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RESEARCH ARTICLE

GLUTAMATE DEHYDROGENASE PLAY MAJOR ROLE IN THE NITROGEN METABOLISM UNDER CADMIUM STRESS IN *SOLANUM LYCOPERSICON*

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ABSTRACT

Glutamate (Glu) metabolism and amino acid translocation were investigated in the control and cadmium stressed leaves of tomato (*Solanum lycopersicon*) using ^{15}N-ammonium and ^{15}N-Glu tracers. Regardless of organ type, ^{15}N-ammonium assimilation occurred via glutamine synthetase (GS; EC 6.1.1.3), both in the control and stressed plants, and it did not depend on Glu dehydrogenase (GDH; EC 1.4.1.2). The ^{15}N-ammonium and ammonium accumulation patterns support the role of GDH in the deamination of ^{15}N-Glu to provide 2-oxoglutarate and ^{15}N-ammonium. In presence of cadmium, excess ^{15}N-ammonium was incorporated into asparagines that served as an additional detoxification molecule. In the presence of MSO, glutamate, alanine and γ-amino butyrate of roots tissue continue to become labelled with ^{15}N under Cd treatment. Free ammonia accumulates rapidly in both leaves and roots in response to MSO. The labelling kinetics of amino acids in roots of tomato plants in the presence of cadmium show that continued assimilation of ^{15}N-ammonium can occur when the GS/GOGAT cycle is inhibited. GDH protein is more abundantly in the mitochondria of cadmium stress plants than control. These findings open, therefore, new perspectives toward a better understanding of the function of GDH, particularly in relation to cadmium stress.

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INTRODUCTION

Ammonium is also produced via internal metabolic reactions, including photorespiration, hydrolysis of nitrogen carrying and storage molecules, and amino acid conversion (Ireland and Lea. 1999). In non leguminous C_3 plants, such as tomato (*Solanum lycopersicon*), the photorespiratory ammonium production by the oxidative decarboxylation of Gly exceeds by about 10-fold the primary nitrate reduction in the vegetative leaves. In the senescing leaves, a large amount of ammonium is produced as a result of protein hydrolysis (Hörteinsteiner and Feller. 2002). Therefore, it is essential that toxic ammonium be immediately reassimilated into organic molecules for nitrogen cycling. Ammonium is assimilated into Gln amide group, which is then transferred to the position of 2-oxoglutarate, yielding two molecules of Glu by the concerted reaction of Gln synthetase (GS; EC 6.1.1.3) and Glu synthase (Fd-GOGAT; EC 1.4.7.1; NADH-GOGAT; EC 1.4.1.14). Nitrogen is then incorporated into Asp, Ala, Asn, and other amides and amino acids. Gln-dependent Asn synthetase (AS; EC 6.3.5.4) provides Asn, which serves as a nitrogen carrier together with Gln and Glu. Numerous studies have been carried out to define the roles of enzymes in nitrogen assimilation and remobilization, tightly interrelated processes during plant growth and development (Miflin and Habash, 2002). It was proposed that ammonium might be directly incorporated into Glu by amination of 2-oxoglutarate via mitochondrial Glu dehydrogenase (NADH-GDH; EC 1.4.1.2) and subsequently into Gln by cytosolic GS1 under particular physiological conditions. Studies on source-sink relations have shown that GDH is induced in plants when nitrogen remobilization is maximal (Masclaux *et al*. 2002). This led to the proposal that the physiological role of GDH is to synthesize Glu for translocation in senescing leaves (Miflin and Habash. 2002). However, there is no evidence to discern a redundant or indispensable role of GDH and GOGAT for Glu synthesis and nitrogen remobilization. In addition, GDH catalyzes the reversible oxidative deamination of Glu to supply 2-oxoglutarate and ammonium (Masclaux *et al*., 2002). To better understand the role of GDH in Glu metabolism in the coordinated reaction with GS, we studied the kinetics of in vivo turnover of ^{15}N-Glu fed to leaf and root samples of cadmium treated tomato plants.

The time course of ^{15}N-ammonium assimilation into the amino acids was then determined in vivo in control and stressed young leaves and roots. To understand the cellular compartmentation of Glu synthesis and amino acid translocation, we investigated the tissue-specific localization of GDH and GS enzymes in tomato plants.

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MATERIAL AND METHODS

Plant material and growth conditions: Seeds of tomato (*Solanum lycopersicon*. Mill cv 63/5F1) were germinated on moistened filter paper at 25ºC in the dark. After 7 days, uniform seedlings were transferred to plastic beakers filled with continuously aerated, basal nutrient solutions of an initial pH 5.8-6, containing 3 mM KNO_3, 0.5 mM Ca $(NO_3)_2$, 2.4 mM KH_2PO_4, 0.5 mM $MgSO_4$, 100 µM Fe-K_2-EDTA, 30 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, and 1 µM $(NH_4)_6Mo_7O_{24}$. Plants were grown in a growth chamber (26ºC/70 % relative humidity during the day, 20ºC/90 % relative humidity during the night). The photoperiod was 16 h daily with a light irradiance of 150 µmol m^{-2}. s^{-1} at the canopy level. At the age of 10 days after transplant, cadmium was added to the medium as $CdCl_2$ at 0 to 50 µM. After one week of Cd treatment, plants were separated into leaves and roots. *^{15}N-Labeling experiments, amino acid analysis, and gas* chromatography-mass spectrometry measurement*:* ^{15}N-labeling experiments were performed using leaves and roots of 17 day-old tomato plants. Samples of leaf and root were prepared from plants treated with 50 µM of cadmium or without cadmium (control plants). Samples were floated on 10 mM MES buffer, pH 6.5, containing 10 mM $CaCl_2$, 40 mM KCl, and 2% (w/v) polyethylene glycol, either in the light (photosynthetic photon flux density, 250 μmol photons m^{-2} s^{-1}) or in the dark, with or without 1 mM MSO for 1 h. Afterward, ^{15}N-ammonium (99% enrichment) or ^{15}N-Glu (99% enrichment; Euriso-top S.A.) was added to the medium. Leaf and root part were dipped into the labeling solutions, quickly transferred, and rinsed with a large volume of water before freezing in liquid nitrogen (time 0). Leaf samples and root part were further incubated in the light and collected at 0, 120, and 240 min. Samples were frozen in liquid nitrogen prior to analysis.

Total amino acids and ammonia were extracted with 2% (w/v) sulfosalicylic acid. Extracts were centrifuged at 17,500*g* for 20 min to eliminate cellular debris. Amino acids were applied to a column (AG 50W-X8 resin, 100-200 mesh, H^+-form, 5 x 0.5 cm; Bio-Rad Laboratories), washed with 4 mL water, and eluted with 2.5 mL of 6 M NH_4OH, then with 1 mL water. Total amino acids were determined by the method of Rosen (1957) and ammonium content by the Berthelot reaction. One-half of the supernatant was adjusted to pH 2.1 with LiOH, and amino acids were separated and quantified by ion-exchange chromatography on a Biotronic LC5001 analyzer using a standard amino acid mixture (Benson standard PANB) by Perkin-Elmer Nelson 2100 software (Rochat and Boutin. 1989). From the remaining supernatant, amino acids were derivatized with either *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide in acetonitrile (Chaves Das Neves and Vasconcelos. 1987). The atom percentage of amide and amino ^{15}N was determined by gas chromatography (GC)-mass spectrometry (MS) analysis (model MD800; Fisons). ^{15}N-Ammonium released from ^{15}N-Glu was determined after purification and derivatization steps as described by Fujihara *et al*. (1986) and Ek *et al*. (1990). The ^{15}N-ammonium extract (100 μL) was mixed with 1 mL of 5% $NaHCO_3$ to adjust pH to 8 before adding 4 μL of pentafluorobenzoyl chloride (PFB-Cl; Sigma). After centrifuging twice for 1 min, the mixture was incubated at room temperature for 30 min. Pentafluorobenzamine (PBFA), a product from the reaction of ammonia on PBF-Cl, was extracted with 1 mL acetate. The organic phase was separated by centrifugation at 2,000*g* for 5 min and mixed with 250 μL of 6% H_3PO_4 to eliminate $NaHCO_3$ and PFB-Cl. Water was eliminated using $CuSO_4$. Then PBFA was derivatized by *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide and analyzed as described for amino acid analysis.

Metabolite extraction and analysis

Amino acid and NH_4^+ were determined after extraction in a 2% solution of 5-sulfosalicylic acid. Total amino acid content was assayed by the Rosen colorimetric method using glutamine as a reference. Individual amino acid composition was determined on pooled samples extracted from an equal dry weight of three plant repeats and phloem and xylem sap, using ionexchange chromatography (Rochat and Boutin. 1989). Free NH_4^+ was determined by the phenol hypochlorite colorimetric method (Berthelot reaction) using $(NH_4)_2SO_4$ as a reference.

Protein Extraction and Enzyme Assays

GS activity was measured by the method reported by O'Neal and Joy (1973). GDH aminating and deaminating activities were assayed as described by Masclaux *et al*. 2000. Total soluble proteins were measured according to Bradford (1976).

Immunolocalization of GDH

For the two control and cadmium treated plants, the middle part of the flag leaves was fixed in freshly prepared 1.5% paraformaldehyde dissolved in sodium phosphate buffer pH 7.4 for 4 h at 4°C. The material was dehydrated in an ethanol series (final concentration 90% ethanol) then embedded in LR White resin (Polysciences, Warrington, PA, USA). Polymerization was carried out in gelatine capsules at 50°C.

For structural investigations, thin sections (1 µm) were stained by the PAS-NBB method as described by Sangwan *et al*. (1992). For immuno-transmission electron microscopy, ultra thin sections of 70 nm were mounted on 400 mesh nickel grids and allowed to dry at 37°C. Sections were first incubated with 5% normal goat serum in Tl buffer (0.05 M Tris-HCl buffer containing 2.5% NaCl, 0.1% BSA and 0.05% Tween 20, pH 7.4) for 1 h at room temperature then with anti-Rubisco (Pinck *et al*. 1984), anti-GS or anti-GDH rabbit serum, in T1 buffer for 14 h at room temperature. Sections were then washed three times with Tl buffer and incubated with 10 nm colloidal gold-goat anti-rabbit immunoglobulin complex (Sigma-Aldrich, St Louis, MO, USA) diluted 1: 70 in T1 buffer for 2 h at room temperature. After several washes, grids were treated with 5% uranyl acetate in water and observed with a CM12 electron microscope (Philips, Eindoven, The Netherlands) at 100 kV. For immunological studies with a light microscope, thin sections of 1 µm were floated on drops of sterile water on slides and essentially the same procedure of labelling (describe above) was performed. Labelling was silver enhanced as described by the supplier (British Biocell, Cardiff, UK) and sections were back-stained with 1% (w/v)

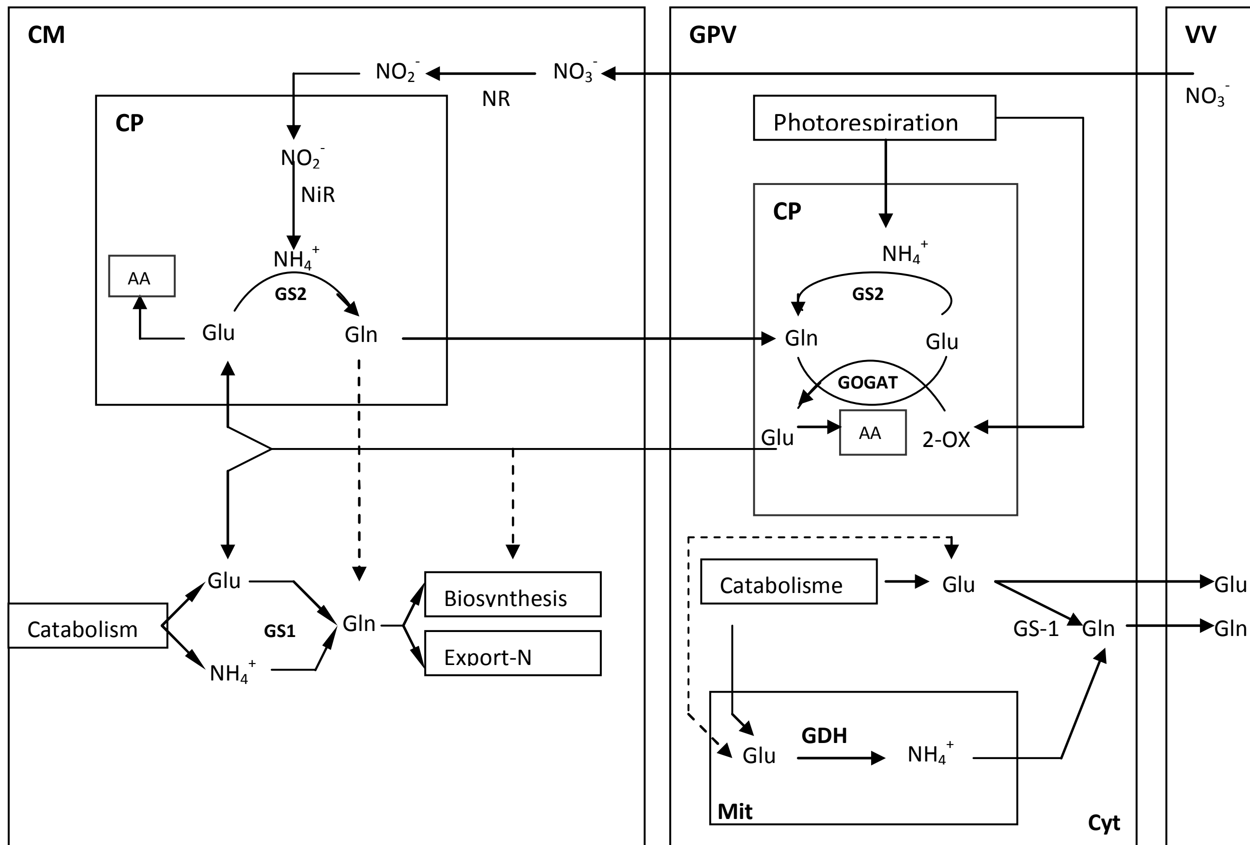

Figure 1. Proposed diagram of the photorespiratory nitrogen cycle involving respiratory Glu metabolism with release of ammonium and 2-oxoglutarate between three subcellular compartments. Ammonium can be produced during photorespiration and oxidative deamination of Glu by GDH and assimilated by the action of the GS-GOGAT pathway. AA: amino acide, CM: cellular mesophyl, CP: chloroplaste, Cyt: cytosol, GDH: glutamate dehydrogenase, GOGAT: glutamate synthase, GS1: cytosolic glutamine synthetase, GS2: plastodic glutamine synthetase, Gln: glutamine, Glu: glutamate, NR: nitrate reductase, NiR: nitrite reductase, 2-OX: 2-oxoglutarate, GPV: prevascular gaine, VV: vascular vaisseau.

fuschine before microscopic observations under bright field plus epipolarized light.

RESULTS

Respiratory Ammonium Release from Glu:In this experiment, we hypothesized that Glu is deaminated in leaves. Glu is involved in the photorespiratory nitrogen cycle during the day and provides Gly and 2-oxoglutarate in the peroxisome through the reaction of Glu:glyoxylate aminotransferase (Fig 1). Two molecules of Gly are in turn converted to CO_2, ammonia, and Ser via the Gly decarboxylase multienzyme complex and Ser hydroxymethyltransferase. Glu deamination through the anaplerotic pathway, involving GDH, could participate in mitochondrial respiration during the day/night cycle (Masclaux-Daubresse *et al*. 2002). Ammonium released in mitochondria by photorespiration and oxidative respiration could then be reassimilated by GS (Fig 1). To investigate Glu behaviour in cadmium treated plants, leaf and root samples were incubated with ^{15}N-Glu either in control or in treated plants. Total ammonium levels were higher in cadmium treated than in control leaves and roots (Fig 2). That a remarkable difference was detected in ammonium levels between 4 hours or 8 hours after ^{15}N-Glu incubation conditions. The addition of Met sulfoximine (MSO) dramatically inhibited GS activity, whereas it did not affect the aminating and deaminating activities of GDH (data not shown). Following MSO treatment, ammonium reassimilation through GS was inhibited and the increase in total ammonium content via photorespiration became apparent as the difference between the control and cadmium treatments both in old and young leaves, except at 30 min in young leaves (Fig 2, B and D). Release of ^{15}N-ammonium from ^{15}N-Glu was higher in the stressed plants compared to the control plants both in leaves and roots (Fig 2). The addition of MSO led to similar rates of ^{15}N-ammonium release from ^{15}N-Glu in treated and not treated leaves with cadmium (Fig 2, A and B). However, the roots accumulated higher amounts of total ammonium than the leaves. In the presence of cadmium, the inhibition of ammonium assimilation by MSO resulted in higher ^{15}N-ammonium accumulation in roots than in leaves after 8 hours (Fig 2, B and D). The results suggest that an extra ^{15}N-ammonium was released from ^{15}N-Glu deamination in the cadmium treated plants by the reaction independent of photorespiration in the leaves.

Kinetics of Ammonium Assimilation into Amide and Amino Nitrogen

The labelling kinetics of amino acids in roots of tomato plants supplied with 1mM MSO for 2h and then with 5mM ^{15}N-Glu for subsequent 12h, shows that continued assimilation of

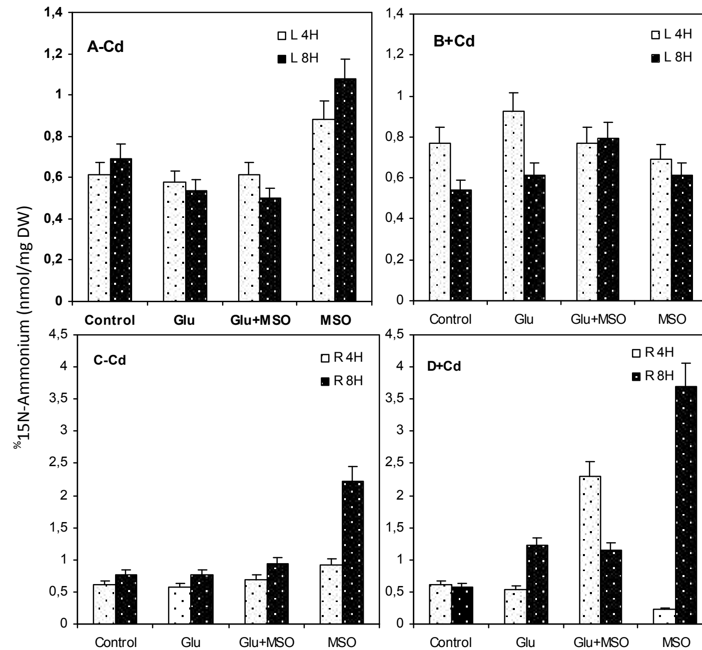

Figure 2. Changes in the level of [^{15}N]ammonium in leaves (A) and roots (C) tissues from control and cadmium treated plants (B) and (D). Leaves and root samples from 17-days-old tomato plants were incubated into the solutions without or with 1 Mm MSO, were harvested at 4 and 8 hours. [^{15}N] Ammonium content was expressed as nmol/g DW. Values represent the means of analysis from five independent plants. DW, dry weight.

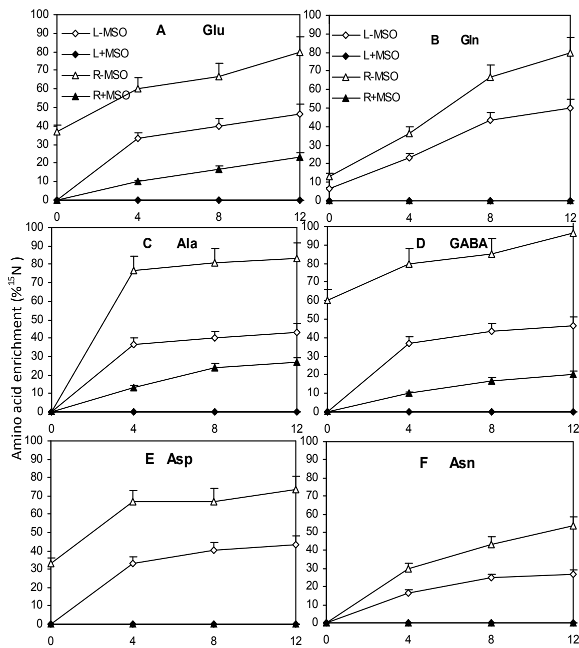

Figure 4. Kinetic analysis of ^{15}N-ammonium incorporation into amides acids in the presence of 20 µM of cadmium in the medium culture. Leaves and roots samples from 17 day-old tomato plants were floated on incubation buffer. After addition ^{15}N-ammonium (time 0), samples were incubated and harvested at 2, 4, 8 and 12h from the solution without or with MSO (GS inhibitor). ^{15}N labelling in amide and amino nitrogen was determined by a GC-MS analyser. Values represent the means of analysis on leaf and root samples from five independent plants.

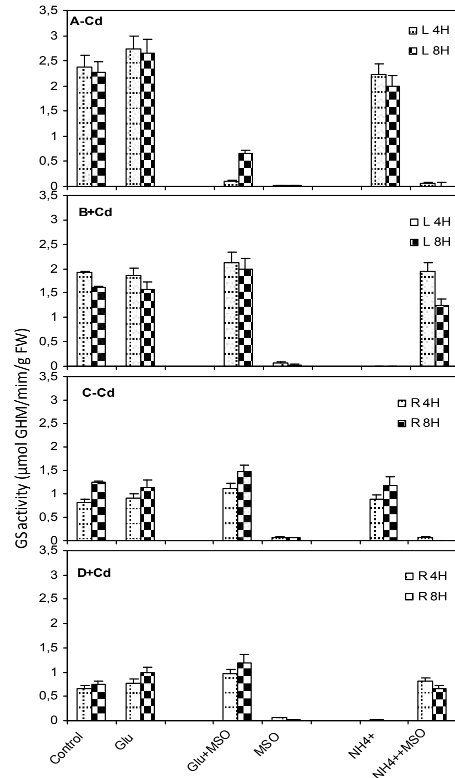

Figure 5. Effect of cadmium on the activity of glutamine synthetase (GS) in leaf and root tissues of tomato plants, which treated or not treated with MSO (GS inhibitor). Glutamine synthetase activity was expressed as µmol GHM/min/g FW. Values represent the means of analysis from five independent plants. FW, fresh weight.

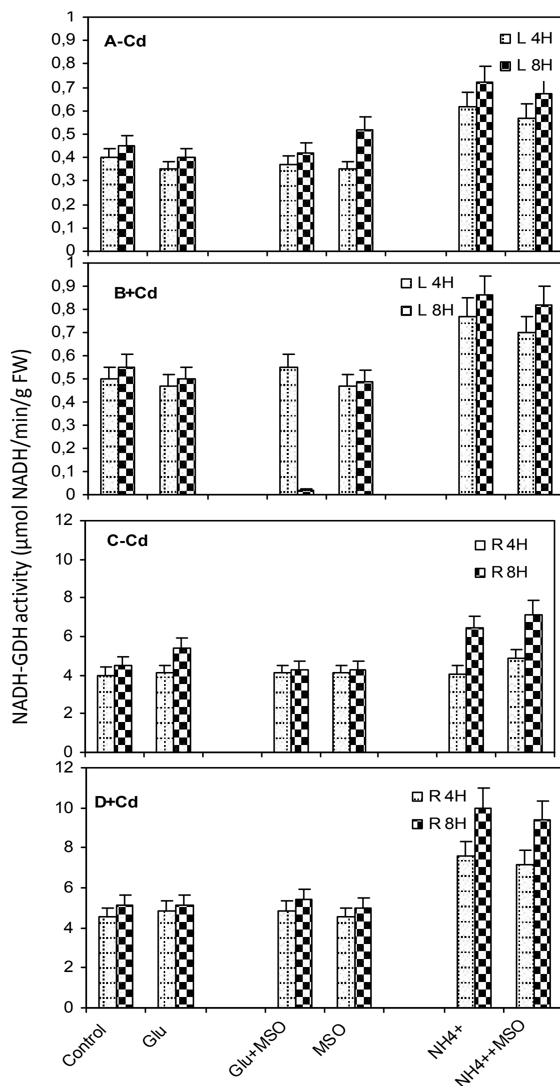

Figure 6. Effect of cadmium on the activity of glutamate dehydrogenase NADH-dependent (NADH-GDH) in leaf and root tissues of tomato plants, which treated or not treated with MSO (GS inhibitor). Glutamate dehydrogenase activity was expressed as µmol NADH/min/g FW. Values represent the means of analysis from five independent plants. FW, fresh weight.

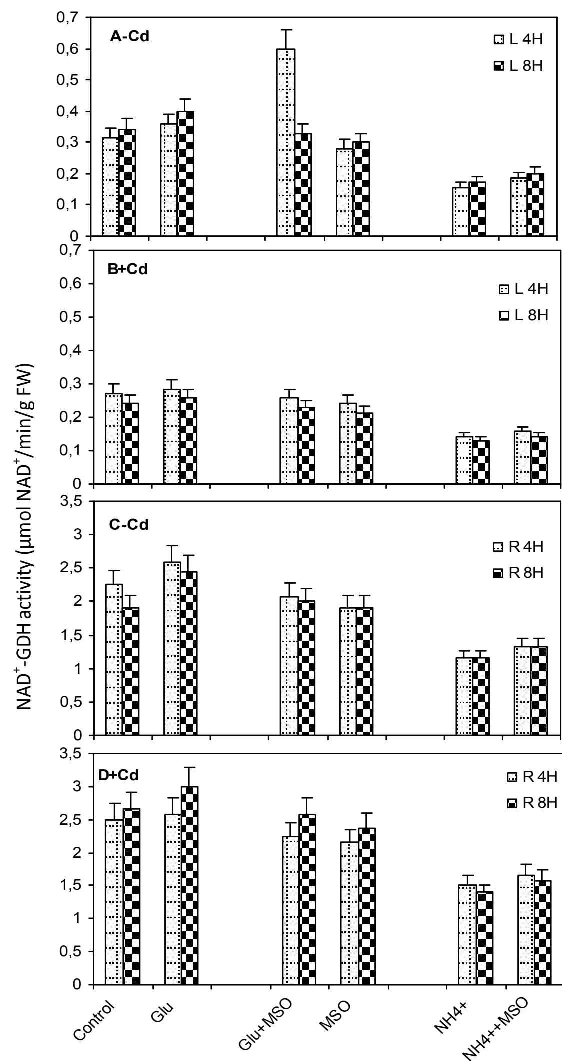

Figure 7. Effect of cadmium on the activity of glutamate dehydrogenase NAD^+-dependent (NADH-GDH) in leaf and root tissues of tomato plants, which treated or not treated with MSO (GS inhibitor). Glutamate dehydrogenase activity was expressed as µmol NAD^+/min/g FW. Values represent the means of analysis from five independent plants. FW, fresh weight.

$(^{15}N)H_4^+$ can occur when the GS/GOGAT cycle is inhibited (Fig 3). In the presence of MSO, three amino acids (glutamate, alanine and γ-aminobutyrate (GABA)) of root tissue continue to become labelled with ^{15}N under conditions where labelling of the amino-N moiety of glutamine is completely inhibited (Fig 3, A-D). The most logical explanation for these observations is that GDH catalyses some synthesis of glutamate in tomato roots, and that this glutamate can be used both for transamination with pyruvate to yied alanine, and decarboxylation to yied GABA. It is worth noting that in roots in the absence of MSO, glutamate and GABA are much more heavily labelled than glutamine amino-N after 2 hours of exposure to ^{15}N-Glu (Fig 3, A, B and D). This would not be inconsistent with some primary ammonia assimilation via GDH. An alternative explanation for these results is that, in the presence of MSO, direct assimilation of ammonia into alanine occurs via an alanine dehydrogenase in tomato roots, with alanine being transaminated to glutamate and/or GABA. However, in the absence of MSO, glutamate and GABA are much more heavily labelled that alanine at early time points (Fig B, C and D), suggesting that it is unlikely that alanine is a precursor of glutamate and GABA. It is evident that is an MSO-insensitive pathway of ammonia assimilation in operation in tomato roots, with GDH remaining a leading candidate for this pathway. I n tomato roots, it was estimated this flux was approximately 30 to 50 nmol/h. g FW. However, as discussed below, this rate could be underestimated as a result of protein turnover and associated isotopic dilution of the free glutamate and alanine pools by ^{15}N-amino acids released from protein, and further underestimated if MSO has secondary effects on photosynthesis and carbon economy with in turn affects the supply of 2-oxoglutarate to GDH in vivo. Free ammonia accumulates rapidly in both leaves and roots. This free ammonia is poorly labelled with ^{15}N (Fig 2) and is probably derived from photorespiratory catabolism of

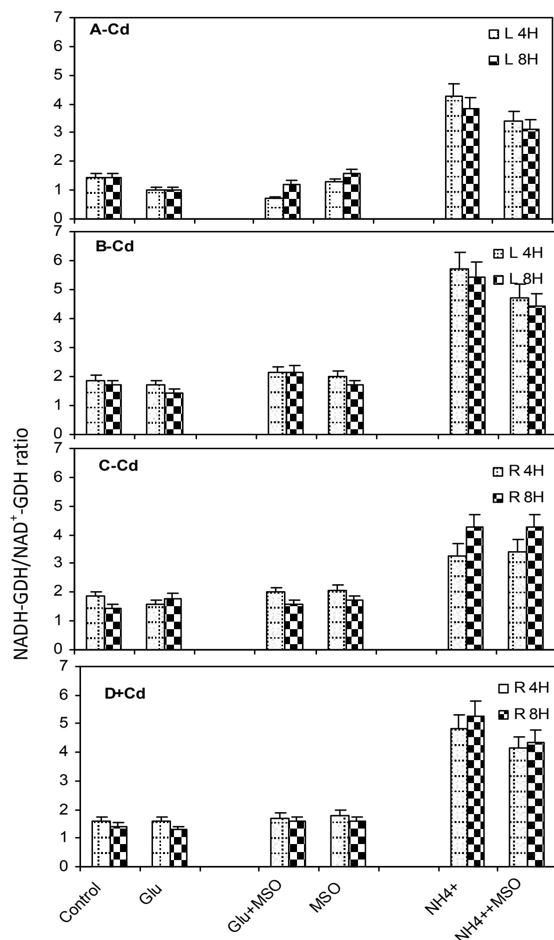

Figure 8. Effect of cadmium on the NADH-GDH/NAD$^+$-GDH ratio in leaf and root tissues of tomato plants, which treated or not treated with MSO (GS inhibitor). Glutamate dehydrogenase activity was expressed as µmol NAD$^+$/min/g FW. Values represent the means of analysis from five independent plants. FW, fresh weight.

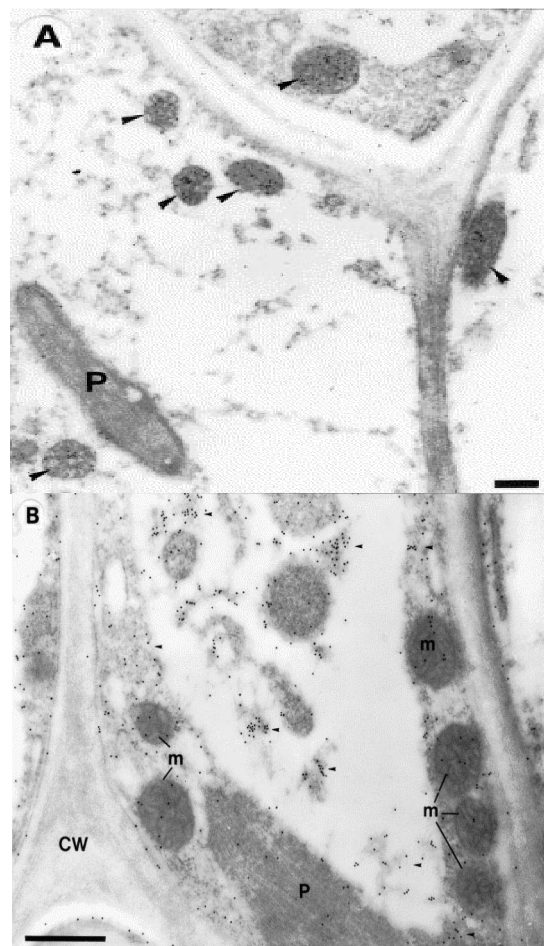

Figure 9. In situ localisation of in leaves of tomato. (A) Control section incubated with anti-GDH serum. Particles are visible in low level only in the mitochondria (arrows). (B) Immunolocalisation of GDH in a mesophyll cell of cadmium treated plants. Gold particles are present both in the cytosol and the mitochondria. P, plastid; CW, cell wall; m, mitochondria. Bars = 0.5 µm. Immunotransmission electron microscopy experiments were performed as described previously using antibodies GDH from grapevine.

unlabeled storage pools of amino acids, and catabolism of amino acids derived from protein turnover. The low ^{15}N abundance of ammonia accumulated in leaves (Fig 2 A-B) means that continued glutamate synthesis from NH_4^+ in the leaf tissue of MSO-treated plants would be difficult to detect because of isotope dilution from $^{15}NH_4^+$. The pools of glutamine, glutamate, alanine, aspartate and asparagines after 12 hours exposure to 15NGlu were, on average , 5- to 10-fold lower in the MSO treated than in the control (-MSO) plants.

The kinetics of ^{15}N-ammonium assimilation were determined in leaves and roots both in presence of cadmium (Fig. 4). When tomato plants were treated with 20 µM of cadmium (Fig 4), the ^{15}N enrichment in Glu and Gln were substantially increased in leaf and root tissues (Fig 4, A and B). MSO inhibit completely GS activity but did not inhibit the GDH aminating and deaminating activities (Fig 5, 6 and 7). As the GDH aminating activity was induced in cadmium treated tomato tissues (Chaffei *et al*. 2010a). The net increase of ^{15}N-Glu in leaves and roots, where tissues were incubated with MSO, suggest that that GDH assimilates ammonium in the tomato tissues especially under stress conditions. In contrast ^{15}N enrichment in Ala and GaBa were highly decreased in cadmium treated roots in parallel to the decrease in the leaves (Fig 4, A and D). These results suggest that MSO did not inhibit the total GOGAT activity and that the difference was due to the higher Glu contents. Moreover, MSO inhibited the labelling of ^{15}N-Ala and ^{15}N-GaBa in the cadmium treated roots (Fig 4, C and D). The labelling patterns correlate with the operation of the GS-GOGAT cycle. The result obtained with Asp and Asn show the substantially decrease in Asp in cadmium treated roots (Fig 4, E). In contrast the Asn content was increased both in leaves and roots (Fig 4, F). The addition of MSO resulted in the complete inhibition of the labelling of ^{15}N-Gln, and the continuously synthesis of ^{15}N-Glu, suggesting that GS not the sole catalyzes the efficient entry of ammonium in our conditions. In cadmium treated plants, the high GDH-aminating activities measured in vitro from leaf and root tissues (Fig 6). The tomato tissues exhibit high in vivo rates of ammonium assimilation in the presence of MSO, a potent inhibitor of GS (Fig 6, B and D). To test this hypothesis, plants were supplied $^{15}NH_4$ in the presence of MSO. Presence of the inhibitor resulted in approximately 90% inhibition of in vitro GS activity (Fig 5, A-D). The insert of figure 7shows the levels of GDH deaminating activities of

