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RESEARCH ARTICLE

GLUTAMATE DEHYDROGENASE OF TOMATO INDUCED IN THE CYTOSOL OF PHLOEM WHEN AMMONIA IS PROVIDED UNDER CADMIUM STRESS CONDITIONS

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ABSTRACT

Glutamate (Glu) dehydrogenase (GDH), enzyme preferentially occurs in the mitochondria of companion cells of a number of plant species grown on nitrate as the sole nitrogen source. For a better understanding of the controversial role of GDH either in ammonium assimilation or in the supply of cadmium, we studied the localization of GDH in tomato (*Solanum* Lycopersicon *63/5F1*) plants grown either on absence of cadmium (AC) or on presence of cadmium (PC). Production of GDH and its activity were strongly induced when plants were grown on presence of cadmium. The induction mainly occurred in highly vascularized organs such as stems and midribs and was likely to be due to accumulation of phloem-translocated ammonium in the sap. GDH was increased in the mitochondria and appeared in the cytosol of companion cells. Taken together, our results suggest that the enzyme plays a dual role in companion cells, either in the mitochondria when mineral nitrogen availability is low or in the cytosol when ammonium concentration increases under cadmium stress conditions.

INTRODUCTION

Ammonium is originate from a wide variety of metabolic processes such as nitrate reduction, photorespiration, phenylpropanoid metabolism, utilization of nitrogen transport compounds, amino acid catabolism, symbiotic nitrogen fixation (Hirel *et al*., 2011). It is then incorporated into an organic molecule, 2-oxoglutarate, by the combined action of the enzymes Gln synthetase (GS) and Glu synthase (Gln-2-oxoglutarate aminotransferase; GOGAT) to allow the synthesis of Gln and Glu. Both amino acids are further used as amino group donors for the synthesis of virtually all the nitrogen-containing molecules including the other amino acids needed for protein synthesis and nucleotides used as basic molecules for RNA and DNA synthesis (Hirel *et al*., 2001). The GS/GOGAT cycle is the major mechanism of ammonium assimilation in higher plants regardless of the various sources of ammonium listed above. However, it has often been argued that other enzymes have the capacity to assimilate ammonium, leading to the hypothesis that alternative pathways might operate under particular physiological conditions when the GS/GOGAT pathway may not be able to fulfill its function (Harrison *et al*., 2003). One of these alternative pathways is the reaction catalyzed by the mitochondrial NAD(H)-dependent Glu dehydrogenase (GDH; EC 1.4.1.2), which possesses the capacity to assimilate ammonium in vitro utilizing the organic molecule 2-oxoglutarate to synthesize Glu. This observation led a number of authors to propose that GDH could operate in the direction of ammonium assimilation (Labboun *et al*., 2009) although all the ^{15}N labeling experiments performed in vivo on a variety of plants demonstrated that GDH operates in the direction of Glu deamination (Labboun, 2009). It was concluded that GDH is involved in the supply of 2-oxoglutarate rather than in assimilation of ammonium when carbon becomes limiting (Miflin and Habash, 2002). The physiological role of GDH in the whole plant context remains speculative given the recent finding that the majority of the GDH protein is located in the mitochondria of companion cells (Dubois *et al*., 2003). Studies on tobacco **(**Nicotiana tabacum**)** leaf source/sink relationships have shown that GDH is induced when plants are grown on ammonium as the sole nitrogen source (Tercé-Laforgue *et al*., 2004). In contrast, both GDH transcripts and activity remain at a very low level when nitrogen remobilization is maximal as the result of nitrogen starvation. It was therefore concluded that GDH does not play a direct role during nitrogen remobilization but is rather induced following an accumulation of ammonium into the leaves when released in the sieve tubes as the result of protein hydrolysis during senescence (Pourteau *et al*., 2006). It was therefore hypothesized that the induction of GDH was not physiologically relevant since the enzyme does not assimilate ammonium in vivo (Yuan *et al*., 2009). As proposed before, the ammonium-dependent induction of the enzyme was the result of a more general response to a metabolic stress due to the toxic and pleiotropic effect of ammonium on cellular metabolism (Desvergne *et al*., 2006). To further investigate

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the putative role of cadmium stress in the regulation of GDH protein synthesis, the subcellular localization of the enzyme was studied in absence or presence of cadmium cultured plants with a particular emphasis on highly vascularized organs where the enzyme is mostly present. In addition, the subcellular localization of GDH was also investigated in tomato plants. In air, the leaves of low Fd-GOGAT expressors accumulate more ammonium than the untransformed controls (Labboun *et al*., 2009). This flexible biological system therefore allows the changes in the subcellular localization of GDH when ammonium is released internally from the leaf mesophyll to be studied.

