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# Cadmium Toxicity Induced Changes in Nitrogen Management in *Lycopersicon esculentum* Leading to a Metabolic Safeguard Through an Amino Acid Storage Strategy

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Tomato (*Lycopersicon esculentum*) seedlings were grown in the presence of cadmium. After 1 week of Cd treatment, a sharp decline in biomass accumulation in the leaves and roots was observed, together with a decrease in the rate of photosynthetic activity due to both Rubisco and chlorophyll degradation and stomata closure. Cadmium induced a significant decrease in nitrate content and inhibition of the activities of nitrate reductase, nitrite reductase, glutamine synthetase (GS) and ferredoxin-glutamate synthase. An increase in NADH-glutamate synthase and NADH-glutamate dehydrogenase activity was observed in parallel. The accumulation of ammonium into the tissues of treated plants was accompanied by a loss of total protein and the accumulation of amino acids. Gln represented the major amino acid transported through xylem sap of Cd-treated and control plants. Cadmium treatment increased the total amino acid content in the phloem, maintaining Gln/Glu ratios. Western and Northern blot analysis of Cd-treated plants showed a decrease in chloroplastic GS protein and mRNA and an increase in cytosolic GS and glutamate dehydrogenase transcripts and proteins. An increase in asparagine synthetase mRNA was observed in roots, in parallel with a strong increase in asparagine. Taken together, these results suggest that the plant response to Cd stress involved newly induced enzymes dedicated to coordinated leaf nitrogen remobilization and root nitrogen storage.

**Keywords:** Cadmium stress — *Lycopersicon esculentum* — Nitrogen — Senescence.

Abbreviations: Cd, cadmium; Fd-GOGAT, ferredoxin-dependent glutamate synthase; GaBa,  $\gamma$ -amino-*n*-butyric acid; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GS1, cytosolic GS; GS2, chloroplastic GS; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; NADH-GOGAT, NADH-dependent GOGAT; NAD<sup>+</sup>-MDH, NAD<sup>+</sup>-dependent MDH; NADH-MDH, NADH-dependent MDH; NADP<sup>+</sup>-MDH, NADP<sup>+</sup>-dependent MDH; NADPH-MDH, NADPH-dependent MDH; NR, nitrate reductase; NiR, nitrite reductase.

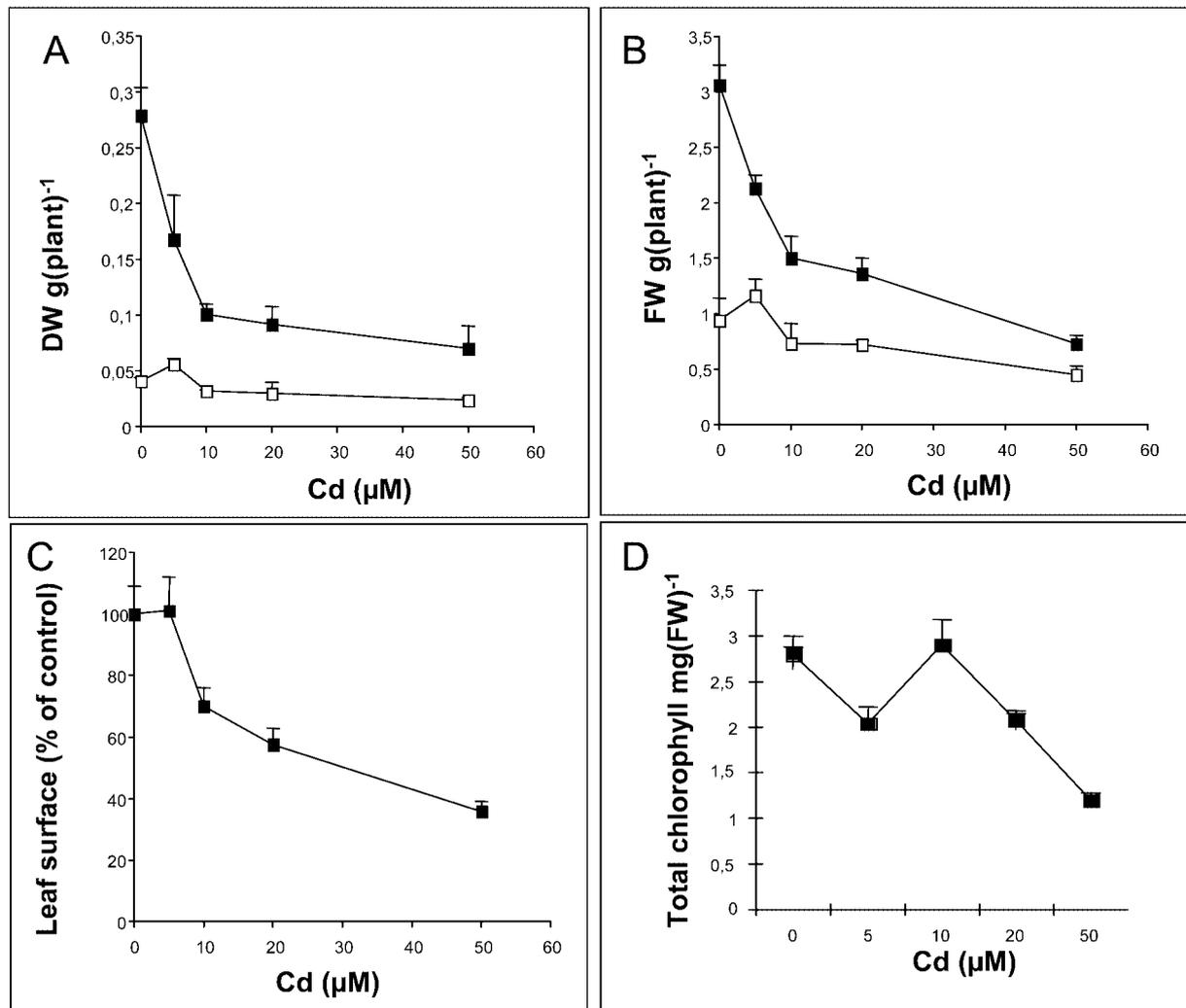
## Introduction

Cadmium is a heavy metal toxic for humans, animals and plants. It is a widespread trace pollutant with a long biological half-life (Wagner 1993). This metal enters agricultural soils mainly from industrial processes, phosphatic fertilizers and atmospheric deposition, and is then transferred to the food chain.

In plants, Cd toxicity is associated with growth inhibition and imbalances in many macro- and micronutrient levels. The presence of Cd in plants results in many physiological alterations affecting both nitrogen and carbohydrate metabolism (Greger and Bertell 1992, Hernandez et al. 1997, Chaffei et al. 2003). One of the most sensitive responses of higher plants to Cd is stomata closure (Barceló and Poschenrieder 1990, Greger and Johansson 1992), followed by the decrease in transpiration water loss and overall inhibition of photosynthesis, through effects on the chlorophyll metabolism (Sheoran et al. 1990, Somashekaraiah et al. 1992, Krupa et al. 1993, Chugh and Swahney 1999). Cadmium induces alterations in the functionality of membranes, by triggering changes in their lipid composition (Fodor et al. 1995, Hernandez and Cooke 1997, Howlett and Avery 1997). It was shown that Cd-induced toxicity involves some other senescence-like processes, such as the participation of oxidative stress, probably mediated by H<sub>2</sub>O<sub>2</sub> and leading to gradual increases in activities of certain antioxidant enzymes, such as catalase and peroxidase, along with increased lipid peroxidation (Chaoui et al. 1997, Sandalio et al. 2001) and proteolytic degradation (Romero-Puertas et al. 2002). Cadmium also induces peroxisome-senescence symptoms in leaves with the induction of the glyoxylate cycle enzymes, malate synthase and isocitrate lyase, as well as peroxisomal peptidases (McCarthy et al. 2001), enzymes that are also known as leaf senescence-associated factors (Buchanan-Wollaston 1997, Buchanan-Wollaston et al. 2003 for a review)

It has been shown that enzymes of N metabolism are differently affected by Cd stress (Petrovic et al. 1990, Chugh et al. 1992, Singh et al. 1994). Nitrate reductase (NR) and nitrite reductase (NiR) activities are significantly decreased, leading to reduced nitrate assimilation by plants. The activities of the glutamine synthetase–glutamate synthase (GS/GOGAT) cycle,

