Plant Cell Physiol. 49(3): 362–374 (2008)
doi:10.1093/pcp/pcn013, available online at www.pcp.oxfordjournals.org

Increase in Ascorbate–Glutathione Metabolism as Local and Precocious Systemic Responses Induced by Cadmium in Durum Wheat Plants

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Durum wheat plants (*Triticum durum* cv Creso) were grown in the presence of cadmium (0–40 μM) and analysed after 3 and 7 d for their growth, oxidative stress markers, phytochelatins, and enzymes and metabolites of the ascorbate (ASC)–glutathione (GSH) cycle. Cd exposure produced a dose-dependent inhibition of growth in both roots and leaves. Lipid peroxidation, protein oxidation and the decrease in the ascorbate redox state indicate the presence of oxidative stress in the roots, where H_2O_2 overproduction and phytochelatin synthesis also occurred. The activity of the ASC–GSH cycle enzymes significantly increased in roots. Consistently, a dose-dependent accumulation of Cd was evident in these organs. On the other hand, no oxidative stress symptoms or phytochelatin synthesis occurred in the leaves; where, at least during the time of our analysis, the levels of Cd remained irrelevant. In spite of this, enzymes of the ASC–GSH cycle significantly increased their activity in the leaves. When ASC biosynthesis was enhanced, by feeding plants with its last precursor, L-galactono-γ-lactone (GL), Cd uptake was not affected. On the other hand, the oxidative stress induced in the roots by the heavy metal was alleviated. GL treatment also inhibited the Cd-dependent phytochelatin biosynthesis. These results suggest that different strategies can successfully cope with heavy metal toxicity. The changes that occurred in the ASC–GSH cycle enzymes of the leaves also suggest that the whole plant improved its antioxidant defense, even in those parts which had not yet been reached by Cd. This precocious increase in the enzymes of the ASC–GSH cycle further highlight the tight regulation and the relevance of this cycle in the defense against heavy metals.

Keywords: Ascorbate–glutathione cycle — Cadmium — L-Galactono-γ-lactone — Hydrogen peroxide — Oxidative stress — Phytochelatins.

Abbreviations: ANOVA, analysis of variance; APX, ascorbate peroxidase; ASC, ascorbate; BSA, bovine seum albumin; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DHR123, dihydrorhodamine 123; DNPH, 2,4-dinitrophenylhydrazine; GL, L-galactono-γ-lactone; GR, glutathione reductase; GSH, glutathione; PC, phytochelatin; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; ROS, reactive oxygen species; TBA, thiobarbituric acid.

Introduction

Cadmium (Cd) is a widespread trace pollutant released into the soil by industrial processes or by the utilization of fertilizers and pesticides containing it (Sanità di Toppi and Gabbrielli 1999, Prasad 2004). Cd is thought to be absorbed by roots through the uptake systems used for the divalent ions necessary for plant mineral nutrition. It can be accumulated in roots, both immobilized in the cell walls, where it is cross-linked by pectin carboxylic groups, and compartmentalized into vacuoles. Plants are also able to load part of the Cd taken up from the soil into the xylem and to transport it to the leaves (Chardonnens et al. 1998). Cd strongly interferes with plant metabolism: it binds sulfydryl groups, altering the activity of several enzymes; on the other hand, it causes deficiency of other essential metals, competing with them for the same carriers. Some data also indicate that Cd induces oxidative stress in plant tissues, since increases in lipid peroxidation, protein oxidation and nucleic acid damage are common events occurring in plants exposed to Cd (Gallego et al. 1996, Romero-Puertas et al. 2002). Althought Cd is not able to trigger Fenton-type reactions (Salin 1988), it can indirectly activate the plasma membrane NADPH oxidase, thus inducing generation of reactive oxygen species (ROS) in the exposed tissues (Piqueras et al. 1999, Romero-Puertas et al. 2004). Moreover, it has been suggested that Cd can also induce oxidative stress by suppressing the antioxidant defenses (Sandalio et al. 2001, Schützendübel et al. 2001). Under these conditions, the concentration of ROS generated as by-products of normal metabolism increases to toxic levels. Recently the production of ROS at the subcellular level was demonstrated in the leaves of *Pisum sativum* plants treated with Cd (Romero Puertas et al. 2004, Rodríguez-Serrano et al. 2006). Resistance or minor sensibility against

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Cd also depends on the capability of a plant to increase its antioxidant defenses, among which the ascorbate (ASC)–glutathione (GSH) cycle plays a pivotal role (Noctor and Foyer 1998, Di Cagno et al. 2001).

Cd also induces the biosynthesis of phytochelatins (PCs), glutathione-derived thiol peptides having the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n is the number of repetitions of the γ-Glu-Cys unit, which can vary from two to eleven (more commonly from two to five) (Grill et al. 1985). PCs form various complexes with Cd, due to the presence of the thiolic groups of cysteine, which chelate Cd and prevent it from circulating as a free ion in the cytosol (Grill et al. 1985).

This heavy metal easily enters the food chain from plant tissues due both to its ability to form complexes with biological macromolecules and peptides, as well to its long biological half-life (Wagner 1993). In order to avoid Cd toxicity by limiting its dietary uptake, the Codex Alimentarius Commission proposed a limit of 0.1 mg Cd per kg of plant biomass (Codex Alimentarius Commission 2000). It is known that cereals and legumes accumulate less Cd in their leaves than other plants, such as lettuce and spinach (Grant et al. 1999). However, large differences in the uptake and/or in the amount and sites of Cd storage have been reported in food plants. Among cereals, durum wheat accumulates a higher amount of Cd than bread wheat (Harris and Taylor 2001). Durum wheat has a great relevance for the agriculture of the Southern region of Italy, being the raw material for pasta. