Glutamate synthase activities and protein changes in relation to nitrogen nutrition in barley: the dependence on different plastidic glucose-6P dehydrogenase isoforms

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Abstract

In barley (Hordeum vulgare L. var. Nure), glutamate synthesis and the production of reducing power by the oxidative pentose phosphate pathway (OPPP) are strictly correlated biochemical processes. NADH-GOGAT was the major root isoform, whose activity increased on a medium supplied with NH$_4^+$ or NO$_3^-$; by contrast, no noticeable variations could be observed in the leaves of plants supplied with nitrogen. In the leaves, the major isoform is Fd-GOGAT, whose activity increased under nitrogen feeding. G6PDH activity increased in the roots supplied with nitrogen; no variations were observed in the leaves. Moreover, an increase of the P2 isoform in the roots was measured, giving 13.6% G6PDH activity localized in the plastids under ammonium, and 25.2% under nitrate feeding conditions. Western blots confirmed that P2-G6PDH protein was induced in the roots by nitrogen. P1-G6PDH protein was absent in the roots and increased in the leaves by nitrogen supply to the plants. The changes measured in cytosolic G6PDH seem correlated to more general cell growth processes, and do not appear to be directly involved in glutamate synthesis. The effects of light on Fd-GOGAT is discussed, together with the possibility for P2-G6PDH to sustain nitrogen assimilation upon illumination.

Key words: Glutamate synthesis, Hordeum vulgare, oxidative pentose phosphate pathway, plastids.

Introduction

Nitrogen assimilation into carbon skeletons represents a physiological process of the utmost importance for plant growth and development. Inorganic nitrogen is assimilated into amino acids, namely glutamate, glutamine, and asparagine which play a pivotal role as N-transport compounds in plants (Lea and Miflin, 2003).

It is now generally accepted that the primary assimilation of NH$_4^+$ into amino acids occurs via the action of glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (2-oxoglutarate aminotransferase, GOGAT). The initial step involves the amination of glutamate to glutamine by glutamine synthetase, with the hydrolysis of ATP. The glutamine formed transfers the amido group to 2-oxoglutarate, yielding two molecules of glutamate in a reaction catalysed by glutamate synthase (GOGAT); the co-operating reactions of these enzymes are referred to as the GS-GOGAT cycle (Lea and Miflin, 1980).

In higher plants GOGAT occurs as two antigenically distinct forms which differ in molecular mass, kinetics, tissue distribution, and physiological role. Different GOGAT isoforms are known, indeed, because of their different specificity towards the reductant: Fd-GOGAT (EC 1.4.7.1), NADH-GOGAT (EC 1.4.1.14) (Suzuki and Gadal, 1982). Another form of GOGAT, NADPH-GOGAT, occurs in bacteria (Vanoni and Curti, 1999).

Most of the Fd-GOGAT activity is located in the leaf chloroplasts; the enzyme is a monomeric iron sulphur flavoprotein made up of a single polypeptide chain (150 kDa) (Vanoni and Curti, 1999). It is involved in the assimilation of NH$_4^+$ derived from nitrate reduction and...
from photorespiration, as well as in amino acid metabolism (Turano and Muhitch, 1999; Pajuelo et al., 1997).

Many studies suggest that Fd-GOGAT expression is mainly controlled by light: Fd-GOGAT enzyme activity and protein both increase in etiolated seedlings exposed to light. This enhancement is accompanied by a parallel increase in Fd-GOGAT mRNA, suggesting a transcriptional control of the Fd-GOGAT gene in response to light (Mattana et al., 1996; Turano and Muhitch, 1999).

NADH-GOGAT is a monomeric iron sulphur flavoprotein with a molecular mass of 200–240 kDa and its activity is located mainly in the root plastids (Gregerson et al., 1993; Lea and Miflin, 2003). NADH-GOGAT seems to be controlled by the N-status of the plant: its activity increases in response to ammonium ions and it has therefore been argued that NADH-GOGAT plays a fundamental role in primary nitrogen assimilation in plants (Lea and Miflin, 1980; Vanoni and Curti, 1999).

Both GOGAT isoforms necessitate electrons to carry on the reaction: in green tissues, photosynthesis provides the reducing equivalents necessary for glutamate synthesis, but in the dark and in non-green tissues the reducing power is supplied by the oxidative pentose phosphate pathway (OPPP) (Bowers et al., 1992; Esposito, et al., 2003).

There is a tight intermingling between OPPP and nitrogen metabolism in plants (Kruger and von Schaewen, 1996; Bogorad et al., 1980), and it has been shown that OPPP is involved in the reduction of NADP+ (Fickenscher and Scheibe, 1986), a chloroplastic isoform of glucose-6-phosphate dehydrogenase (G6PDH) which is insensitive to reductant regulation (Kumar and Scheibe, 1986). A plastidic isoform of G6PDH (P2-G6PDH), more resistant to NADPH levels, was detected (Fickenscher and Scheibe, 1986). The homogenate was then filtered through four layers of muslin and centrifuged at 3000 rpm for 20 min at 4 °C. The supernatant (fraction designated as crude extract) was used for G6PDH assays. Alternately, the supernatant (crude extract) was desalted on a Sephadex G25 column (Amersham Biosciences PD-10) equilibrated in buffer A.

Glucose-6P dehydrogenase (G6PDH) was extracted in 100 mM TRIS–HCl pH 7.5, 10 mM MgCl2, 5 mM EDTA, 1 mM PMSF, 10% glycerol, 15 μM NADP+, 1 mM DTT, and 1.5 mM mercaptoethanol. The homogenate was then filtered through four layers of muslin and centrifuged at 20 000 g for 20 min at 4 °C. The supernatant (fraction designated as the crude extract) was used for G6PDH assays. Alternately, the supernatant (crude extract) was desalted on a Sephadex G25 column (Amersham Biosciences PD-10) equilibrated in extraction buffer without PMSF and mercaptoethanol, giving no appreciable change in measurable G6PDH activity with respect to the untreated samples.

**Materials and methods**

**Growth conditions of plants**

Seeds of barley (*Hordeum vulgare* L. var. Nure) were supplied by the Istituto Sperimentale di Cerealicoltura di Fiorenzuola d’Arda (PC) and germinated in the dark for 3–5 d on moistened paper. Seedlings were then transferred to a nitrogen-free medium (Riganò et al., 1996a) in a controlled cabinet, according to Esposito et al. (2001a), at 21 °C under a 16/8 h light/dark regime with approximately 180 μmol photons m−2 s−1. Nitrogen was supplied either as 10 mM ammonium phosphate or 10 mM potassium nitrate after 7 d of hydroculture. The nutrient solutions were controlled for pH and daily adjusted to keep the initial ammonium/nitrate concentration.

**Preparation of crude extracts for enzyme activities**

Barley plants were harvested; roots and leaves were separated, quickly frozen in liquid nitrogen, and powdered in a mortar with a pestle.

Glutamate synthase isoforms were extracted with buffer A (50 mM KH2PO4–KOH pH 7.5, 100 mM KCl, 5 mM EDTA, 2 mM 2-oxoglutarate, 1 mM DTT, 1 mM phenylmethylsulphonylfluoride, PMSF), and 0.1% PVPP was added to the extraction buffer for the preparation of the crude extract of leaves, in order to avoid interference due to polyphenolic compounds. The homogenate was then filtered through four layers of muslin and centrifuged at 13 000 rpm for 30 min at 4 °C. The supernatant (fraction designated as crude extract) was desalted on Sephadex G25 column (Amersham Biosciences PD-10) equilibrated in buffer A.

Glucose-6P dehydrogenase (G6PDH) was extracted in 100 mM TRIS–HCl pH 7.5, 10 mM MgCl2, 5 mM EDTA, 1 mM PMSF, 10% glycerol, 15 μM NADP+ , 1 mM DTT, and 1.5 mM mercaptoethanol. The homogenate was then filtered through four layers of muslin and centrifuged at 20 000 g for 20 min at 4 °C. The supernatant (fraction designated as the crude extract) was used for G6PDH assays. Alternately, the supernatant (crude extract) was desalted on a Sephadex G25 column (Amersham Biosciences PD-10) equilibrated in extraction buffer without PMSF and mercaptoethanol, giving no appreciable change in measurable G6PDH activity with respect to the untreated samples.

**Organelle preparations from roots and leaves of barley plants**

Plastids from barley roots were isolated using the method previously described in detail by Esposito et al. (2003). Chloroplasts were prepared from plants collected within 2 h from the beginning of the light period. Barley leaves (30–70 g) were macerated in a mortar using a cold buffer (4:1 v/w) containing 50 mM HEPES–KOH pH 7.5, 330 mM sorbitol, 1 mM MgCl2, 2 mM EDTA, and 1 mM MnCl2, 1% (w/v) sodium ascorbate was added immediately before use. The whole subsequent procedure was carried out at 4 °C.

The homogenate was filtered through four layers of muslin and one layer of Miracloth and then centrifuged at 2500 g for 4 min at 4 °C. The pellet was resuspended in 6 ml of extraction buffer and layered on 17.5 ml of buffer (50 mM HEPES–KOH pH 7.5, 330 mM sorbitol, 1 mM MgCl2, 2 mM EDTA, 1 mM MnCl2) containing 50% Percoll (v/v). It was subsequently centrifuged at 9000 g for 4 °C for 15 min. Intact chloroplasts were withdrawn, 30 ml of extraction buffer were added, and then the suspension was centrifuged at 2500 g for 4 min at 4 °C. The pellet was designated as the plastoplast fraction.

**Organelle yield**

The yield of isolated plastids was calculated as the percentage of the plastidic marker activity in the plastid fraction with respect to the activity of the same marker enzyme in the crude extract. Similarly, contamination from other subcellular fractions was calculated as the percentage of marker enzyme activities in the plastid fraction with respect to marker activities measured in the crude extract.