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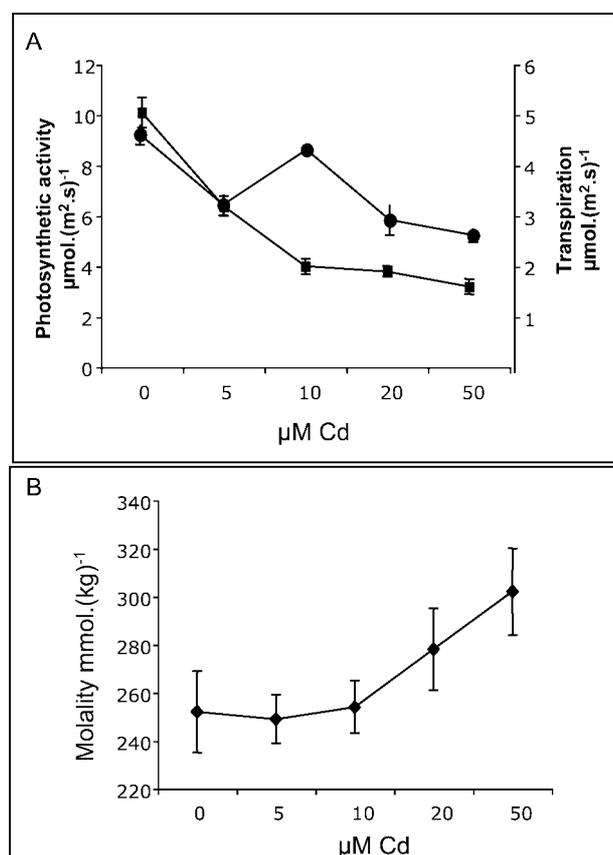
**Fig. 1** Effect of Cd stress on the dry weight (A) and fresh weight (B) of leaves (black squares) and roots (open squares) of tomato plants. Effect of Cd on the leaf surface (C) and on the total chlorophyll content (D) of tomato plants. Values are mean  $\pm$  SE of five individual plants. Standard errors are not shown when they are smaller than the symbol.

which operates in ammonium incorporation into carbon skeletons, are also affected (Ouariti et al. 1997, Gouia et al. 2000). In contrast, glutamate dehydrogenase (NADH-GDH) activity showed a substantial rise under Cd stress (Boussama et al. 1999). GDH enzyme has also been associated with other stresses, such as pathogenic stress (K. Pageau, M. Reisdorf-Cren, J.-F. Morot-Gaudry and C. Masclaux-Daubresse unpublished, Osuji and Madu 1996, Srivastava and Singh 1987, for a review) as well as leaf senescence (Masclaux et al. 2000). All these data suggest that the changes observed in nitrogen metabolism in Cd-stressed plants are similar to changes observed during natural senescence (Mae et al. 1983, Masclaux et al. 2000, Buchanan-Wollaston 1997, for a review).

The main function of leaf senescence is the recycling of nutrients. For example, up to 80% of the nitrogen contained in *Arabidopsis*, rice or maize leaves is exported during senescence

from source leaves to the new emerging organs (Himelblau and Amasino 2001, Masclaux et al. 2001, for a review). Cytosolic glutamine synthetase (GS1) and GDH enzymes are likely to play an important role in this process (Masclaux et al. 2001). However, Cd-stressed plants show dramatic growth inhibition and sink emergence is reduced. Therefore, the role of a nitrogen mobilization-like process has to be questioned.

In this work, the main changes related to nitrogen metabolism in tomato plants submitted to increasing doses of Cd in their culture medium are presented. Metabolic markers such as N metabolites, enzyme activities and transcripts encoding key enzymes were examined. We report an integrative study that shows that a Cd pollutant provoked senescence-like symptoms in tomato plants, leading to nitrogen mobilization and a storage strategy. Results are discussed with regard to overall symptoms associated with senescence in plants, as well as recovery strategy.



**Fig. 2** Effect of Cd stress on (A) the photosynthetic activity (black squares) and transpiration (black circles) of tomato leaves, (B) the molality of the leaf fluids of Cd-treated plants. Values are mean  $\pm$  SE of five individual plants. Standard errors are not shown when they are smaller than the symbol.

## Results

### *Biomass determination and senescence biomarker analysis*

We observed that the biomass of both roots and leaves decreased when increasing doses of Cd were added to the culture solution (Fig. 1A, B). The decline in fresh and dry weights was more dramatic for leaves than for roots, and was observed even at the lowest Cd concentration used. An external Cd concentration of 50  $\mu\text{M}$  triggered 75% and 30% of reduction in leaf and root growth, respectively (Fig. 1A, B). A progressive decrease in leaf surface was detected for plants treated with a 10  $\mu\text{M}$  or higher concentration of Cd. Leaf surface was reduced to 60% under the 50  $\mu\text{M}$  Cd treatment (Fig. 1C). The total soluble chlorophyll content of leaves decreased slightly, particularly when the Cd concentration was higher than 10  $\mu\text{M}$  (Fig. 1D).

### *Gas exchange measurements*

The decrease in chlorophyll content was parallel to the decrease in photosynthetic activity (Fig. 2A). Change in  $\text{CO}_2$  absorption by Cd treatment was also accompanied by a decrease in transpiration rates, indicating that a partial stomata closure occurred (Fig. 2A). Cd could have induced severe water stress in the tomato plants. To check this possibility, we measured the molality of internal leaf fluids, and we observed that their molality was weakly increased when the Cd concentration was higher than 10  $\mu\text{M}$  (Fig. 2B).

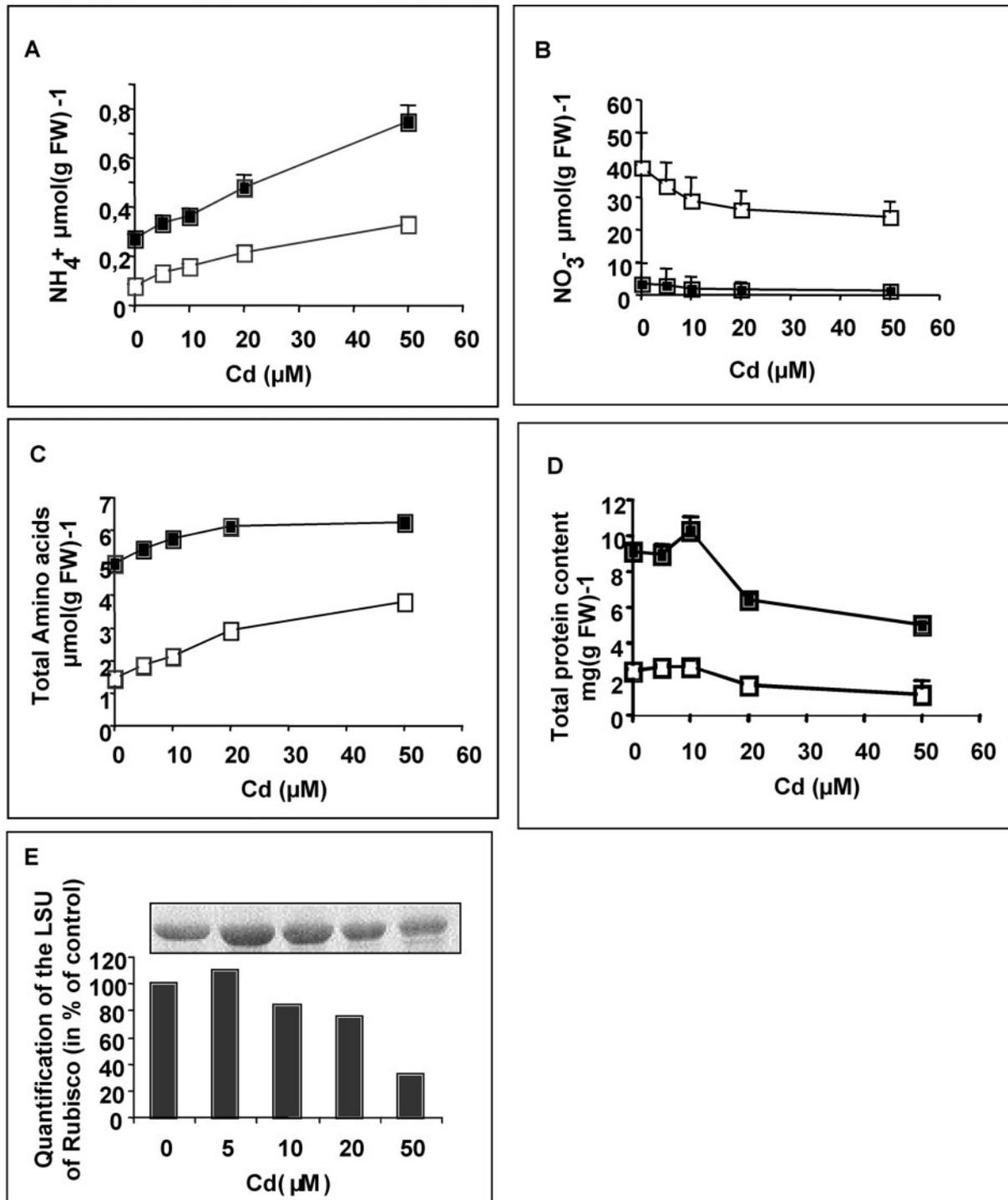
### *Changes in nitrogen distribution in Cd-treated plants*