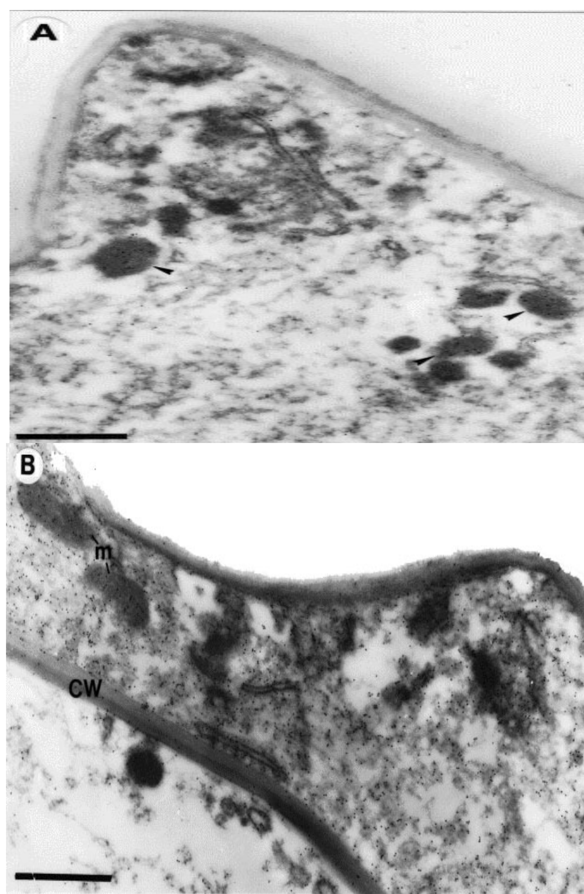

Figure 10. In situ localisation of GDH in tomato epidermis root tips cells. A positive control was performed using anti-GDH serum (A), GDH was only detected in the mitochondria (arrows). As expected, the labelling is restricted to the cytosol (arrows). (B) Immunolocalisation of GDH in cadmium cells located in the root hair zone. A strong labelling is observed both in the cytosol and in the mitochondria. CW, cell wall; m, mitochondria. Bars = 1 µm. Immunotransmission electron microscopy experiments were performed as described previously using antibodies against GDH from grapevine.

tomato leaves and roots, with or without MSO. These results support that GDH strongly aminates 2-oxoglutarate in Glu (Fig 6, B and D) but also exerts low deaminating activity (Fig 7, B and D) under stress growth conditions. In addition, it is shown that the in vitro the increase of aminating / deaminating GDH ratio in cadmium treated tissues with or without MSO (Fig 8, A-D) reflect the in vitro implication of aminating GDH activities in the balancing of the cellular levels of three majorcomponents: the ammonium ions, 2-oxoglutarate, and Glu, under physiological stressed growth conditions, if GDH operates in the aminating direction, it may assimilate excessive ammonium ions in concert with GS/Glu synthase cycle reactions and/or assimilate some photorespiratory ammonium (Tobin and Yamaya. 2001). The cadmium treated tomato tissues overexpressing the *gdh* gene encoding for the α-subunit of GDH showed increased tolerance to heavy metal stress (Chaffei *et al*. 2010a) (Fig 11).

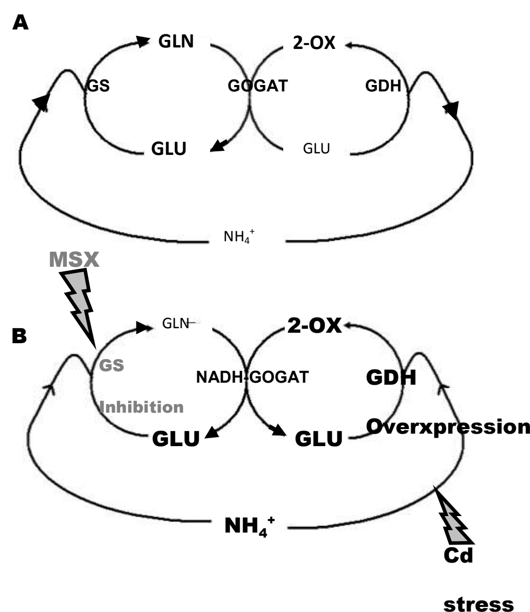

Figure 11. Scheme of ammonium recycling in tomato plants showing the occurrence of a futile cycle implying the GS/GOGAT cycle and the deamination of Glu by GDH. (A) Under standard physiological conditions of plants. (B) In the presence of the inhibitor of GS, MSO, under cadmium stress conditions. The font size of the characters for an enzyme activity or a metabolite is proportional to its activity or its concentration, respectively.

Recent findings on the cellular and subcellular localisation of GDH

In a recent study, the subcellular localisation of GDH was investigated in tomato leaves and roots using immunogold-labelling experiments. The protein was found to be preferentially located in the mitochondria of the phloem companion cells in both organs (Fig 9 and Fig 10). As shown in figure 9B, the presence of GDH protein in the cytosol with the highly content in the mitochodria were obtained when cytoimmunolocalisation was performed on cadmium stressed leaves of tomato. A control using antibodies against glutamate dehydrogenase (GDH) clearly shows that the protein is only present in the mitochondria (Fig 9A). Although the function of GDH in the cytosol is induced in this cellular compartment, it plays an important role in the recycling of carbon and nitrogen molecules in source organs and/or at specific stress conditions. This may be the case when the enzyme activity is high, like in mature roots (Fig 10A), or induced in response to either internal or external supply of ammonium.