The yield of isolated chloroplasts was calculated as the percentage of chlorophyll (determined according to Moran, 1982) in the plastid fraction with respect to the activity of the same marker enzyme in the crude extract. Controls on chloroplast yield were performed using alkaline pyrophosphorylase (APPase) or glyceraldehyde-3P dehydrogenase as markers enzymes, giving no appreciable difference with respect to the chloroplast fraction.
Plastid integrity and G6PDH latency

Plastid integrity was measured by calculating the latencies of APPase and G6PDH in intact organelles broken by different methods. Intact plastids were broken by freeze-thawing (Tettlow et al., 1993), by Triton X-100 detergent (McDonald and ap Rees, 1983), or by osmotic shock (Esposito et al., 2003).

Enzyme assays

Fd-GOGAT activity was determined using reduced methyl viologen as the electron donor (Lea et al., 1990). The reaction mixture consisted of 200 mM KH2PO4–KOH pH 7.5, 10 mM glutamine (Gln), 10 mM 2-ketoglutarate, 15 mM methyl viologen, 1 mM amino-oxyacetic acid (transaminase inhibitor), and extract. After 5 min of pre-incubation at 30 °C, the reaction was started by the addition of reductant solution (47 mg Na2S2O4, 50 mg NaHCO3 in 1 ml of water). After 30 min of incubation at 30 °C, the reaction was terminated by adding 1 ml of ethanol and then shaking vigorously.

Fd-GOGAT activity was determined by the quantitative measurement of glutamate using High Performance Liquid Chromatography (HPLC) as described previously (Esposito et al., 2003). The activity was expressed as nmol glutamate formed min−1 mg−1 protein.

NADH-GOGAT activity was assayed consistently within 2 h of extraction, monitoring NADH oxidation at 340 nm and using two control mixtures (minus 2-ketoglutarate and minus glutamine) to correct for endogenous NADH oxidation. The assay mixture contained: 50 mM KH2PO4–KOH pH 7.5, 10 mM KGA, 10 mM Gln, 10 mM 2-ketoglutarate, 15 mM methyl viologen, 1 mM amino-oxyacetic acid (transaminase inhibitor), and extract. After 5 min of pre-incubation at 30 °C, the reaction was started by the addition of reductant solution (47 mg Na2S2O4, 50 mg NaHCO3 in 1 ml of water). After 30 min of incubation at 30 °C, the reaction was terminated by adding 1 ml of ethanol and then shaking vigorously.

Fd-GOGAT activity was determined by the quantitative measurement of glutamate using High Performance Liquid Chromatography (HPLC) as described previously (Esposito et al., 2003). The activity was expressed as nmol glutamate formed min−1 mg−1 protein.

G6PDH activity was assayed monitoring NADP+ reduction at 340 nm (Esposito et al., 1998). The assay mixture contained: 50 mM TRIS–HCl pH 8.0, 10 mM MgCl2, 0.15 mM NADP+, 3 mM glucose-6P, and extract. The activity was expressed as nmol reduced NADP+ min−1 mg−1 protein.

Other enzymes as markers during organelles isolation were assayed as described in the references indicated: phosphoenolpyruvate carboxylase (PEPCase, cytosolic marker) (EC 4.1.1.31), (Esposito et al., 1998), cytochrome c oxidase (Cyt-c ox, mitochondrial marker) (EC 1.9.3.1), (McDonald and ap Rees 1983), APPase, (plastidic marker) (EC 3.6.1.1), (Tettlow et al., 1993), glyceraldehyde-3P dehydrogenase (EC 1.2.1.9), (Kang and Rawsthorne, 1996).

Electrophoresis and western blotting

Fd-GOGAT and G6PDH isoforms from barley leaves and roots were resolved by SDS–PAGE performed in 10% gel (4% stacking gel), loaded with approximately 10–40 μg protein and run according to Laemmli (1970) for 90 min at 40 mA/180 V. The separated polypeptides were transferred to a nitrocellulose membrane (0.2 μm pore size), then incubated for 2 h at room temperature with antibodies raised against barley Fd-GOGAT, as described by Pajuelo et al. (2000), or against cytosolic, P1 and P2-G6PDH (Wendt et al., 2000). After incubating the membrane with secondary antibodies, cross-reacting polypeptides were identified and stained for alkaline phosphatase activity.

Blotted membranes were scanned on a personal computer and evaluated using Image J software (NIH, USA).

Results

GOGAT activities and proteins in barley plants

NADH-GOGAT activities

NADH-GOGAT activities were higher in the roots than in the leaves of barley plants grown for different days after nitrogen supply.

Roots of plants grown without nitrogen exhibited a NADH-GOGAT activity of 7.1±0.4 nmol min−1 mg−1 protein (Fig. 1A); this value was about 6-fold higher than the activities measured in the leaves (see below).

NADH-GOGAT activity did not change in roots of plants grown without nitrogen during the experimental period (7 d), remaining at rate values between 6.17–7.64 nmol min−1 mg−1 protein (P >0.1).

Supply of 10 mM NH4+ caused a sudden increase of NADH-GOGAT activity up to 20.1±4 nmol min−1 mg−1 protein (2.8-fold, P <3×10−5) 1 d after nitrogen supply, which further increased up to 45.4±2.7 nmol min−1 mg−1 protein 5 d after ammonium supply (Fig. 1A).