The partition of nitrogen between mineral compounds, amino acids and soluble proteins was investigated. Fig. 3A shows that ammonium accumulation in Cd-treated plants was parallel to the Cd concentration applied to the nutritional

**Table 1** Amount of individual amino acids in the leaves and roots of tomato plants treated with increasing doses of Cd (percentage of total amino acids)

CdCl <sub>2</sub> (mM)	Individual amino acid proportion (percentage of total amino acids)										
	Asp	Asn	Glu	Gln	Gly	Ser	Pro	Ala	GaBa	Lys	Arg
Leaves of tomato											
0	23.7	0.6	28.0	11.4	1.7	4.8	1.0	12.9	7.3	0.4	1.7
5	18.8	0.8	23.7	10.0	1.3	4.1	1.6	13.1	12.8	0.4	0.3
10	20.4	0.8	24.1	13.0	1.8	3.6	1.5	12.4	10.3	0.4	0.2
20	19.4	0.6	25.6	13.6	1.1	3.9	1.5	9.0	9.0	0.6	0.1
50	17.8	1.7	23.8	18.0	0.5	3.5	1.6	5.6	13.4	0.8	0.3
Roots of tomato											
0	9.6	2.0	15.0	18.3	2.3	3.6	0	3.4	20.0	1.4	1.3
5	8.7	2.0	15.0	18.8	2.1	3.6	0	2.9	19.1	1.8	1.5
10	6.8	2.1	12.5	19.1	1.9	3.3	0	2.0	23.7	2.2	1.9
20	5.2	6.3	10.2	21.3	1.3	3.4	1.4	1.7	22.9	2.3	2.8
50	8.0	17.8	6.9	21.7	1.4	2.9	1.4	1.1	12.9	3.1	2.5

Individual amino acid composition was determined on pooled samples extracted from an equal dry weight of three plant repeats.



**Fig. 3** Changes in ammonium (A), nitrate (B), total amino acids (C), total protein (D) and the large subunit of Rubisco (LSU) (E), in leaves (black squares) and roots (open squares). Values are mean  $\pm$  SE of five individual plants. Standard errors were not shown for total amino acids.

medium. In contrast, the amount of nitrate contained in root and leaf tissues was dramatically reduced by Cd treatment (Fig. 3B). The total nitrate in plants treated with 50  $\mu\text{M}$  Cd was 24% and 62% of the nitrate content measured in leaves and roots of control plants, respectively. Total amino acid content increased

progressively in leaves and roots of plants when treated by Cd (Fig. 3C). This increase was higher in roots.

Individual amino acid content was also determined and the results are presented in Table 1. In control plants the main amino acids encountered in leaves were glutamate (Glu), aspar-

**Table 2** Individual amino acid amounts in xylem and phloem saps extracted from tomato plants treated or not with 20  $\mu\text{M}$  Cd (as a percentage of total amino acids)

	Amino acids (% of control)		F-value
	Untreated control	Treated plants (20 $\mu\text{M}$ Cd)	
In xylem sap			
Gln	66.06 $\pm$ 1.9	60.40 $\pm$ 3.73	0.024
Val	4.40 $\pm$ 0.1	5.14 $\pm$ 1.19	0.265
Lys	3.80 $\pm$ 1.1	6.61 $\pm$ 1.31	0.008
Asn	3.13 $\pm$ 0.3	7.45 $\pm$ 1.80	0.002
GaBa	3.10 $\pm$ 1.4	1.28 $\pm$ 0.17	0.013
Leu	2.66 $\pm$ 0.3	2.47 $\pm$ 0.18	0.190
Arg	2.59 $\pm$ 0.5	3.68 $\pm$ 0.84	0.047
Thr	2.01 $\pm$ 0.2	1.59 $\pm$ 0.25	0.029
His	1.94 $\pm$ 0.3	3.72 $\pm$ 0.45	0.000
Ala	1.84 $\pm$ 1.7	0.27 $\pm$ 0.08	0.048
Ile	1.81 $\pm$ 0.1	2.14 $\pm$ 0.38	0.138
Ser	1.18 $\pm$ 0.8	0.32 $\pm$ 0.04	0.033
Glu	1.10 $\pm$ 0.3	0.91 $\pm$ 0.13	0.239
Asp	0.93 $\pm$ 0.3	0.68 $\pm$ 0.15	0.114
Total	96.6	96.7	
In phloem sap			
Gln	26.42 $\pm$ 6.32	35.72 $\pm$ 9.92	0.129
Glu	21.21 $\pm$ 3.68	18.31 $\pm$ 5.70	0.383
Asp	19.97 $\pm$ 2.89	6.61 $\pm$ 4.04	0.0007
GaBa	6.59 $\pm$ 0.44	0.20 $\pm$ 0.22	0.001
Gly	4.18 $\pm$ 1.27	5.03 $\pm$ 5.64	0.751
Ser	3.95 $\pm$ 0.27	2.94 $\pm$ 1.64	0.210
Ala	3.21 $\pm$ 0.16	3.96 $\pm$ 0.15	0.0002
Asn	2.74 $\pm$ 0.30	4.19 $\pm$ 0.68	0.004
Ile	2.32 $\pm$ 0.53	1.81 $\pm$ 1.58	0.521
Thr	1.54 $\pm$ 0.18	1.46 $\pm$ 0.15	0.500
Val	1.52 $\pm$ 0.22	1.51 $\pm$ 1.07	0.980
Lys	1.27 $\pm$ 0.08	1.58 $\pm$ 0.49	0.203
His	0.73 $\pm$ 0.13	2.36 $\pm$ 1.18	0.017
Arg	0.40 $\pm$ 0.25	3.45 $\pm$ 2.31	0.0204
Total	96.0	90.0	

Individual amino acid composition was determined separately on six control and six treated plants. Mean  $\pm$  standard deviation of six repeats are presented. ANOVA was calculated on data from six repeats.

tate (Asp), glutamine (Gln), alanine (Ala) and  $\gamma$ -amino-*n*-butyric acid (GaBa). GaBa as well as Gln, Glu and Asp are the major amino acids represented in roots. In Cd-treated leaf tissues, we observed that Gln and proline (Pro) accumulated whereas Ala, serine (Ser) and Asp decreased. In addition, in the leaves of the 50  $\mu\text{M}$  Cd-treated plants, an increase in asparagine (Asn) and lysine (Lys) was observed concomitantly with the glycine (Gly) decrease. However, the more drastic changes in amino acid proportions were observed in roots. In contrast to leaves, Glu in roots decreased dramatically with Cd increase in

the culture medium. Asp and Gln remained stable whereas Asn and Pro proportions were increased by 8- and 14-fold, respectively. The proportion of GaBa was decreased 2-fold by the 50  $\mu\text{M}$  treatment.

The decrease in nitrate content and the increase in both free amino acids and ammonia might suggest that (i) nitrogen assimilation is higher in the treated plants than in controls or (ii) nitrate assimilation is affected and both ammonia and amino acids are released by protein degradation and hydrolysis. Since previous studies have already demonstrated that Cd affected nitrogen uptake as well as nitrate reduction (Gouia et al. 2000), we first tested the proteolysis hypothesis by measuring the total soluble protein content of leaves and roots (Fig. 3D). It is now well accepted that Rubisco is the major nitrogen storage protein in leaves (Masclaux et al. 2001 for review), so we estimated Rubisco content by quantification of the large subunit of this enzyme on SDS-PAGE stained gels (Fig. 3E). The results showed that both total soluble protein and Rubisco decreased in treated leaves. This decrease reached 70% of the control value in plants treated with 50  $\mu\text{M}$  Cd.

