

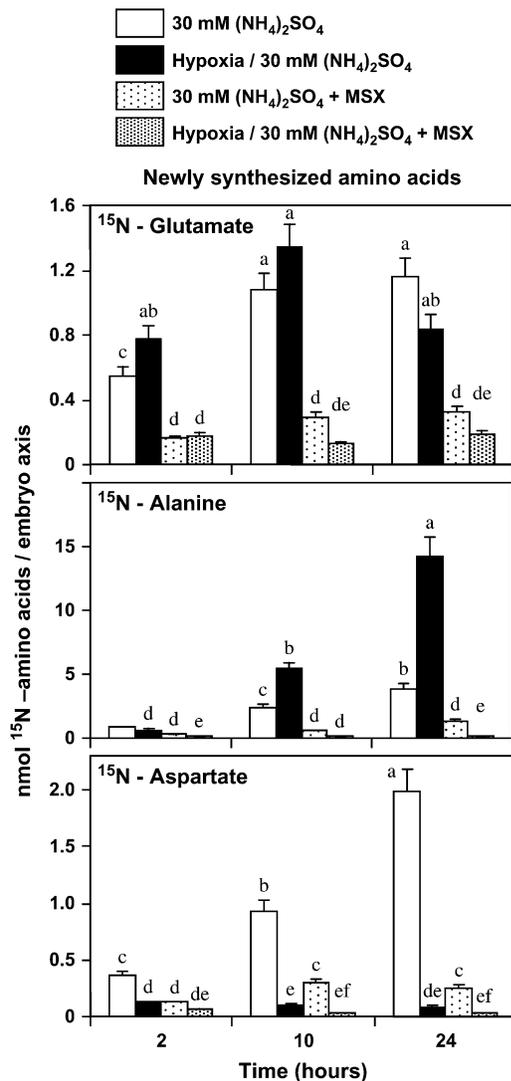
isoform. The second GDH  $\beta$  isoform was not revealed, due probably to its low abundance in axis tissue (Fig. 5b).

## Discussion

### *Concerted modulation of glutamate and alanine synthesis achieved as an adaptive reaction to hypoxic stress in young M. truncatula seedlings*

The regeneration of NAD<sup>+</sup> from NADH under low oxygen conditions by lactate (LDH) and ethanol (PDC/ADH) fermentative pathways contributes to the survival of cells by maintaining glycolysis (Ismond *et al.*, 2003; Kursteiner *et al.*, 2003). However, over a long time-scale the products of these reactions, lactate, acetaldehyde, and ethanol may have damaging consequences on cell integrity and survival (see Introduction). Thus, counteracting the damaging effects of the lactate and ethanol fermentative pathways during hypoxia is an important issue, to which amino acid metabolism and, in particular, alanine accumulation may contribute. In *M. truncatula*, hypoxic stress resulted in up-regulation of the gene coding for the mitochondrial isoform of alanine aminotransferase (*mAlaAT*) (Ricoult *et al.*, 2006). Following this, ammonium incorporation into alanine increased and newly synthesized alanine during the 24 h <sup>15</sup>NH<sub>4</sub><sup>+</sup> labelling period was twice as high in hypoxic embryo axes than in

the control and alanine accumulated as the major amino acid instead of asparagine. Feeding embryo axes <sup>15</sup>N-glutamate or <sup>15</sup>N-alanine showed that AlaAT *in vivo* activity was directed towards alanine synthesis under hypoxic stress, while the reaction was reversible in the normoxic control (Ricoult *et al.*, 2006). However, how the alanine fermentative pathway provides plants with a mechanism to tolerate hypoxic stress is still not clear. Altogether, the present results and those presented in Ricoult *et al.* (2005, 2006) have provided new insights to this issue. The data suggest that rather than just an activation of alanine synthesis, the adaptive reaction of the plant to hypoxic stress consists of a concerted modulation of nitrogen flux through both glutamate and alanine synthesis. The incorporation of <sup>15</sup>NH<sub>4</sub><sup>+</sup> and amino acid synthesis during hypoxic stress in comparison to the control has highlighted for the first time the central role of glutamate in the adaptive reaction of plants to hypoxic stress. The pools of newly synthesized glutamate (<sup>15</sup>N-glutamate) in normoxic and hypoxic embryo axes were very similar (Fig. 1) indicating that the glutamate content was subject to a very tight control. The importance of glutamate homeostasis in plant tissues has recently been reviewed (Forde and Lea, 2007). It appears as if the decrease in glutamate utilization by GS and asparagine synthetase, due, probably, to the lack of ATP, was compensated for by increased NADH-GOGAT and



**Fig. 4.**  $^{15}\text{NH}_4^+$  labelling experiment for the determination of the fate of ammonium in *M. truncatula* seedlings fed excess ammonium under normoxia or hypoxic stress. Nineteen-hour germinated seedlings were split into two batches and maintained for 5 h either on sterile deionized water or aqueous MSX (5 mM), a glutamine synthetase inhibitor. Young seedlings (24 h) were fed with 30 mM ( $^{15}\text{NH}_4$ )<sub>2</sub>SO<sub>4</sub> (99%  $^{15}\text{N}$  atom excess) for 2, 10, and 24 h under either normoxia or hypoxic stress conditions. The results are the mean of three replicates, corresponding to  $^{15}\text{N}$  atom excess in glutamate, alanine, aspartate, glutamine (single and double labelled), and asparagine (single and double labelled) (%  $^{15}\text{N}$ -amino acids) and the amounts of newly synthesized amino acids during the labelling period, expressed as nmol  $^{15}\text{N}$ -amino acids per embryo axis. Values followed by different letters, are significantly different according to ANOVA analysis and Newman-Keuls test ( $\alpha = 5\%$ ).