When barley plants were grown on 10 mM NO3−, the levels of NADH-GOGAT activity in the roots increased up to 18.2±0.9 nmol min−1 mg−1 protein on the third day (2.6-fold, P <5×10−4) (Fig. 1B), slowly decreasing during the following experimental period (Fig. 1B).

Proteins and chlorophyll determination

Proteins were estimated by the method of Bradford (1976) based on the colorimetric assay with blue-Coomassie R-250. Bovine serum albumin was used as the standard.

Chlorophyll was extracted in N,N’-dimethylformamide and the concentration of pigment was calculated according to Moran (1982).

Fig. 1. NADH-GOGAT activities in barley roots (closed circles) and leaves (open circles) measured in plants grown for 7 d in a N-free medium; then nitrogen as 10 mM (NH4)2PO4 (A), or KNO3 (B) was supplied (arrow), and enzyme activities were assayed on the given days. Data are means of three independent determinations ±SE. Other details are in the Materials and methods.
In the leaves, NADH-GOGAT activity remained constant at values near to 1–1.4 (P > 0.3) nmol min\(^{-1}\) mg\(^{-1}\) protein following the supply of 10 mM ammonium or 10 mM nitrate, in comparison with plants grown on a nitrogen-free medium (Fig. 1A, B). No change in NADH-GOGAT activity was observed in leaves during the growth on a nitrogen-free medium for 7 d (1.1±0.15 nmol min\(^{-1}\) mg\(^{-1}\) protein, P > 0.3).

**Fd-GOGAT activities**: Fd-GOGAT activities have been measured in both roots and leaves of barley plants grown for different days after nitrogen supply. Fd-GOGAT activity was higher in the leaves than in the roots and it was slightly enhanced by exogenous nitrogen.

In the roots of plants grown without nitrogen the activity was 171.9±2.6 nmol min\(^{-1}\) mg\(^{-1}\) protein, and did not change in the first 7 d of growth on a nitrogen-free medium; Fd-GOGAT activity slightly increased to 215±20.1 nmol min\(^{-1}\) mg\(^{-1}\) protein 1 d after NH\(_4^+\) supply (+25%, P <0.02), then it returned to the initial value after 3 d (Fig. 2A). Roots of plants did not show any appreciable (P >0.13) variation in Fd-GOGAT activity (182±22 nmol min\(^{-1}\) mg\(^{-1}\) protein) following nitrate supply (Fig. 2B).

The western blotting analysis, using monoclonal antibodies raised against barley leaves Fd-GOGAT, did not show appreciable variations in detectable Fd-GOGAT protein in barley roots upon different nitrogen feeding conditions (not shown).

In the leaves of plants grown without nitrogen, Fd-GOGAT activity was 265.2±39.8 nmol min\(^{-1}\) mg\(^{-1}\) protein; these activity rates remained unchanged for at least 7 d (not shown); a 2-fold increase (P <0.03) over 520 nmol min\(^{-1}\) mg\(^{-1}\) protein was observed 1 d after ammonium supply, then Fd-GOGAT activity gradually returned to the control value in 7 d (Fig. 2A). In the leaves of plants grown on NO\(_3^-\) the activity increased up to 390 nmol min\(^{-1}\) mg\(^{-1}\) protein (+47%, P <0.1) 3 d after nitrate supply (Fig. 2B).

In the leaves there was an increase of the polypeptides reacting with Fd-GOGAT antibodies when external nitrogen was available: the increase of stained bands was greater with NH\(_4^+\) after 1 d, whereas it was less evident with NO\(_3^-\) (Fig. 3).

To investigate the effect of light on Fd-GOGAT protein, 7 d-etiolated barley seedlings were exposed to the light, leaves were detached in the first 5 h upon illumination, crude extracts were assayed for Fd-GOGAT activity and processed by Western blotting.

Fd-GOGAT activity doubled within 1 h upon illumination and a further 4-fold increase in comparison with the dark value was reached within 5 h (data not shown).

Western blots showed no appreciable change (densimetric values were ±15%) in the signal measured in crude extracts from dark-grown leaves with respect to that measured after illumination, both in nitrogen-free grown and in nitrate-fed grown plants (Fig. 4); similar results were obtained in ammonium-fed plants (not shown).

**Fig. 2.** Fd-GOGAT activities in barley roots (closed circles) and leaves (open circles) measured in plants grown for 7 d in a N-free medium; then nitrogen as 10 mM (NH\(_4\))\(_2\)PO\(_4\) (A), or KNO\(_3\) (B) was supplied (arrow), and enzyme activities were assayed on the given days. Data are means of three independent determinations ± SE. Other details are in the Materials and methods.

**Fig. 3.** Western blots of Fd-GOGAT in crude extracts from leaves of barley plants grown for 7 d in a N-free medium (–N); then nitrogen as 10 mM (NH\(_4\))\(_2\)PO\(_4\), or KNO\(_3\) was supplied, and proteins were detected by SDS-PAGE/western blotting using antibodies raised versus barley Fd-GOGAT (Pajuelo et al., 1997) on the given days. Data are representative of at least three experiments. Other details are in the Materials and methods.
Effects of inhibitors on GOGAT activities: To investigate the occurrence of de novo GOGAT formation, inhibitors of protein synthesis were added to the medium together with nitrogen sources on the seventh day and GOGAT activities were measured 24 h later.

Cycloheximidé (CHX) (4 mg l⁻¹), a powerful inhibitor of eukaryotic protein synthesis, suppressed NADH-GOGAT activity to less than 10% of the control value, whereas chloramphenicol (Cm) (50 mg l⁻¹), an inhibitor of 50S ribosome subunits (70S ribosomes), did not affect the enzyme rate (Table 1).

Similarly, CHX provoked a complete disappearance of the Fd-GOGAT activity in the roots and a 90% decrease in the leaves after 24 h (Table 1); similar results were obtained on detectable Fd-GOGAT protein in western blots, where densitometric analysis confirmed an 80–90% disappearance of proteins reacting versus Fd-GOGAT antibodies in the presence of CHX in the presence of ammonium or nitrate, respectively (Fig. 5A).

Cm did not affect Fd-GOGAT activities after 24 h, which were 80–93% of the control (Table 1); accordingly, western blots, subjected to densitometric analysis, did not show any appreciable changes in Fd-GOGAT protein content both in leaves (Fig. 5) and in roots (not shown).

G6PDH activities and G6PDH isoforms in barley

G6PDH activities have been measured in both roots and leaves of barley after nitrogen supply. G6PDH activity was higher in the roots than in the leaves; moreover, the effects of N supply were measured only in heterotrophic tissues, being the photosynthetic cells which were essentially unaffected by the supply of a nitrogen source.

In the leaves of plants grown without nitrogen, G6PDH activity was 42.2 ± 4.1 nmol min⁻¹ mg⁻¹ protein; G6PDH levels remained unchanged around a value of 42.3 ± 0.7 nmol min⁻¹ mg⁻¹ protein three days after ammonium supply, or 42.8 ± 2.4 nmol min⁻¹ mg⁻¹ protein after nitrate supply (Fig. 6).

In the roots of plants grown without nitrogen, G6PDH activity was 111 ± 6.4 nmol min⁻¹ mg⁻¹ protein and an increase up to 192 ± 7.9 nmol min⁻¹ mg⁻¹ protein (+73%, \( P < 3 \times 10^{-5} \)) was observed after 3 d of growth on a medium supplied with NH₄⁺. Roots of plants supplied with NO₃⁻ showed an activity of 201 ± 6.6 nmol min⁻¹ mg⁻¹ protein (+81%, \( P < 1.5 \times 10^{-5} \)), a slightly higher value compared with that measured in ammonium supplied plants (Fig. 6).

Effects of protein synthesis inhibitors: The occurrence of de novo synthesis of G6PDH was investigated by adding cycloheximidé together with nitrogen sources on the 7th day, measuring G6PDH activities 24 h later.

CHX provoked a 50% inhibition of G6PDH activity in the roots of both ammonium-fed and nitrate-fed plants with respect to the control plants. G6PDH activities measured in nitrogen-fed roots in the presence of protein synthesis inhibitor thus appear identical to those measured in roots grown in a medium lacking nitrogen sources. No changes were recorded in the leaves after 24 h of CHX exposition (Table 1).

**Table 1. Effects of inhibitors of protein synthesis on NADH-GOGAT (A), Fd-GOGAT (B), and G6PDH (C) activities in roots and leaves of barley plants under different nitrogen feeding conditions**

| CHX, cycloheximidé; Cm, chloramphenicol. Data are expressed as nmol min⁻¹ mg⁻¹ protein; in parenthesis the percentage of activity with respect to the control plants.
| n.d., not determined.

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<th>Root</th>
<th>Leaf</th>
<th>Root</th>
<th>Leaf</th>
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<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>157 (100%)</td>
<td>358 (100%)</td>
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<td><strong>(B) Fd-GOGAT</strong></td>
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<td>Control</td>
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Fig. 4. Western blots of Fd-GOGAT in crude extracts from leaves of etiolated (7 d) barley plants grown on a nitrogen-free medium (-N) or supplied with 10 mM KNO₃ (NO₃⁻). Leaves were detached at the end of the dark period (0 time) and after the given hours of illumination. Antibodies are raised versus barley Fd-GOGAT (Pajuelo et al., 1997). Other details are in the Materials and methods.
Western blots of G6PDH: Crude extracts of barley roots were probed with G6PDH antibodies specific for the cytosolic (Cy-G6PDH), chloroplastic (P1-G6PDH), and plastidic/chloroplastic (P2-G6PDH) isoforms. A densitometric analysis allowed the changes in G6PDH proteins on the blots to be estimated. Western blots showed a very faint band of the P2 isoform in extracts from N-free roots, whereas the cytosolic isoform was evident. An increase of the cytosolic enzyme was observed after nitrogen supply (+76%); moreover, P2-G6PDH became clearly visible after nitrogen supply. The increase of the P2-G6PDH isoform was more conspicuous (+20%) with nitrate than with ammonium. The P1-G6PDH was not detectable in root extracts, regardless of the experimental growing conditions (Fig. 7).