#### *Changes in nitrogen distribution in xylem and phloem saps of treated and untreated plants*

The partition of nitrogen between mineral compounds and amino acids in xylem and phloem saps was measured (Table 2).

The content of total amino acids in the xylem sap extracted from control plants ( $2.48 \pm 0.6 \text{ nmol } \mu\text{l}^{-1}$ ) and Cd-treated plants ( $2.25 \pm 1.04 \text{ nmol } \mu\text{l}^{-1}$ ) were similar (ANOVA *F*-value = 0.7). However, some individual amino acid proportions were altered by Cd treatment. Gln represented 66% of the total amino acids translocated through the xylem sap of control plants, and decreased to 60% in Cd-treated plants. In parallel, GaBa and Ala were decreased by 2.4- and 6.7-fold, respectively. Cadmium treatment increased Asn, Lys, His and Arg proportions by 2.4-, 1.7-, 1.9- and 1.4-fold, respectively. Glu and Asp proportions in xylem sap were minor and unchanged by Cd stress. In parallel, nitrate and ammonia contents were measured. Ammonia slightly increased in xylem sap with Cd treatment, from 2.3 ( $\pm 0.7$ )  $\text{nmol } \mu\text{l}^{-1}$  to 3.5 ( $\pm 0.7$ )  $\text{nmol } \mu\text{l}^{-1}$ , whereas nitrate decreased from 24.1 ( $\pm 1.9$ )  $\text{nmol } \mu\text{l}^{-1}$  to 15.5 ( $\pm 1.0$ )  $\text{nmol } \mu\text{l}^{-1}$ .

Individual amino acid content in phloem sap was determined. The content of amino acids in the phloem sap extracted from Cd-treated plants ( $0.317 \pm 0.17 \text{ nmol } \mu\text{l}^{-1}$ ) was significantly increased compared with control plants ( $0.184 \pm 0.05 \text{ nmol } \mu\text{l}^{-1}$ ) (ANOVA *F*-value = 0.056). The proportions of Gln and Glu in phloem sap were not significantly modified by Cd stress. In contrast we observed that major changes occurred, since GaBa was decreased by 32-fold whereas Asn, His and Arg were increased by 1.5-, 3.2- and 8.6-fold, respectively. Moreover, minor amino acids, which represented 4% of total amino acids in control phloem sap rose to 10% in Cd-treated plants.

**Table 3** NR, NiR and GS activities are repressed in leaves and roots of tomato plants treated with increasing doses of Cd

CdCl <sub>2</sub> (mM)	Enzymatic activity (as percentage of control)					
	NR		NiR		GS	
	Shoots	Roots	Shoots	Roots	Shoots	Roots
0	100	100	100	100	100	100
5	100	81	94	100	60	87
10	85	36	83	97	45	78
20	52	28	61	88	38	70
50	35	23	33	68	35	67

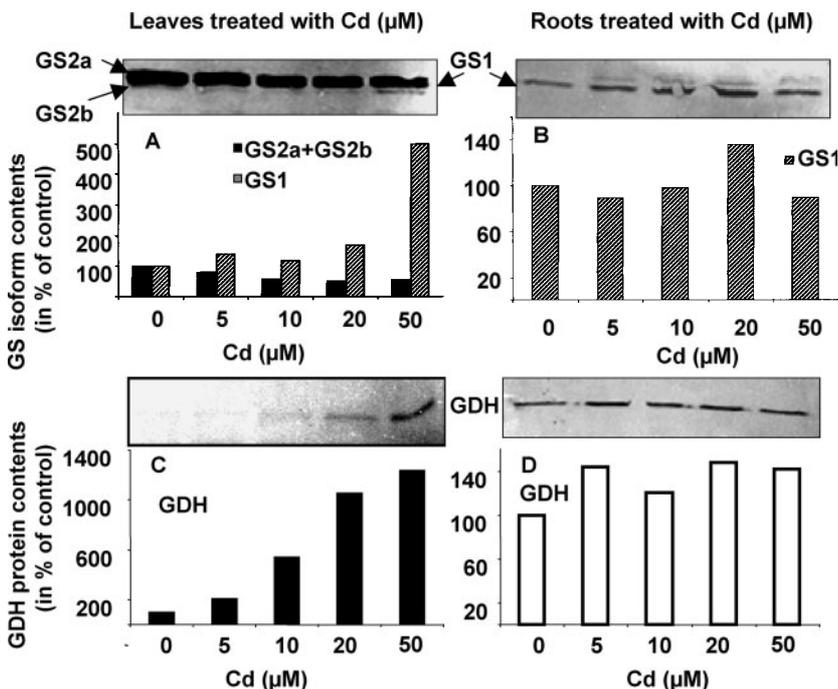
#### Changes in activity and protein content of key enzymes involved in nitrogen management

In order to investigate the influence of Cd on nitrogen assimilation and nitrogen recycling we measured activity and protein content of enzymes involved in primary nitrogen assimilation (nitrate reductase (NR), nitrite reductase (NiR), chloroplastic glutamine synthetase (GS2) and GOGAT) and nitrogen mobilization (GS1, GDH and NADH-GOGAT) (Masclaux et al. 2001 for review). The presence of Cd in the culture medium resulted in a decrease in NR, NiR and GS activities in both leaves and roots (Table 3, Chaffei et al. 2003). Inhibition of NR activity was similar in leaves and roots. In contrast, GS and NiR activities were altered to a higher extent in leaves than in roots.

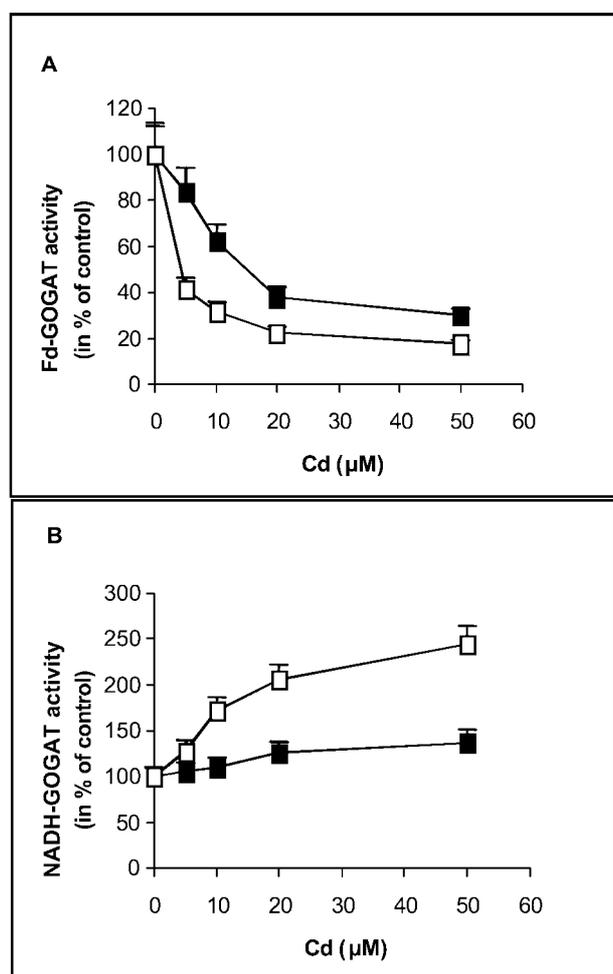
GS activity is the sum of GS2 and GS1 activities. In order to estimate the effect of Cd on isoforms, protein content of GS1

and GS2 was investigated by western blot analysis (Fig. 4A, B). The molecular masses of the two isoenzymes are different. Three bands corresponding to either GS1 (40 kDa), GS2b (42 kDa) or GS2a (44 kDa) proteins can be unambiguously visualized on western blots using antibodies raised against GS2 of tobacco (Becker et al. 1992). In the leaves of the stressed tomato plants, the level of GS1 protein was clearly increased up to approximately 6-fold in the 50  $\mu$ M treated plants. Immunoblot observation and signal quantification showed that a large amount of the two GS2 isoforms (GS2a and GS2b) was detected in leaf protein extracts, but could not be clearly detected in root extracts. However, their amount continuously decreased with Cd concentration. In contrast, Cd treatment induced the appearance of the GS1 isoform, which cannot be detected in untreated leaf tissues. In roots, the major isoform was GS1, and its amount was almost unchanged whatever Cd treatment applied. GS2 signals were low and under the quantification threshold allowed by the analyser. The sharp decrease in GS activity in leaves might then be due to the decrease in GS2.

GS and Fd-GOGAT both cooperate in ammonia assimilation in higher plants; therefore, Fd-GOGAT activity was assayed, and a decrease in Fd-GOGAT activity was clearly observed in treated plants (Fig. 5A). Surprisingly the decrease in Fd-GOGAT activity was more important in roots than in leaves. Consequently, in treated plants, it seems unlikely that the remaining Fd-GOGAT activity can ensure efficient synthesis of glutamate, illustrated by the decrease in glutamate content previously described in roots. An alternative to glutamate Fd-GOGAT-dependent synthesis is the involvement of NADH-



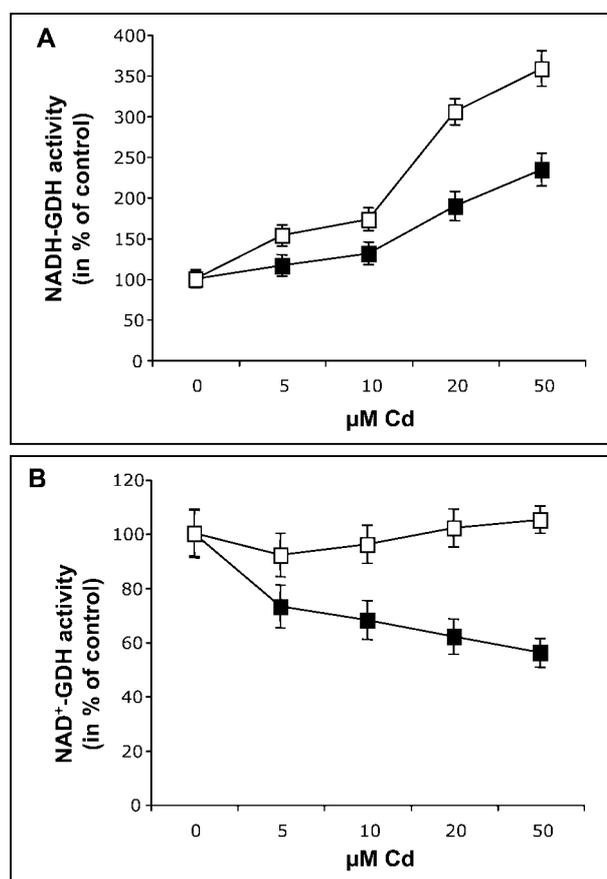
**Fig. 4** Changes in GS (A, B) and GDH (C, D) in leaves (A, C) and roots (B, D) of Cd-treated plants. Protein extracts were obtained from roots and leaves from four independent plants. Ten micrograms of a mix of the four plant samples were loaded in each track for SDS-PAGE separation. Western blot signals were quantified and protein contents expressed as a percentage of the untreated control (0  $\mu$ M).



**Fig. 5** Changes in Fd-GOGAT (A) and NADH-GOGAT (B) activity in leaves (filled squares) and roots (open squares) of Cd-treated tomato plants. Values are mean  $\pm$  SE of five individual plants. Standard errors are not shown when they are smaller than the symbol. The Fd-GOGAT and NADH-GOGAT activity [units in  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ ] of the control values (100%) represented in leaves 13 units and 1.4 units and in roots 1.4 units and 0.2 units, respectively.

GOGAT activity. In the Cd-treated plants, NADH-GOGAT activity was increased, especially in roots, by three fold compared with control plants (Fig. 5B). However, the NADH-GOGAT specific activity represents only 16% of the Fd-GOGAT activity in roots.

Another putative source of glutamate is the reaction catalysed by the mitochondrial NADH-dependent GDH. The change in NAD(H)-GDH activities was investigated. NADH-GDH activity increased by 3- and 4-fold under Cd treatment in roots and leaves, respectively (Fig. 6A; Chaffei et al. 2003). Moreover, the NAD<sup>+</sup>/NADH ratio was decreased by Cd treatment (Fig. 6A, B). In the 50  $\mu\text{M}$  treated plants, the ratio was equal to 25% and 34% of the control for leaves and roots, respectively, indicating that the change in the GDH aminating and deaminating activities is in favour of glutamate synthesis.



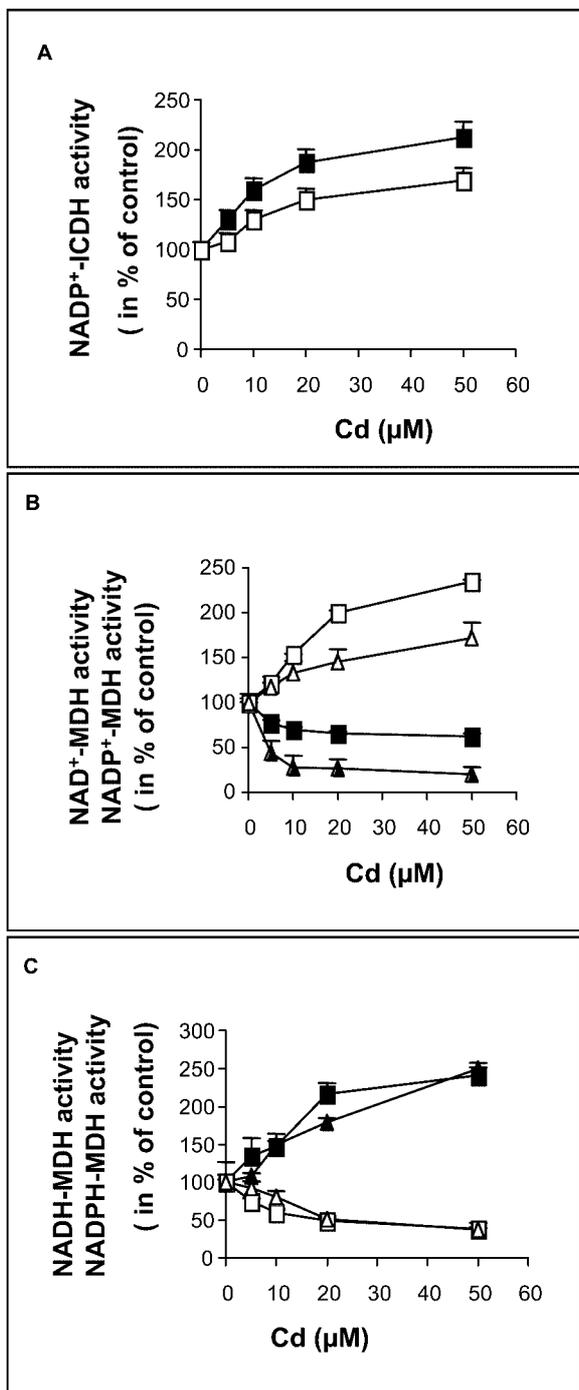
**Fig. 6** Changes in NAD<sup>+</sup>-GDH (A) and NADH-GDH (B) activity in leaves (black squares) and roots (open squares) of Cd-treated tomato plants. Values are mean  $\pm$  SE of five individual plants. Standard errors are not shown when they are smaller than the symbol.

The amount of GDH protein was not similar in the leaves and roots; GDH protein content was lower in leaves than in roots. With increasing doses of Cd, the amount of GDH protein increased in the leaves by approximately 14-fold (Fig. 4C). No significant change in GDH protein content was observed in the roots (Fig. 4D).

#### *Changes in activities of key enzymes catalysing the availability of the carbon skeleton devoted to amino acid synthesis*

NADP<sup>+</sup>-dependent isocitrate dehydrogenase (ICDH) belongs to a multi-isoenzymic family, whose members exist in several cellular compartments. Although an ICDH isoenzyme was found in the plastid, it was proposed that cytosolic ICDH might play a major role in 2-oxoglutarate production for use in amino acid synthesis (Gálvez et al. 1994). The effect of Cd treatment on ICDH activity is presented in Fig. 7A, which shows that exogenously applied Cd clearly stimulated NADP<sup>+</sup>-ICDH activity in leaves and roots.

NAD(P)H is a key reducing factor for nitrogen management, since it is used by enzymes such as nitrate reductase,



**Fig. 7** Changes in NADP<sup>+</sup>-ICDH (A), NAD<sup>+</sup>-MDH (squares) and NADP<sup>+</sup>-MDH (triangles) (B) and NADH-MDH (squares) and NADPH-MDH (triangles) (C) activity in leaves (filled squares and triangles) and roots (open squares and triangles) of Cd-treated tomato plants. Values are mean  $\pm$  SE of five individual plants. Standard errors are not shown when they are smaller than the symbol.

glutamate dehydrogenase, NADH-dependent GOGAT and also by ICDH. It is known that NAD(P)H cannot cross the membrane directly and uses metabolic channels and specially the

malate/oxaloacetate channel, controlled by the malate dehydrogenase enzyme. We measured both NAD(P)<sup>+</sup> and NAD(P)H MDH activities. The NAD(P)<sup>+</sup>-MDH activities increased in roots, whereas the NAD(P)H-dependent activities were decreased (Fig. 7B, C). This suggests that MDH isoforms preferentially catalysed the synthesis of oxaloacetate (OAA) and reducing power in roots. Interestingly the opposite was observed in leaves, suggesting that MDH preferentially catalysed malate synthesis in leaves and then constituted a supplementary sink for reducing power in leaves.

#### *Transcript levels of enzymes implicated in nitrogen metabolism*

To determine whether the change observed in enzyme activity is related to transcriptional control by Cd treatment, we measured the steady-state levels of the cognate transcripts (Fig. 8). 18S rRNA was used as a constitutive control, and a *Glubas* probe, which encodes a basic  $\beta$ -1,3-glucanase, served as a stress-related control (Van Kan et al. 1992).

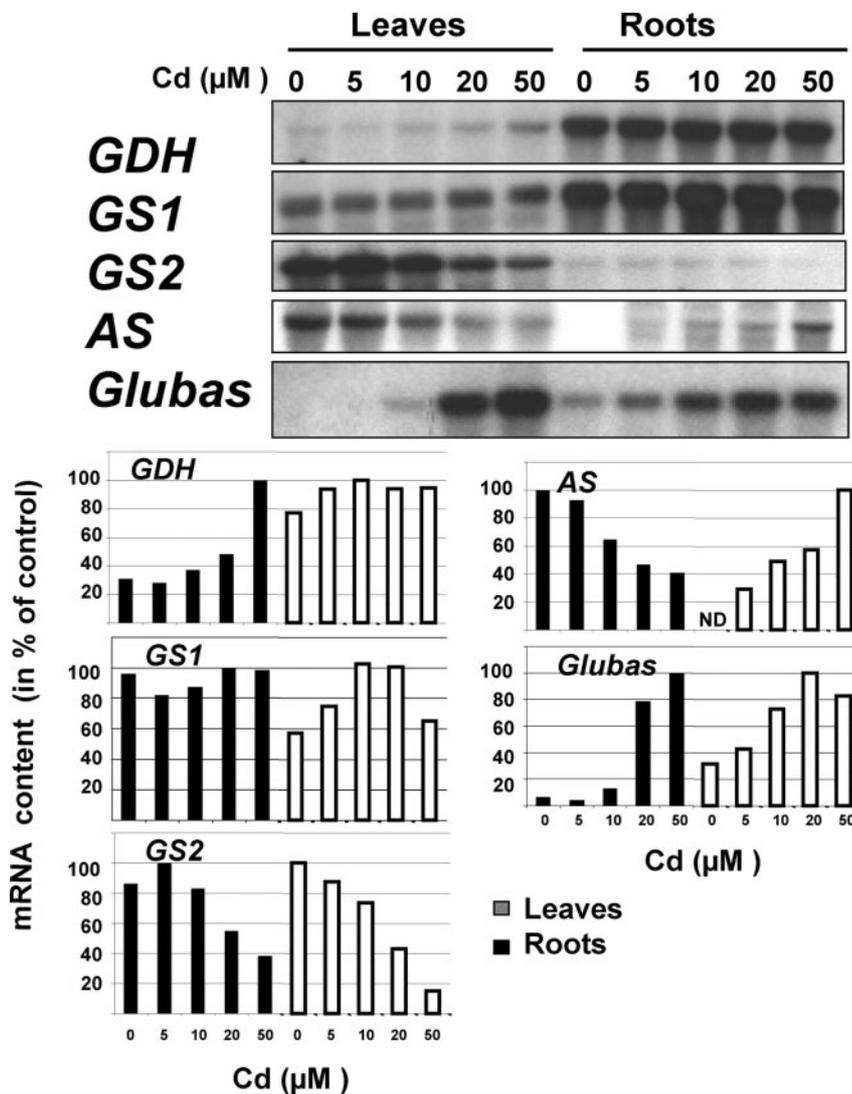
The GDH mRNA steady-state level was increased by 4-fold in the leaves of tomato, when plants were treated with increasing doses of Cd (Fig. 8). In roots, the increase in GDH mRNA accumulation triggered by Cd treatment appeared smaller because of the pre-existing high level of transcripts in the roots of the control plants.

No difference in the abundance of transcripts encoding GS1 was clearly observed in the leaves of control and Cd-treated plants. In contrast, GS1 mRNA content was increased in roots of plants treated with Cd and a typical Gaussian pattern showed the dose-dependent effect of Cd on GS1 mRNA accumulation.

Cadmium treatment led also to a decrease in the steady-state level of GS2 in both roots and leaves of tomato. At 50  $\mu$ M Cd, GS2 mRNA content was lowered by 60% in the leaves and 85% in the roots, compared with the control plants. Using heterologous asparagine synthetase (AS) probe from *Lotus japonicus*, as well as low stringency conditions, we were able to observe one band located at about 2 kb (the size attended for AS mRNA) that showed a differential expression pattern in leaves and roots of tomato plants. The expression of the AS gene was strongly increased in roots of tomato plants when treated with Cd, whereas AS mRNA content decreased in the leaves of the same plants. *Glubas* was used as a stress-related control and mRNA levels showed a continuous increase in leaves and roots with increasing doses of Cd in the culture medium. Maximum *Glubas* mRNA content was obtained with 50 and 20  $\mu$ M Cd treatments and represented 16-fold and 3-fold the value of the leaves and roots of control plants, respectively.

## Discussion

In this study, tomato plants were exposed to Cd toxicity by introducing increasing doses of this pollutant into the culture medium. The morphology of tomato plants was dramatically



**Fig. 8** Changes in the steady-state level of transcripts encoding key enzymes involved in nitrogen metabolism. Total RNA from leaves and roots was obtained from a mix of four plants and an equal amount (10  $\mu\text{g}$ ) was loaded in each track. The 18S rRNA probe was used as a loading control. *Glubas* probe was used as a stress-sensitive control. The relative values of each mRNA in roots (white bars) and leaves (black bars) were presented on histograms as a percentage of maximum.

affected by Cd. We observed that the emergence of new organs was altered and that newly emerged leaves were chlorotic. Leaf transpiration and the molality of leaf fluids were both affected, showing that the Cd treatments we applied induced stomatal closure, symptoms previously described for Cd syndrome (Barceló and Poschenrieder 1990, Greger and Johansson 1992). In parallel we observed inhibition of photosynthesis, and as a consequence, a decrease in dry weight, fresh weight and leaf surface. We observed that roots from Cd-stressed plants were shorter and less hairy than those from controls. However, treated roots were thicker and stronger, which might have compensated in part for the loss of biomass. Indeed, it appeared that root biomass was affected to a lesser extent compared with leaves. From all these observations, it can be concluded that fresh weight, dry weight, total chlorophyll content and leaf surface are good biomarkers for estimating the senescence-like phenomena related to the stress trauma triggered by a heavy metal (Smart 1994, Masclaux et al. 2000).

This model was then used to investigate the effect of Cd toxicity on nitrogen management in plants. We observed that Cd-treated plants accumulated ammonium and free amino acids in both leaves and roots. They progressively lost nitrate. The decrease in nitrate in roots and leaves might have been a consequence of efficient reduction activity. However, a concomitant decrease in total soluble protein and in the activity of enzymes involved in primary nitrogen assimilation (NR, NiR and GS/GOGAT) was observed in both leaves and roots (Chaffei et al. 2003 and this work). Inhibition of NR, NiR and GS activity has also been described in maize, pea and barley seedlings (Nussbaum et al. 1988, Chugh et al. 1992, Boussama et al. 1999). The decrease in nitrate might then be due to a deleterious effect of Cd on mineral absorption, maybe through the alteration of transpiration. In leaves, Rubisco is the most abundant soluble protein, representing up to 50% of the total leaf soluble protein pool (Masclaux et al. 2001, for review). Therefore, the decrease in the abundance of the Rubisco large subunit

in tomato leaves during Cd stress might contribute largely to the net loss of total leaf protein observed. The loss of Rubisco during senescence is well documented. We have shown in previous work that nitrogen remobilization is a senescence-associated process that takes place in source organs such as senescing leaves (Masclaux et al. 2001). Nitrogen remobilization is of major importance in plant nitrogen economy since it provides nitrogen to young, developing organs. Nitrogen remobilization is usually associated with the induction of the expression of enzymes such as specific proteases, the cytosolic GS1 isoform and GDH. We have thus investigated whether these enzymes are expressed in leaves and roots of tomato when submitted to Cd stress.

We showed that the total GS activity was decreased in both roots and leaves of Cd-treated plants, with higher inhibition in the leaves. Indeed, protein analysis and transcript steady-state level study showed that both GS2 proteins and mRNA decreased in Cd-treated plants. In parallel, we observed that GS1 mRNA accumulated mainly in the roots of treated plants. The GS1 protein remained constant in roots except at 20  $\mu$ M Cd, and the GS1 isoform increased in leaves of Cd-treated plants. This suggested that when Cd affected the GS2 activity, plants induced the cytosolic isoform to compensate and continue glutamine biosynthesis.

Moreover, concomitantly with the increase in GS1 protein in leaf crude extracts we also observed a sharp increase in GDH protein in both roots and leaves, which paralleled changes in *GDH* mRNA levels. Induction of *GDH* gene expression appeared to be higher in leaves than in roots. Both NAD<sup>+</sup>- and NADH-dependent GDH activities can be measured (Chafefi et al. 2003). The NAD<sup>+</sup>/NADH ratio decreased as Cd concentration in the culture medium increased. This suggested that tomato plants can express several GDH isoenzymes with differential deaminating and aminating activities, and that Cd-treated plants preferentially expressed isoenzymes with higher in vitro aminating capacity, i.e. preferentially involved in glutamate synthesis. However, until now, there has been no evidence that the functionality of GDH activity in vitro is representative of in vivo reality. The role of the induction of GDH during stress as well as during senescence still remains and it is attractive to suppose that both GDH and NADH-GOGAT are induced in order to compensate for the lack of Fd-GOGAT (Masclaux et al. 2000). We observed in this work that NADH-GOGAT activity was increased in the Cd-treated roots although NADH-GOGAT activity represents only 10% of the Fd-GOGAT activity. These results taken together confirmed that when stressed by Cd, plant nitrogen metabolism shifted from primary assimilation to recycling of ammonia and organic nitrogen remobilization (see Feller and Fischer 1994 for review).

During natural leaf senescence, nitrogen remobilization is essential to support new organ emergence and growth (Masclaux et al. 2001). Interestingly, we observed in this work, that the nitrogen remobilization process is favoured in Cd-treated

plants in which the growth and the emergence of new organs is considerably compromised, compared with untreated plants. This then suggests that GS1, GDH and proteolysis are involved for another purpose than the building of new organs.

Studying the qualitative and quantitative changes in the content of individual amino acids can thus be informative. We observed that total amino acid content was increased by Cd in both leaves and roots (Fig. 3C). This increase was 2-fold in roots than leaves, even if leaves lost their proteins more rapidly than roots. Moreover, amino acids were twice as plentiful in the phloem sap of Cd-treated plants, but unchanged in their xylem sap, suggesting that the roots can be used as a storage element where amino acids are transported from leaves.

In leaves, we observed that the proportion of Glu remained unchanged (Table 1). This might be a consequence of buffering effects through modulations in GaBa or Pro, which are increasing. Indeed, these amino acids are direct products of Glu metabolism. Pro and GaBa are known to be two stress markers (Narayan and Nair 1990, Bown and Shelp 1997), and their increase in stressed leaves might be considered as such. Among the main amino acids represented (i.e. Glu, Asp and Gln), Gln was highly increased by Cd stress. This amide (high N/C ratio) is known to be highly reactive and to serve as the major nitrogen transport form in plants. Indeed, Gln is the more important amino acid contained in phloem sap (about 30%; Table 2). Glu, Asp and GaBa represented about 20%, 20% and 6.5%, respectively, of the total amino acids. Glu and Gln were not significantly affected by Cd stress, but Asp and GaBa were strongly decreased. In contrast, the proportions of Asn, His, Arg and minor amino acid bulk were strongly increased, thus suggesting that some products of proteolysis might be exported directly through the phloem to the roots whereas GaBa and Asp export might be affected by Cd.

In roots, the total amino acid content (Fig. 3C) was highly increased. Gln, Glu, GaBa and Asp were the major amino acids found in roots. Unlike leaves, the proportion of Glu was strongly affected by Cd treatment, whereas Gln remained relatively stable. The most important changes observed are the strong decrease in Glu (2-fold) and the strong increase in Asn (9-fold). Asn, which is also an amide providing a high N/C ratio, is however, less reactive than Gln and can be used by the plant as a nitrogen storage compound. The nitrogen storage status of roots in plants treated with a high Cd dose is also confirmed by the increase in Pro and Arg. Indeed, Arg is a nitrogen storage compound, mainly in trees, and this increase in a herbaceous species should be mentioned. The amino acid content in xylem sap was unchanged by Cd treatment. We observed that Gln represents about 60% of total amino acids. The Gln content in xylem sap was slightly but significantly decreased by Cd stress, thus showing that Gln increase in roots did not influence xylem sap composition. The main changes were a strong decrease in GaBa and Ala content and a strong increase in Asn and Lys, reflecting changes parallel to those in roots. This sug-

gests that except for Gln, changes occurring in root tissues influenced widely xylem sap composition.

The sharp increase in Asn in roots led us to question AS expression in both roots and leaves of tomato plants. The use of the AS probe facilitated this study. It is interesting to note that the 3-fold increase in AS mRNA in roots of treated plants was the highest induction observed among the transcripts studied. AS is an ATPase and the active implication of Cd-stressed roots in Asn synthesis implied then the availability of dedicated energy.

In the plant, the cytosolic ATP pool is provided mainly through the photosynthetic and oxidative phosphorylation pathways (Krömer 1995). In roots and in photosynthetic cells maintained under low light input, the mitochondrial respiratory pathway is the major source of energy. Glycine decarboxylation catalysed by glycine decarboxylase in leaf mitochondria is the major respiratory pathway dedicated to ATP synthesis (Wiskich et al. 1990). However, we have seen that both photosynthesis and photorespiration were altered by Cd treatment, suggesting that these energy sources were a limiting factor in leaves of Cd-stressed plants. In treated leaves we observed, that ICDH activity was increased. This suggests that the synthesis of 2-OG dedicated to amino acid synthesis and especially to Glu maintenance continued and that the availability of reducing power remained, sufficient.

In roots, the mitochondrial phosphorylation pathway usually consumes carbon skeletons provided by sugar mobilization from leaves. When stressed by Cd, we observed that Glu and GaBa were strongly decreased. This suggested that because of a decrease in sugar production in leaves, and *per se* a decrease in sugar import into roots, Glu might be used in roots as a respiratory substrate for mitochondria. Two ways could then be implied. Glu might be (i) converted into GaBa, which will then provide succinate to sustain respiration, or (ii) deaminated in 2-OG by GDH into mitochondria. Since NAD(P)<sup>+</sup>-MDH activity was favoured in roots, whereas NAD(P)H-MDH is favoured in leaves, the data showed that a transfer of reducing power from leaves to roots occurred *via* the mobilization of organic acids such as malate.

In conclusion, the data presented in this study showed that Cd treatment induced a differential but coordinated response of the carbon and nitrogen management pathways between leaves and roots. Total and individual amino acid content as well as GS1, GDH and AS expression suggest that nitrogen is recycled and translocated from leaves to roots. While leaf biomass was strongly affected by Cd toxicity, biomass of roots was less affected. The increase in the proportions of Asn, Pro and Arg in roots indicated that these organs store high N/C amino acids and nitrogen *per se*. We suppose that such a strategy sacrifices the aerial organs of the plants to preserve roots as a nutritional safeguard organ to ensure future recovery.

## Materials and Methods

### *Plant material and growth conditions*

The plant material used in this work was tomato (*Lycopersicon esculentum*, Mill. cv. 63/5F1). Seeds were sterilized in 10% (v/v) hydrogen peroxide for 20 min, and washed abundantly in distilled water afterwards. After imbibition, the seeds were germinated on moistened filter paper at 25°C in the dark. After 7 d, the uniform seedlings were transferred to 6 litre plastic beakers (eight plants per beaker) filled with continuously aerated, basal nutrient solutions of an initial pH 5.8–6, containing 3 mM KNO<sub>3</sub>, 0.5 mM Ca (NO<sub>3</sub>)<sub>2</sub>, 2.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 100 μM Fe-K<sub>2</sub>-EDTA, 30 μM H<sub>3</sub>BO<sub>3</sub>, 5 μM MnSO<sub>4</sub>, 1 μM CuSO<sub>4</sub>, 1 μM ZnSO<sub>4</sub> and 1 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>. Plants were grown in a growth chamber (26°C/70% relative humidity during the day, 20°C/90% relative humidity during the night). The photoperiod was 16 h with a light irradiance of 150 μmol m<sup>-2</sup> s<sup>-1</sup> at the canopy level. At the age of 10 d after transplant, Cd was added to the medium as CdCl<sub>2</sub> at 0–50 μM. Control plants were untreated plants (0 μM). After 1 week of Cd treatment, plants were separated into leaves and roots. Roots were washed rapidly three times in 1 litre of distilled water, then samples were stored in liquid nitrogen for subsequent analysis or dried at 70°C for at least 3 d in order to determine both dry material and ionic content.

### *Chlorophyll and total protein determinations*

Chlorophyll content was estimated on crude leaf extracts (Arnon 1949). Soluble protein was determined using a commercially available kit (Coomassie Protein assay reagent; Bio-Rad, Hercules, CA, U.S.A.).

### *Phloem and xylem sap collection*

Phloem and xylem exudates were collected from tomato plants treated or not with Cd. Phloem exudates were obtained using the technique described by King and Zeevaart (1974). Stems were cut off and recut under water before rapid immersion in the collection buffer. For each experiment, stems of six untreated plants and six Cd-treated plants were placed separately in 1 ml of the collection buffer (10 mM HEPES and 10 mM EDTA, adjusted to pH 7.5 with NaOH). Exudates were collected during 8 h in a humid chamber (relative humidity >90%) in the dark. Phloem exudates were then adjusted at pH 2.0, centrifuged to remove debris and concentrated. Exudates were stored at –80°C before analysis.

For xylem sap collection, stems of plants were cut 2 cm above the root system. The cut stem was rinsed with water and blotted dry. Bleeding sap was collected in a latex-made microtube well fitted around the stem sections. Samples were stored at –80°C. Amino acid analysis was performed as described below.

### *Metabolite extraction and analysis*

Nitrate was colorimetrically determined on an automatic analyser following diazotation of the nitrite obtained by reduction on a Cd column (Henriksen and Selmer-Olsen 1970). The reduced nitrogen content of dried plant material was determined with the Kjeldahl method.

Amino acid and NH<sub>4</sub><sup>+</sup> were determined after extraction in a 2% solution of 5-sulfosalicylic acid (50 mg FW ml<sup>-1</sup>). Total amino acid content was assayed by the Rosen colorimetric method using glutamine as a reference. Individual amino acid composition was determined on (i) pooled samples extracted from an equal dry weight of three plant repeats and (ii) phloem and xylem sap, using ion-exchange chromatography (Rochat and Boutin 1989). Free NH<sub>4</sub><sup>+</sup> was determined by the phenol hypochlorite colorimetric method (Berthelot reaction) using (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as a reference.

### Photosynthesis and transpiration determination

Net CO<sub>2</sub> assimilation and transpiration rates of attached tomato leaves were measured by IRGA techniques (model LCA4, Leaf Chamber Analyser). During analyses, plants were maintained in their corresponding culture conditions.

In order to obtain leaf fluids, freshly harvested leaf tissues were immediately mashed and introduced into microcentrifuge tube polypropylene filters (0.45 µm; Whatman, Maidstone, U.K.). The molality of the leaf fluids collected was measured using the Vapor Pressure Osmometer 5500 manufactured by Wescor Inc. (Logan, UT, U.S.A.).

### Enzyme assays

Enzymes were extracted from frozen leaf and root material stored at -80°C. All extractions were performed at 4°C. Nitrate reductase was extracted and the maximal extractable activity measured (Ferrario-Méry et al. 1997). Nitrite reductase activity was measured as the reduction in the amount of nitrite in the assay mixture by incubating an aliquot of the extract for 30 min at 30°C with 75 mM Tris-HCl buffer (pH 7.5), 2 mM NaNO<sub>2</sub> and 750 µM methyl viologen (MV). The reaction was initiated by sodium dithionite and stopped by vortexing (Chérel et al. 1986). GS was measured according to the method of O'Neal and Joy (1973). The NADH- and NAD<sup>+</sup>-dependent glutamate dehydrogenases (NADH-GDH and NAD<sup>+</sup>-GDH) were measured as described by Turano et al. (1996), except that the extraction buffer was the same as for GS. The activities of ferredoxin and NADH-dependent glutamate synthases (Fd-GOGAT) were measured as described by Suzuki et al. (1994), using MV or NADH as electron donor. NAD(H)-MDH and NADP(H)-MDH malate dehydrogenase activity was measured as described by Johnson (1970). Total ICDH activity was measured spectrophotometrically by following the reduction of NADP at 340 nm as described by Gálvez et al. (1994). Enzyme activities were measured per min and per g FW and then expressed as a percentage of the control.

### Gel electrophoresis, Western blot analysis and gel staining procedure

Proteins were extracted as described above for GS and GDH assays. Proteins were separated by SDS-PAGE (Laemmli 1970). An equal amount of protein was loaded in each track. The percentage of polyacrylamide in the running gels was 10% for GDH, GS and Rubisco. Denatured proteins were electrophoretically transferred to nitrocellulose membranes or directly stained with Coomassie Blue for Rubisco detection. Polypeptide detection was performed using polyclonal antiserum raised against grape leaf GDH (Loulakakis and Roubelakis-Angelakis 1990) and tobacco GS2 (Becker et al. 1992). Relative GS2, GS1, GDH and Rubisco protein amounts were determined by densitometric scanning of Western blot membranes or stained gels and quantification using the NIH image 1.63 application, public domain.

### Extraction of total RNA and Northern blot analysis

Total RNA was extracted as described by Verwoerd et al. (1989) from plant material stored at -80°C. Northern blot analysis was performed as described previously (Masclaux et al. 2000). The following <sup>32</sup>P-labelled probes were used for mRNA detection: GS2 from *Nicotiana tabacum* (Becker et al. 1992), GS1 from *N. tabacum* (Dubois et al. 1996), GDH cDNA from *N. tabacum* (Masclaux-Daubresse et al. 2002) and asparagine synthetase *Ljas1* (accession number X89409) and *Ljas2* (accession number X94184) from *L. japonicum*, kindly provided by Dr. R.N. Waterhouse. The 18S rRNA probe (Deng et al. 1991) was used as a constitutive control and the *Glubas* probe, which encodes a basic β-1,3-glucanase, as a stress-related control (Van Kan et al. 1992).

Since tobacco and tomato genes are highly homologous, hybridization with *GS1*, *GS2* and *GDH* probes was performed under high stringency conditions at 65°C according to Church and Gilbert (1984). Filters were washed with 2× SET (0.06 M Tris-HCl pH 8, 0.3 M NaCl, 4 mM EDTA) at room temperature for 5 min and at 65°C for 10 min. Additional washing was performed successively using 1× SET and 0.5× SET at 65°C for 15 min before drying and exposure to X-ray film. Heterologous hybridization with a mix of *Ljas1* and *Ljas2* probes was performed in low stringency conditions at 55°C. Filters were washed with 2× SET (0.06 M Tris-HCl pH 8, 0.3 M NaCl, 4 mM EDTA) at room temperature for 5 min and at 55°C for 10 min. Additional washings were performed using 1× SET at 55°C for 15 min, before drying and exposure to X-ray film. The relative amounts of mRNA were determined by densitometric scanning of Northern blot autoradiograms and quantified using the NIH image 1.63 application (public domain).

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