MATERIALS AND METHODS

Plant material and growth condition

seeds of tomato (*Solanum lycopersicon*. Mill cv 63/5F1) were sterilized and seedlings were grown in a growth chamber (Chaffei *et al*., 2004). At the age of 10 days after transplant, cadmium was added to the medium as $CdCl_2$ at 0 and 100 µM until one week. Six plants were used for each feeding condition. One week after sowing, leaves were numbered 8, 9, 11, 13, 15, and 20 (from bottom to top) for both PC and AC plants. Leaf 10 that corresponds to an old source leaf (in which GDH induction is maximum) and leaf 18 that corresponds to a young sink leaf were used for both biochemical and cytoimmunochemical experiments. The stem sections were collected between leaf 16 and 20 for the top and between leaf 9 and 12 for the bottom. Stem, midrib, and mesophyll tissue (in which the midrib and small veins were removed) were harvested and pooled in two groups. One was weighed and then lyophilized to determine fresh and dry weights. The other was weighed, frozen, and used to determine both the quantity of protein and GDH activity. These tissues were frozen in liquid nitrogen and immediately reduced to a homogenous powder that was stored at -80°C and used for all the further experiments. All the harvesting of fresh material was done between 1 and 5 pm.

Metabolite Extraction and Analyses

Lyophilized plant material was used for metabolite extraction. Free ammonium was extracted and its concentration determined according to methods of Ferrario *et al* (1998), total amino acids were analyzed by the Rosen colorimetric method (Rosen, 1957). Free amino acid composition of phloem exudates was determined by ion-exchange chromatography (Rochat and Boutin, 1989).

Phloem Sap Collection: Phloem exudates were collected from 10-week-old wild-type tomato plants in the light period as described previously (Chaffei *et al*., 2004).

Enzymatic Assay and Determination of Total Soluble Protein

Enzymes were extracted from frozen leaf material stored at -80°C. All extractions were performed at 4°C. GDH was measured as described by Masclaux *et al*, (2000), except that the extraction buffer was the same as for GS (O'Neal and Joy, 1973).

Statistic Analysis

For metabolite analyses and measurement of enzyme activities, results are presented as mean values for six plants with ses (se = sd/ $\sqrt{n-1}$, where sd is the standard deviation and **n** the number of replicates).

Cytoimmunochemical Studies

Leaf fragments (2-3 mm^2), mesophyll, midribs, or stems were fixed in freshly prepared 1.5% (w/v) paraformaldehyde in phosphate buffer 0.1 m, pH 7.4, for 4 h at 4°C. For immunolocalization, material was dehydrated in an ethanol series (final concentration 90% [v/v] ethanol) then embedded in London Resin white resin (Polysciences, Warrington, PA). Polymerization was carried out in gelatin capsules during 10 h at 54°C. For immunotransmission electron microscopy studies, ultra thin sections were mounted on 400-µm mesh nickel grids and allowed to dry at 37°C. Sections were first incubated with 5% (v/v) normal goat serum in T1 buffer (0.05 m Tris-HCl buffer containing 2.5% [w/v] NaCl, 0.1% [w/v] BSA, and 0.05% [v/v] Tween 20, pH 7.4) for 1 h at room temperature then with anti-GDH rabbit serum (Loulakakis and Roubelakis-Angelakis, 1990), diluted 70 times in T1 buffer for 6 h at room temperature. Sections were then washed 5 times with T1 buffer, 2 times with T2 buffer (0.02 m Tris-HCl buffer containing 2% [w/v] NaCl, 0.1% [w/v] BSA, and 0.05% [v/v] Tween 20, pH 8) and incubated for 2 h at room temperature with 10 nm colloidal gold-goat anti-rabbit immunoglobulin complex (Sigma, St. Louis) diluted 50 times in T2 buffer. After several washes, grids were treated with 5% (w/v) uranyl acetate in water and observed with a Philips CM12 electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV. Polyclonal antiserum raised against tobacco GS2 (Hirel *et al*., 1984) and tobacco IDH (Lancien *et al*., 1998) were used as controls for cytoimmunochemistry. For both techniques, additional controls were conducted either by omitting the primary

RESULTS

To determine the concentration of ammonium and amino acids in the phloem, samples of sap were collected in AC and PC plants, using the same young leaf in all experiments. This young leaf was used in both AC and PC plants because older leaves were not able to exude. The ammonium concentration of the phloem sap was 16 times higher in PC plants compared to AC plants (Table 1). The total concentration of free amino acids in the phloem sap was 3 times higher in PC plants due to a preferential accumulation of Pro and Ser in response to the excess of ammonium (data not shown)

GDH Activity and Protein Levels in Stems and Midribs of Plants Grown on absence or on presence of cadmium

Ammonium is one of the main effectors responsible for the increase or the induction of GDH at the activity, protein, and gene expression levels, and that the protein is mostly if not exclusively localized in the CCs. Both GDH aminating and deaminating activities were therefore measured in stems of AC and PC treated plants following manual separation of the central cylinder from the cortical parenchyma cell layers. Using this technique, it was possible to demonstrate that GDH aminating activity was always higher in the central cylinder compared to the cortical cell layers, along the stem length in both AC and PC plants (Table 2). In addition, highest activity was measured in the central cylinder in the basal stem part of PC treated plants, whereas at the top of the

Table 1. Ammonium and amino acid concentrations in the phloem sap. Ammonium and aminoacids concentrations (nmol mL^{-1}) in the phloem sap of AC and PC plants. Values nd, not detectable.

	AC	PC
NH_4^+	13.8 ± 3.2	236.1 ± 30.1
Asp	15.1 ± 2.8	7.7 ± 2.5
Asn	nd	8.4 ± 0.4
Ser	25.5 ± 2.7	116.2 ± 16.2
Glu	15.3 ± 2.3	30.4 ± 1.9
Gln	1.0 ± 0.1	34.7 ± 4.4
Pro	nd	112.5 ± 15.6
Others	75.7 ± 16.4	115.8 ± 17.4
Total	138.0 ± 28.9	434 ± 53.1

Table 2. NADH-GDH activity in organs containing vascular tissue. NADH-GDH activity (nmol min^{-1} mg $protein^{-1}$) in midribs and stems of AC and PC plants. Values are the means ± sd.

	Midribs	Stems			
		Cortex		Central cylinder	
		Base	Top	Base	Top
PC	220.8 ± 21.3	103.6 ± 2.9	35.0 ± 10.8	322.4 ± 39.3	88.4 ± 12.3
AC	78.6 ± 26.3	72.3 ± 3.3	47.4 ± 2.7	186.5 ± 12.6	72.1 ± 4.3

Table 3. Quantification of GDH protein in different tissue sections of tomato plants. Immunolocalization of the enzyme was performed using transmission electron microscopy on plants grown on presence or absence of Cd. Values are the means ± sd of gold particles counted on 3 to 20 different sections. See footnotes for induction levels of GDH protein. [a]Significant [b]Not significant at 0.05 probability level. [c]nd, Not detectable

	Number of Gold Particles/μm^2					
	Mitochondria		Cytosol		Vacuole	
	CA	PC	AC	PC	AC	PC
Leaf mesophyll cells	3.82 ± 1.1	5.1 ±1.2[b]	0.55 ±0.1	0.87 ± 0.26[b]	nd[c]	nd[c]
Minor veins CC	27.9 ± 3.9	43 ± 6.8[b]	4.73 ±0.8	12.6 ± 2.9[a]	6.9 ± 2	26± 8[a]
Midribs CC	21 ± 3.6	45 ± 3.6[a]	5.4 ±0.45	13 ± 3.5[a]	20 ± 7	61±13[a]
Base stems CC	53 ± 7.7	104±4.3[a]	5.8 ±0.7	20 ± 4.1[a]	14 ± 5	31±10[a]
Top stems CC	30 ± 4.5	54 ±8.1[a]	6.3 ± 1	9.5 ± 0.34[a]	nd[c]	nd[c]

stems its activity was 3 times lower regardless of the presence or absence of cadmium in the medium culture. Although this technique was not fully accurate in terms of tissue separation, it demonstrated that the enzyme is more active in the zone of the stem containing vascular tissue. A similar pattern was observed for GDH deaminating reaction, but its activity was approximately 10 times lower compared to its aminating counterpart (data not shown). GDH aminating activity was also much higher in the leaf midrib of PC plants (Table 2). Both GDH aminating and deaminating activities were therefore measured in stems of AC and PC treated plants following manual separation of the central cylinder from the cortical parenchyma cell layers. Using this technique, it was possible to demonstrate that GDH aminating activity was always higher in the central cylinder compared to the cortical cell layers, along the stem length in both AC and PC treated plants (Table 2).

Subcellular Localization of GDH by Immunogold Transmission Electron Microscopy

To refine the localization of GDH in phloem-rich tissues such as stems, petioles, and midribs and to determine whether ammonium provided internally released during cadmium stress conditions had an impact on the subcellular distribution of the protein, immunocytochemical electron microscopy experiments were conducted. In previous physiological studies experiments were performed on leaves that still contained some minor veins in the mesophyll tissue (Sack *et al*., 2003). Therefore, in this study the localization of GDH was investigated in deribbed leaf fragments. Figure 1A shows a partial view of a mature leaf mesophyll cell of Cd treated plants after incubation with the GDH antiserum. The section was devoid of gold particles, indicating the absence of GDH protein. However, it is possible that very low amounts of protein were present in the mesophyll cells and therefore at the limit of detection of the immunocytochemical technique. Quantification of gold particles confirmed the absence of GDH both in the mitochondria and cytosol of leaf mesophyll, considering that the background level is around 4 particles/μm^2. For the cytosol, the labeling was close to the background level, and the changes in GDH protein content observed both in the mitochondria and in the cytosol of Cd treated plants were not significant (Table 3). In contrast, in the CCs of the minor veins of Cd treated plants gold particles were mostly present in the mitochondria. However, some labeling was detected in the cytosol and the vacuolar material (Fig 1B). In the CCs of minor veins of AC plants the labeling was weak and mostly visible in the mitochondria (Fig 1C). Quantification of gold particles showed that in the minor vein CCs the increase in GDH protein content occurred both in the cytosol and in the vacuole of Cd treated plants (Table 3). In the leaf midrib (Fig 2A) of Cd treated plants, GDH protein was present both in the cytosol and the mitochondria of the CCs, but the strongest labeling was detected inside the vacuole. In the vacuole, the variations observed between organs and treatments were not only due to the density of labeling in the vacuolar material but also dependent on the

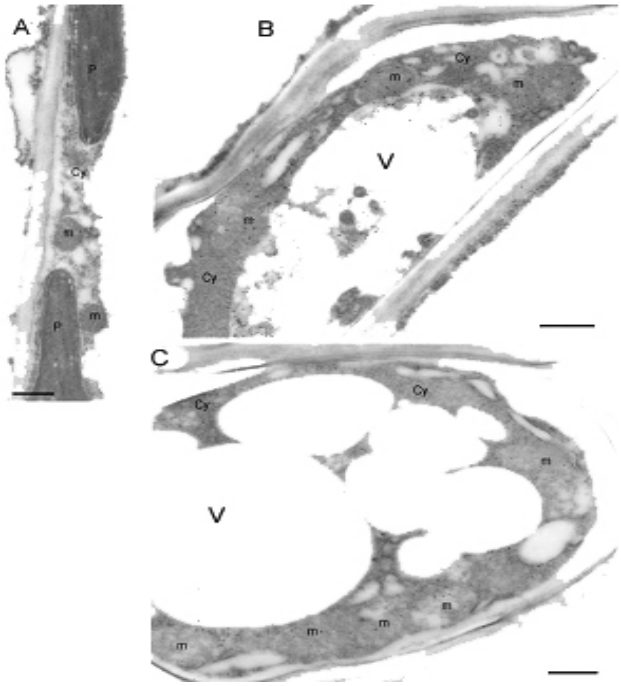

Fig. 1. Immunolocalization of GDH in mature tomato leaf tissues of PC (presence of cadmium) and AC (absence of cadmium) plants. A, mesophyll cell of mature leaf of PC plants. B, CC of a leaf minor vein of PC plants. Arrows indicate labelling in the vascular material. C, CC of a leaf minor vein of AC plants. Cys, cytosol; m, mitochondria; P, plastids; S, starch granule; V, vacuole. Bars = 1μm.

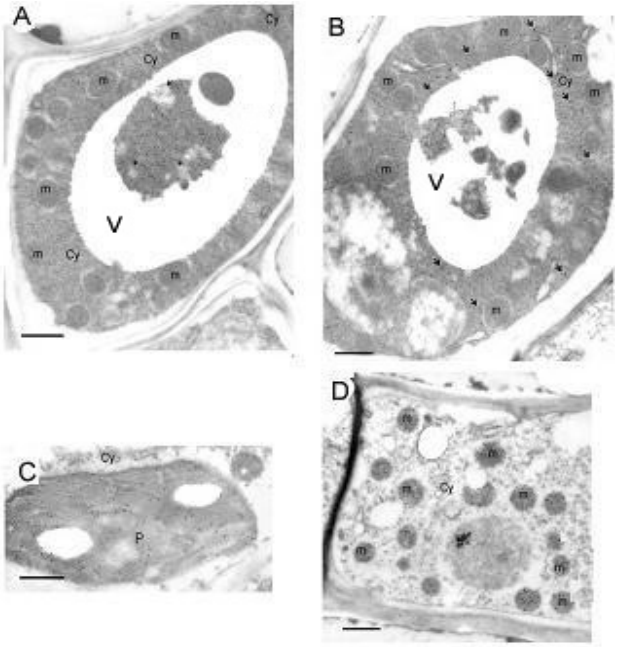

Fig. 2. Immunolocalization of GDH in a leaf midribs and petiole of PC plants. A, CC of a leaf midrib. Large arrows indicate labelling inside the vacuole (V) and arrowheads indicate the presence of scarce labelling area in this vacuolar material. B, Control section of a CC in a leaf midrib of PC plants and treated with GS antibodies (arrows indicate the presence of the protein in the cytosol of CC). C, Leaf mesophyll cell treated with GS antibodies. D, Control section of a CC in the leaf midrib of Cd treated plants and treated with IDH antibodies. Cy, Cytosol; m, mitochondria; P, plastids; V, vacuole. Bars = 1 μm.

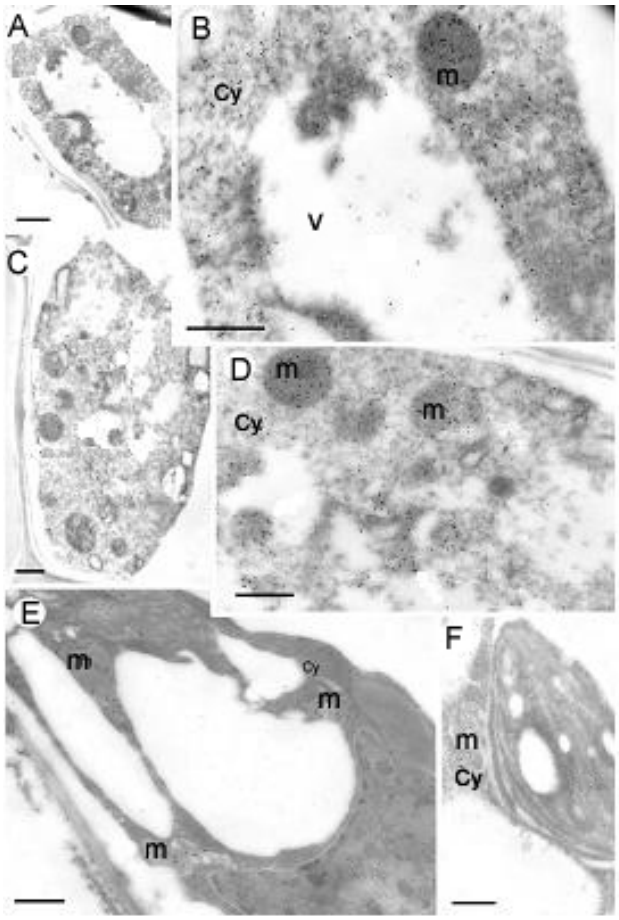

Fig. 3. Immunolocalization of GDH in the CCs of PC and AC basal tomato stem internodes. A, CC of a basal internode of PC plants. B, detail of a basal internode CC of PC plants. C, CC of a basal internode of PC treated plants. C, CC of a basal internode of Cd treated plants showing the presence of labeling in the vacuolar material (arrows). D, Detail of a basal internode CC in which labeling was detected in the vacuolar material (arrows). E, CC of a basal internode of AC plants. F, Cortical parenchyma cell of PC treated plants. Cy, Cytosol; m, mitochondria; P, plastids; V, vacuole. Bars = 2 μm (A), 1 μm (B–F).

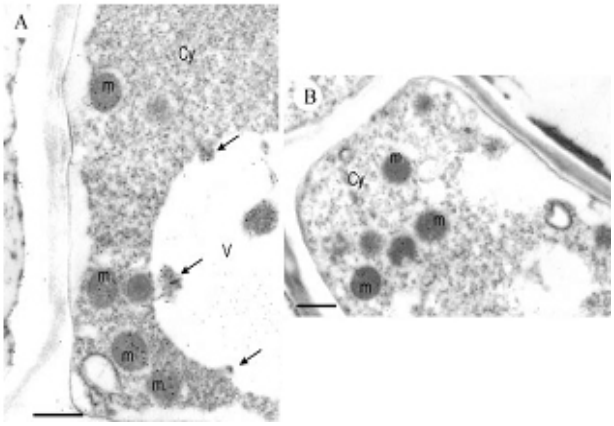

Fig. 4. Immunolocalization of GDH in the CCs of top stem parts of PC and AC plants. A, CC of top stem part of PC plants. Arrows indicate the presence of labelling in vacuolar inclusions originating. B, CC of top stem part of AC plants. Cy, Cytosol; m, mitochondria; P, plastids; V, vacuole. Bars = 1 μm.

proportion of cellular material present inside the vacuole. Therefore, the data presented in table 3 correspond to an average density of gold particle present in the whole vacuolar area. Although much lower than in the cytosol and the vacuole, an increase in GDH protein in the mitochondria of midribs CCs (Table 3) was also observed. Controls for the specificity of the labeling were also performed with GS and isocitrate dehydrogenase (IDH) antiserum. Figure 2C shows a partial view of a mature leaf mesophyll cell of Cd treated plants after incubation with the GS antiserum. Gold particles were only present in the plastids. When minor vein or midrib sections of Cd treated plants were treated with the GS antiserum, gold particles were only detected in the cytosol of the CCs (Fig 2B). The other control using antibodies against IDH clearly shows that the protein is only present in the mitochondria (Fig 2D). In the three controls we did not observe any labeling above the background level in the vacuolar material of the CCs (Fig 2, B and C). At the base of the stem of Cd treated plants large amounts of GDH protein were present both in the mitochondria and the cytosol of the CCs (Fig 3, A-B). Some labeling was also detected in the vacuolar material of these cells (Fig 3, C-D). However, the density of gold particles was much lower compared to that found in the vacuole of the leaf midrib CCs (Fig 2A and table 3). In an equivalent stem section of AC and PC plants GDH protein was mostly present in the mitochondria of the CCs. However, compared to PC plants the labelling was much weaker (Fig 3E). Quantification of gold particles in the basal stem part CCs of plants fed with Cd confirmed a stronger induction of GDH in the cytosol compared to that seen in the mitochondria (Table 3). This quantification also shows that there is more protein in the stems compared to midribs. A weak background of gold particles could be seen in the basal stem part cortical parenchyma cells of PC plants (Fig 3E). In these cells, the number of gold particles was very low compared to CCs containing tissue and not significantly different in AC and PC plants. Moreover, their density was similar to that measured in leaf mesophyll cells (data not shown). A similar investigation was conducted using the top stem part of both AC and PC treated plants. A very weak labeling was only observed in the top stem CCs of PC plants in both the cytosol and in the mitochondria (Fig 4A). At the top of the stem of AC plants, the labeling was mostly present in the mitochondria of CCs and was much weaker in comparison to that of PC treated plants (Fig 4B, Table 3). Compared to AC plants there was a significant increase in the number of gold particles both in the mitochondria and the cytosol of PC plants (Table 3). However, the signal was much lower when compared to that observed in the basal part of the stem, and the vacuole was unlabeled (Fig. 3, A-B, Table 3).

DISCUSSION

High concentrations of ammonium either provided released into the sieve tube from protein hydrolysis under senescence or stress conditions (Gao *et al*., 2012) generally lead to an increase in leaf GDH activity. To our knowledge, this is the first demonstration that GDH increase occurs mainly in stems and midribs and is restricted to the CCs. Subcellular localization of the enzyme using immunogold labeling demonstrates that the amount of GDH increases mainly in the cytosol and vacuolar material and in the mitochondria of the CCs. This phenomenon occurs when ammonia is internally released during cadmium application process. This conclusion was corroborated by higher amounts of ammonium translocated through the phloem stream, irrespective of its origin. The increase in both NADH and NAD-dependent activity and GDH content was proportional to the increase in ammonium content of the phloem sap and not directly correlated to the concentration of a particular amino acid. It is well established that cycling of nitrogen molecules from xylem to phloem largely contributes to nitrogen transport via the phloem (Yan *et al*., 2012). In contrast, photorespiratory ammonium that is produced in the leaf mesophyll cells is likely to be loaded in the sieve tubes and accumulated along the phloem pathway into the CCs (Van Bel, 2003). Both deductions imply that ammonium itself or one of its derivates induces GDH production in the CCs. Since there is an apoplastic diffusion of ammonium within the leaf, it is likely that the extracellular flux of ammonium is not able to induce GDH in the mitochondria or in the cytosol of the mesophyll cells. Under standard plant growth conditions, the basic level of GDH activity in the CCs, which remains confined to the mitochondria, may have a house-keeping role in a tissue that has to cope with a low oxygen concentration (Foyer *et al.,* 2003). Under these conditions, the lack of reducing equivalents such as NADH is in favor of the enzyme having a function in oxidizing Glu as a respiratory substrate, as already demonstrated to occur in vivo (Mayevsky and Rogatsky, 2007). In ammonium-fed plants, the increase in GDH observed in the mitochondria of CCs may be related to a higher demand in reducing equivalents when inorganic nitrogen and particularly NH_4^+ become available in large amounts. The appearance of GDH in the cytosol of CCs of PC plants suggests that the enzyme is able to assimilate ammonium when its concentration reaches a certain level. The picture arising from the immunogold labeling studies is that the increase in the cytosolic GDH is not only due to a higher ammonium level under cadmium stress conditions but is also dependent of the anatomy of the organ. GDH induction was observed to be higher in basal parts of the stem, which may be explained by the fact that they are the main site for xylem-phloem exchange of nitrogen (Sharathchandra *et al*., 2012).

Consistently, we have previously shown that both the ammonium and the free amino acid content is higher in cadmium-fed plants at the base of the plant. Conversely, at the top of the stem where ammonium is loaded directly into the phloem, we observed a lower induction of GDH in both the cytosol and the mitochondria of the CCs. Interestingly, we also found some organ specificities in the distribution of GDH protein, particularly in the leaf midrib, in which the vacuolar material was strongly labeled. It is unlikely that this labeling is an artifact since two positive controls performed either with GS antibodies or IDH antibodies and a negative control performed with preimmune serum confirmed the specificity of the labeling in the cytosol (GS) and in the mitochondria (IDH) of CCs but not in the vacuole. In addition, other experiments showed that the vacuolar labeling was specific to the CCs in the leaf midrib because little vacuolar labeling was observed in other cell types and CCs in minor veins, top stem internodes, and mesophyll cells. The high levels of GDH in the vacuole remain enigmatic, although GDH has been found previously localized inside

multivesicular bodies associated with vacuolar autophagic activity. This lytic activity of the vacuole already reported in the stressed leaves is the result of carbon starvation (Miyashita and Good, 2008). These results suggest that GDH is sensitive to the C status of the plant via the vascular tissue if we consider the well-established fact that the expression of GDH is repressed when the cellular level of sugars is high (Miyashita and Good, 2008). In conclusion, our results suggest that GDH plays a dual role in CCs, either in the mitochondria when mineral nitrogen availability is low or in the cytosol when ammonium concentration increases under cadmium stress conditions. An attractive hypothesis is that GDH, when induced by ammonium in the cytosol, may act as a sensor to evaluate the carbon/nitrogen status of the plant particularly with respect to ammonium and sugar concentration and/or fluxes through the phloem stream. A putative sensing role for GDH under cadmium stress conditions is the continuity between CCs and sieve tubes is one of

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