In the roots the presence of CHX in the nutrient medium produced a smaller increase in the cytosolic protein (96% of control minus N) and very faint bands of P2-G6PDH could be observed under nitrogen nutrition (15–18% of the control without CHX) (Fig. 7).

Crude extracts of barley leaves showed the presence of all the three G6PDH isoforms: the cytosolic isoform slightly increased (+35%) with ammonium. There was a basic and constant presence of P2-G6PDH in all the conditions examined (changes were ±15%) (Fig. 7); P1-G6PDH isoform, which was barely detectable in N-free leaves, was induced both by ammonium and nitrate (Fig. 7).

Supply of CHX to the plants did not cause any noticeable changes (changes were ±10%) in the different G6PDH isoforms in the leaf extracts. Only P2-G6PDH signal seemed to be weaker in ammonium plus CHX (45% of control) (Fig. 7).

Compartmentation of G6PDH

To investigate the different activities of G6PDH isoforms in plastids and chloroplasts, enzyme assays were conducted.
on isolated organelles and the percentage of activity present within plastids was calculated taking account of yield and contamination of the preparations by other subcellular fractions.

The preparation methods for plastids from barley roots provided a plastid yield of 35.2±4.2%, with a negligible (0.39±0.14%) cytosolic contamination; the root plastids showed an integrity of 73.5±4.9% (Table 2). The chloroplast yield from barley leaves was 63±1.8%; the cytosolic contamination was maintained around 0.58±0.13%; chloroplast preparations exhibited an integrity of 49.4±8.2% (Table 2).

The recoveries of the enzyme activities in the different fractions obtained during the preparations suggested no significant loss of activity (Table 2).

G6PDH activity in root plastids was 13.6±3.5% of the total activity in ammonium-fed plants. When plants were fed with nitrate, an increase in the percentage of plastidic isoform(s) was measured, reaching a level of 25.2±4.9% of the total G6PDH activity.

A one-tailed t-test of the data indicating the different distribution of the P2-G6PDH, gave a P value of 8×10⁻³, producing a statistical difference of the results between roots fed with nitrate or ammonium.

The increase in cytosolic G6PDH activity in nitrogen-fed roots can be estimated in view of the appearance of the P2-G6PDH isoform and its different relative abundance following nitrate or ammonium feeding. Western blots suggest that in roots grown without nitrogen the P2-G6PDH protein is not detectable; therefore, it could be calculated that in roots grown without nitrogen the P2-G6PDH protein accounts for 82 nmol min⁻¹ mg⁻¹ protein; if P2-G6PDH represents 13.6% of the total G6PDH activity, this plastidic activity accounts for 26 nmol min⁻¹ mg⁻¹ protein; therefore, the increase of the cytosolic isoform under ammonium is about 55 nmol min⁻¹ mg⁻¹ protein.

G6PDH activity in isolated chloroplasts from N-fed plants accounted for 16.4±5.5% of the total activity measured in the leaf crude extract. In these experiments no significant differences in the percentage of G6PDH in the leaf chloroplasts isolated from nitrate-fed or ammonium-fed plants could be observed, but this result needs further investigation.

Discussion

The results presented in this study indicate that inorganic nitrogen supplied to barley plants considerably increased NADH-GOGAT activity in the roots: NO₃⁻ increased NADH-GOGAT activity 2.5-fold in 24 h, and NH₄⁺ led to a 6-fold increase 3 d after supply. In rice roots, ammonium supply provoked NADH-GOGAT expression in 3–6 h, and protein detection in 24 h (Hirose et al., 1997; Ishiyama et al., 2003); this effect is not directly induced by ammonium, but probably by one of its derivatives (Ishiyama et al., 2003).

Accordingly, nitrogen supply to barley plants provoked a 2-fold increase in total G6PDH activity in the roots in the first 3 d. It should be underlined here that this result is in agreement with the ammonium-induced appearance of the P2-G6PDH isoform in barley roots reported previously (Esposito et al., 2001b) and suggests a close link between P2-G6PDH-mediated OPPP, and NADH-GOGAT activity in the roots.

The OPPP only produces NADPH, and different mechanisms could be involved in the transfer of the reductants from NADPH to electron donors for GOGAT activities. The existence of FNR enzyme in the roots to produce reduced ferredoxin from NADPH for Fd-GOGAT has been established (Oji et al., 1985). By contrast, the source of reductants for NADH-GOGAT must be discussed; the presence of a putative trans-hydrogenase has been suggested (Bowsher et al., 1992), even if it is still not proven in root plastids; or reductants can be provided by other enzyme activities involved in the ‘malate valve’, the main regulatory mechanism to balance reductants and energy in the plant cell (Scheibe, 2004). Anyway, further research is required to determine the source of reductant for NADH-GOGAT in non-photosynthetic plastids.

It cannot be ruled out that an effect of glutamine levels is in increasing NADH-GOGAT synthesis (Hirose et al., 1997), as previously demonstrated for G6PDH (Esposito et al., 2001b). The increased amino acid synthesis after nitrogen supply supports this hypothesis: glutamine levels in the roots increased 50-fold within 2 d after ammonium feeding.