Moreover, the fact that we observed an increase in the amount of protein in the mitochondria in roots of cadmium treated plants (Fig 10B). This increase was often combined with changes in the enzymatic activities pattern observed following leaves and roots of cadmium treated tomato plants (Fig 6, B and D). When tomato plants were treated with 20 µM of cadmium, GDH activity increased. Further work is now required to assess the role of the enzyme especially in the phloem mitochondria of cadmium stressed leaves and roots. In particular, it remains to determine if the two genes, each encoding one of the two polypeptides which combine in different ratios to form the seven NADH-GDH isoenzymes

(Myashita and Good. 2008), are expressed simultaneously or independently depending on the physiological status of the mitochondria phloem and of the cytosol.

DISCUSSION

Nitrogen metabolism in senescing tomato tissues induced by cadmium is characterized by a progressive hydrolysis of stromal proteins and degradation of chloroplasts (Hörteinsteiner and Feller. 2002). Since the main metabolic process in tomato plant senescence consists of nutrient remobilization, toxic free ammonium should be rapidly refixed into the amino acids to avoid deteriorating effects and provide nitrogenous forms suitable for source-sink transport. Our ^{15}N-labeling study provides evidence that significant ^{15}N-ammonium was released from ^{15}N-Glu deamination by GDH in the dark to higher extents in old than in young leaves. This is consistent with the higher GDH activity in response to natural senescence (Masclaux-Daubresse *et al*. 2002). It is estimated that up to one-third of the Glu-dependent respiratory rates in isolated mitochondria can be attributed to the GDH deamination reaction (Miyashita and Good. 2008). The carbon flow from Glu oxidation becomes important under conditions of carbon limitation in cadmium stress conditions (Chaffei *et al*. 2004) and carbohydrate starvation (Miyashita and Good. 2008). Also, the transition of control to old stressed tissues is characterized in part by a decrease in Suc (Masclaux *et al*., 2000). Therefore, it is conceivable that GDH supplies 2-oxoglutarate by Glu oxidation for the nitrogen and carbon cycle in Cd treated leaves.

Label of ^{15}N-ammonium was rapidly incorporated into ^{15}N-Gln by GS in the light and in the presence of cadmium regardless of different tissues of tomato plants. Kinetics of ^{15}N-ammonium assimilation clearly showed that Fd-GOGAT and/or NADH-GOGAT transferred ^{15}N of Gln to ^{15}N-Glu as soon as 2 hours after ^{15}N-ammonium feeding in tabsence of cadmium, while the ^{15}N-Glu labeling by GOGAT was slightly delayed in presence of heavy metal. Despite the induction of cytosolic GS1 and a partial degradation of chloroplasts in both leaf and root of stressed plants, the chloroplastic GS2 protein remains predominant over the cytosolic GS1 protein in tomato leaves (ratio of 95%:5% in leaves; Chaffei *et al*., 2004). This implies that both the chloroplastic GS2 and cytosolic GS1 are involved in ammonium assimilation. Our in vivo ^{15}N-labeling data clearly contrast with the proposal that GDH and cytosolic GS1 play the major role for the synthesis and reallocation of amino acids in senescing leaves (Habash, 2002). Indeed, Glu synthesis from ammonium and 2-oxoglutarate in isolated plant mitochondria has been reported (Yamaya *et al*., 1986), but the rate of Glu formation was as low as 0.2% of photorespiratory ^{15}N-ammonium release or 1.2% of the oxidative deamination rate of Glu (Aubert *et al*., 2001).

Moreover, MSO completely blocked ^{15}N transfer from ^{15}N-ammonium to the amide and amino groups of Glu and Gln in leaves and roots, but Glu content not affected in roots. These data provide strong evidence that the GDH plays the primary route of ammonium assimilation in cadmium stress conditions and GS-GOGAT cycle plays a minor role. The role of amino acid transport was first assigned for NADH-GOGAT in tomato plants because it was found in the vascular tissues, particularly in the metaphloem, metaxylem-parenchyma, and mestome sheath cells of the vascular bundles, whereas it was not detected in the mesophyll, CCs, and SEs (Tobin and Yamaya, 2001). In concert with the GS1 located in the CC-SE complex, Fd-GOGAT presumably plays a complementary role to NADH-GOGAT during development because NADH-GOGAT occurred at higher control plants and decreased with cadmium stress (Chaffei *et al*. 2004). Consistently, the low level of Fd-GOGAT (15% of wild-type activity) supplies Glu for the normal growth of the transgenic tobacco lines in which NADH-GOGAT is not detected in leaves and roots (Feraud *et al*. 2005). Taken together, the data support the notion that the GDH is induced takes place in mitochodria and cytosol cells for the biosynthesis of Glu prior to the cycle of amino acids, and that GS-GOGAT cycle does not play a role in Glu supply even in tomato tissues. These localization studies show that the GS/GOGAT cycle GDH voice are highly compartmentalized at both the subcellular and cellular levels. Regardless of the effect of ammonium, increases in GDH activity have also been observed following carbohydrate starvation, a process that could be reversed by the presence of cadmium in culture medium. Recently, this consensus was again questioned following the discovery that GDH is mainly if not exclusively localized in the phloem companion cells (Dubois *et al*. 2003). In particular, the finding that an increase in GDH immunolocalization was induced especially in mitochondria of the companion cells when the ammonium concentration increased, to hypothesize that the enzyme may act as a sensor to evaluate the C/N status of the plant. In many studies both the metabolic environment and the tissue localization of GDH were not really taken into account, since the catalytic function of the enzyme in higher plants was studied in vivo by providing ^{15}N-labeled ammonium or ^{15}N-labeled Glu to leaf or root organs.

A study performed on tomato organs treated with cadmium tended to indicate that roots were able to incorporate less ^{15}N ammonium into total reduced nitrogen. However, the response of the plant in addition to inhibition of GS activity (MSO) and its lower leaf/root ratio did allow the conclusion that GDH is solely responsible for the difference found in the rate of ammonium assimilation in stress conditions, when ammonium becomes toxically accumulated.

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