AlaAT activities, as revealed by increased amounts of newly synthesized alanine ( $^{15}\text{N}$ -alanine) during hypoxic stress.

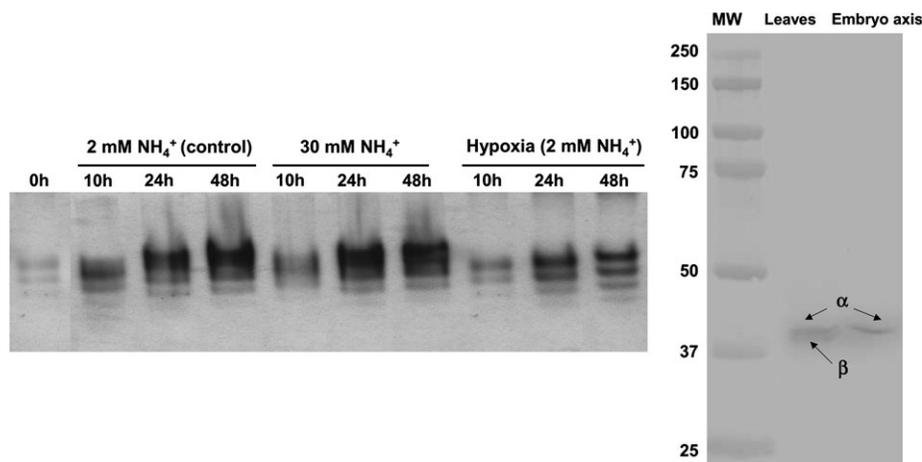
Therefore, it is likely that the reductive amination of 2-oxoglutarate by NADH-GOGAT during hypoxic stress fulfils two major roles. The first is the synthesis of glutamate, the substrate of AlaAT, and the second is the

oxidation of NADH when oxidative phosphorylation is totally or partially inhibited by the lack of oxygen, thus making  $\text{NAD}^+$  available to enable glycolysis to proceed. Unlike proline that is proposed to play a protective role in osmotic stress, alanine intrinsically may not play a protective role in hypoxic stress, rather it can be considered as a N storage form, the synthesis of which drains excess glutamate and contributes to maintaining homeostasis. Alanine may also play an indirect role in the protection of a plant against the damaging effects of the lactate and ethanol fermentative pathways. Through competition with ethanol fermentation from pyruvate, alanine synthesis saves C3 skeletons, thus avoiding a shortage in carbon availability and limiting the accumulation of acetaldehyde, a toxic compound. In addition, an increase in alanine synthesis, by competing with lactic fermentation for pyruvate, intervenes in cytosolic pH regulation. The synthesis of alanine, arising from the decarboxylation of malate to pyruvate as a result of malic enzyme activity, along with the decarboxylation of glutamate to GABA, are proton-consuming reactions (Carroll *et al.*, 1994; Edwards *et al.*, 1998; Ricoult *et al.*, 2005).

#### *GDH does not contribute to ammonium assimilation/ NAD<sup>+</sup> regeneration in hypoxia-stressed M. truncatula seedlings*

Under aerobic conditions, the expression of *GDH1* increased 2–3 times in embryo axes after the completion of germination and during early post-germination growth. The same pattern has been reported previously by Glévarec *et al.* (2004). This increase in expression was correlated with an increase in enzyme activity measured either *in vitro* or by activity staining on native PAGE. The increase in both gene expression and enzyme activity may be related to the proposed role of GDH in the mobilization of N stores during the germination of *M. truncatula*, i.e. oxidation of glutamate that allows for the production of 2-oxoglutarate and reducing power (NADH). Furthermore, in the absence of nitrate reduction, oxidative deamination of glutamate is a source of ammonium for GS. *GDH1* expression was up-regulated by hypoxic stress, while GDH enzyme activity determined either *in vitro* or by native PAGE staining, was down-regulated compared with the control. The discrepancy between the level of gene expression and enzyme activity indicates that GDH was submitted to post-translational down-regulation by hypoxic stress.

However, studies by Glévarec *et al.* (2004) indicated that *in vitro* GDH activity does not reflect whether the enzyme catalyses an amination or deamination reaction. In order to solve this problem, *in vivo*  $^{15}\text{NH}_4^+$  labelling in the presence and absence of the GS inhibitor, MSX, was used in combination with GC-MS amino acids analyses. Irrespective of labelling conditions (normoxia or hypoxia),



**Fig. 5.** (a) GDH isoenzyme patterns revealed by activity staining after native PAGE of extracts of *M. truncatula* seedlings grown under normoxia and hypoxic stress conditions. Young seedlings (24 h) were transferred for 10, 24, or 48 h on 2 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (control), excess ammonium, 30 mM or 2 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> under hypoxia. At each sampling time, proteins were extracted from embryo axes and separated on a 7% native polyacrylamide gel and stained for NAD-GDH activity. An equal amount of proteins (50 µg) was used for each lane. (b) GDH immunoblot analysis. *M. truncatula* seeds were germinated for 19 h and seedlings maintained for 21 h on sterile deionized water before proteins were extracted from embryo axes. Heat-denatured proteins were separated on a 11% SDS-PAGE gel, blotted and reacted with antibodies raised against GDH. Proteins extracted from the leaves of 2-month-old plants were used for comparison.

when embryo axes were fed ammonium at low concentration (2 mM), <sup>15</sup>N was not incorporated into amino acids in the presence of MSX. At high exogenous concentrations of ammonium (30 mM), in the presence of MSX under normoxia and to a lesser extent under hypoxia, <sup>15</sup>N was incorporated into three amino acids, glutamate, aspartate, and alanine. This result suggests that, in the absence of GS activity, under excess ammonium, glutamate would be synthesized through GDH aminating activity. In similar conditions i.e. *A. thaliana* *gdh1-1* null mutant submitted to excess exogenous ammonium (20 mM NH<sub>4</sub><sup>+</sup>) (Melo-Oliveira *et al.*, 1996) or tobacco submitted to 20 mM NH<sub>4</sub><sup>+</sup> and excess exogenous salt (250 mM NaCl) (Skopelitis *et al.*, 2006), GDH was proposed to play a non-redundant role in ammonia assimilation. However, in the work of Melo-Oliveira *et al.* (1996), the *gdh1-1* mutant plants were not analysed for their capacity to assimilate ammonium. The conclusion was drawn on the basis that the mutant showed stunted growth under artificial conditions including low light, sucrose, and high exogenous ammonium. In the work of Skopelitis *et al.* (2006), where GDH *in vivo* aminating activity was assessed in tobacco by <sup>15</sup>NH<sub>4</sub><sup>+</sup> labelling, <sup>15</sup>N was shown to be incorporated into only two amino acids, glutamate and proline, when GS was inhibited by MSX under salt stress.

In our opinion, the present results, as well as those cited in the literature, are not enough to support unequivocally the involvement of GDH in ammonium assimilation either under normoxia or hypoxic stress. At high and probably saturating concentrations of <sup>15</sup>NH<sub>4</sub><sup>+</sup>, a non enzymatic <sup>15</sup>N/<sup>14</sup>N isotopic exchange reaction cannot be ruled out, as the capacity of GDH to catalyse the exchange of

labelled ammonium with glutamate has previously been demonstrated (Aubert *et al.*, 2001). The hypothesis of isotopic exchange is further supported by the fact that the incorporation of <sup>15</sup>N into glutamate occurred only when ammonium was supplied at high exogenous concentrations ranging between 20 mM and 30 mM. In these experiments the label was never shown to be incorporated beyond glutamate and one or two other amino acids. The physiological assimilation of ammonium, even under stress conditions, implies the incorporation of inorganic nitrogen for both amino acid and protein synthesis.

Therefore, what might be the significance of the up-regulation of the expression of *GDH1* in *M. truncatula* immediately following hypoxic stress? It has been suggested that the return to aerobic conditions is anticipated in plants subjected to hypoxic stress by expressing genes whose products have functions during the subsequent recovery period (Drew, 1997). Here, it is proposed that *GDH1* was up-regulated by hypoxia induced carbon-stress, in anticipation that the product of its transcription would regenerate 2-oxoglutarate by deaminating glutamate during the subsequent post-hypoxic recovery period. Higher rates of glycolysis and ethanolic fermentation are known to lead to carbon stress due to faster depletion of sugar stores in hypoxic tissues (Ismond *et al.*, 2003). Up-regulation of the expression of *GDH* genes has been observed under various conditions associated with carbon stress, i.e. senescing leaves, low light and dark and C/N imbalance due to excess ammonium nutrition (Melo-Oliveira *et al.*, 1996; Masclaux-Daubresse *et al.*, 2005; Skopelitis *et al.*, 2006). The function of GDH in these conditions is assumed to be the oxidative deamination of glutamate that provides C

skeletons (2-oxoglutarate) and reducing power (for a review see Forde and Lea, 2007). The same pattern of regulation was observed for the hypoxia inducible *AlaATI* in *A. thaliana* (Miyashita et al., 2007). *AlaATI* was up-regulated at the transcriptional level during hypoxic stress while the major role of the encoded enzyme was shown to be the conversion of alanine into glutamate during the post-hypoxic period.

### Supplementary data

One supplementary table giving the sequences for PCR amplification of genes studied in this article is available at *JXB* online.

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