Repeating the same calculus for NO₃⁻-fed roots, where P2-G6PDH represents 25.2% of the total activity, it appears that the increase of cytosolic isoform is less than 40 nmol min⁻¹ mg⁻¹ protein, giving a 36% increase with nitrate feeding.

G6PDH activity in isolated chloroplasts from N-fed plants accounted for 16.4±5.5% of the total activity measured in the leaf crude extract. In these experiments no significant differences in the percentage of G6PDH in the leaf chloroplasts isolated from nitrate-fed or ammonium-fed plants could be observed, but this result needs further investigation.

Table 2. Properties of plastids and chloroplasts preparations

The data are expressed as a percentage of marker activities (see Materials and methods) measured in organelle fractions with respect to the crude extract. In the lower part of the table, the recoveries of soluble proteins, marker enzymes, and chlorophyll are shown as a percentage of activity found in plastid plus cytosol fractions with respect to crude extract ± SE. n, number of separate determinations used to calculate mean values. Other details in Materials and methods; n.d., not determined.

<table>
<thead>
<tr>
<th>Plastids</th>
<th>Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>35.2±4.2</td>
</tr>
<tr>
<td>Integrity</td>
<td>73.5±4.9</td>
</tr>
<tr>
<td>Cytosolic contamination</td>
<td>0.39±0.14</td>
</tr>
<tr>
<td>Mitochondrial contamination</td>
<td>2.33±0.6</td>
</tr>
<tr>
<td>Recoveries</td>
<td></td>
</tr>
<tr>
<td>PEPCase</td>
<td>108±5.9</td>
</tr>
<tr>
<td>APase</td>
<td>97.1±4.7</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>80.1±4.6</td>
</tr>
<tr>
<td>G6PDH</td>
<td>99.8±2.6</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>–</td>
</tr>
</tbody>
</table>
supply to nitrogen-starved barley plants (Rigano et al., 1996a,b). Complex mechanisms were activated in order to avoid the inhibition of the GS-GOGAT cycle: (i) glutamine was promptly stored in the vacuole (Dietz et al., 1990) or (ii) exported to the shoot (Rigano et al., 1996a), or (iii) the amide-group was transferred to aspartate to synthesize asparagine, the main nitrogen compound transported through the xylem in barley, whose levels strongly increased following glutamine raise (Rigano et al., 1996b).

The increase in G6PDH rate upon nitrogen feeding in the roots can be partially ascribed to the rise in cytosolic enzyme activity and, in part, to the appearance of a plastidic G6PDH isoform (Esposito et al., 2001b), which shows the properties of P2-G6PDH described in potato (Wendt et al., 2000), barley roots (Esposito et al., 2001a), and spinach leaves (Debnam et al., 2004); this isoform appears to be strictly correlated to glutamate synthesis in barley root plastids (Esposito et al., 2003).

In higher plants the P2-G6PDH represents a consistent percentage of total activity in the root cell, ranging between 15% in pea to 35% in maize (Debnam and Emes, 1999). These results suggest that the percentage of P2-G6PDH in the roots is about 25% with nitrate, and about 14% with ammonium, accounting for an increased request of electrons for N assimilation when nitrate reductase/nitrite reductase system is induced (Wright et al., 1997). These results are in accordance with previous findings (calculated on a chromatographic elution percentage of cytosolic and plastidic isoforms), in which 17% of plastidic G6PDH was located in the root plastid 7 d after ammonium supply (Esposito et al., 2001a).

The increase in total G6PDH activity in the roots under nitrogen must be discussed, due to the appearance of P2-G6PDH isoform: in barley roots grown without nitrogen the P2-G6PDH protein is not detectable, confirming previous reports (Esposito et al., 2001b). Moreover, it could be calculated that ammonium feeding caused a 50% rise in cytosolic activity, whereas P2-G6PDH represents 14% of total activity; this indicates an effectively increased synthesis of asparagine and glutamine which are promptly translocated to the aerial part of the plant through the xylem flux (Rigano et al., 1996a,b). In NO3–-fed roots, P2-G6PDH represents 25% of the total activity, and cytosolic isoform increased of 36% under nitrate feeding. This would reflect a major control of the nitrate carrier/NR/NiR systems on the whole cell metabolic processes.

Accordingly, the synthesis of the inducible P2 isoform, and of the part of cytosolic G6PDH subsequently produced upon NH4+ feeding (Esposito et al., 2001b), are stopped together with de novo protein synthesis. A constitutive G6PDH activity remained unchanged under inhibitory effect on protein synthesis, suggesting that cytosolic G6PDH has a low turnover rate; the possibility that the cytosolic G6PDH may be blocked at its N-terminal amino acid sequence cannot be excluded (unpublished data).

The data suggest that plastidic OPPP in the roots produces reductants for NADH-GOGAT activity, whereas Fd-GOGAT activity and protein remained substantially unchanged in the roots during nitrogen assimilation.

The induction of NADH-GOGAT activity is correlated with the de novo synthesis of the enzyme: blocking protein synthesis with cycloheximide results in a lack of increase in NADH-GOGAT activity.

By contrast, a stimulatory effect of exogenous nitrogen on Fd-GOGAT activities in the leaves was observed. One day after ammonium supply Fd-GOGAT activity doubled and a 50% increase was also observed 3 d after nitrate feeding.

Interestingly, the prompt increase in Fd-GOGAT activities upon nitrogen supply is not coupled with similar changes in detectable Fd-GOGAT protein in western blots (Mattana et al., 1996): only in ammonium-fed plants a noticeable increase of stained band was observed 1 d after nitrogen supply in the leaves. In both cases, activities and proteins (not shown) returned to the basal levels in 7–10 d suggesting the achievement of a new metabolic state of the photosynthetic cell.

It is well known that Fd-GOGAT expression is induced by light in maize (Suzuki et al., 2001), A. thaliana (Ziegler et al., 2003), barley (Pajuelo et al., 1997), and this effect can be mimicked by red light, (Suzuki et al., 2001; Ziegler et al., 2003), and/or sucrose (Ziegler et al., 2003); these results support the hypothesis that light is not the primary effector on Fd-GOGAT expression, and suggest that Fd-GOGAT activity in green leaves is only partly regulated by the light (Pajuelo et al., 1997).

Evidence is provided for the light-regulation of Fd-GOGAT activity, even if the amount of Fd-GOGAT protein did not seem to change significantly in the first 24 h upon illumination suggesting that a basal level of protein is present in etiolated leaves and ready to activate upon switching on of the light.

Accordingly, no change in Fd-GOGAT protein was observed in maize after switching off the light for at least 48–72 h (Suzuki et al., 1996), despite measurable changes in the activity (Pajuelo et al., 1997).

The possibility of different Fd-GOGAT isoforms occurring has been suggested in a number of papers, some proposing distinct Fd-GOGAT isoforms for leaves and roots (Avila et al., 1987), others supporting the idea of a constitutive Fd-GOGAT whose activity is in part modulated by light, (Suzuki et al., 2001) and a ‘supporter’ enzyme able to sustain nitrogen assimilation in the dark or under stress conditions (Coshigano et al., 1998). It cannot be ruled out that Fd-GOGAT antibodies used in this study (Pajuelo et al., 1997) might detect a root isoform different from leaf Fd-GOGAT, and this supposition needs further investigation.

In leaves, total G6PDH activities remained unchanged; this result would suggest a lack of correlation, in photosynthetic cells of higher plants, between OPPP and nitrogen
assimilation. Immunoblotting analysis showed changes both in the amount of cytosolic and P1-G6PDH isoforms following nitrogen feeding. This would suggest post-translational modifications of the enzymes or metabolites effects to control the activity (Hauschild and von Schaewen, 2003; Debnam et al., 2004).

In the leaves the change in P1-G6PDH appears to be similar to that noted for Fd-GOGAT, whereas P2-G6PDH remained at a constitutive level, similar to NADH-GOGAT.

In illuminated green leaves, P2-G6PDH exhibits a constant measurable activity and a clearly detectable amount of protein, despite changes in nitrogen supply to the plants. This might suggest that NADH-GOGAT would maintain a constant rate of nitrogen assimilation, regardless of light and nitrogen status of the leaf, sustained by P2-G6PDH which, in its turn, would ensure a constant furnishing of reductants both in the dark and in the light, when photosynthetic electron flux could be partially inhibited by stomata closure and/or other oxidative stresses. It is necessary to underline that P2-G6PDH is able to uphold a considerable part of its maximum activity in the presence of high NADPH/NADP+ ratios (Esposito et al., 2001a, 2003), which have been proved to occur in leaves under illumination (Lendzian, 1980; Forti et al., 2003); this implies the activity of P2-G6PDH in the light supports nitrogen assimilation. The possibility of P2-G6PDH functioning in the light has been recently proposed (Debnam et al., 2004) following light stress in spinach leaves.

As already known, P1-G6PDH would not be able to work under the light because of the high NADPH/NADP+ ratio (Lendzian, 1980), and it would be activated after the first phase of transition from light to darkness; in the darkness, the NADPH/NADP+ ratio quickly decreased to one-third of the light value within 3 min upon darkening of barley leaves (Forti et al., 2003), therefore P1-G6PDH, no longer inhibited, would provide the reducing power required by Fd-GOGAT activity during the dark nitrogen assimilation. According to this study’s experiments, P1-G6PDH was insensitive to protein synthesis inhibition by cycloheximide, suggesting, apart from the control by reductants, a post-translational modulation of enzyme activity; in a recent study, similar results for potato chloroplast P1-G6PDH have been reported and a regulation of the chloroplast P1-G6PDH by protein phosphorylation has been suggested (Hauschild and von Schaewen, 2003).

The cytosolic isofrom of G6PDH is not directly influenced by the N-status: it is involved in cellular replication and homeostasis, thus playing a central role in the overall basal metabolism (Kruger and von Schaewen, 2003). As recently proposed, the cytosolic G6PDH appears to be regulated by glucose levels, in a sugar-sensing pattern (Hauschild and von Schaewen, 2003), indicating an involvement of this isofrom in a wide network of cellular processes.

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References


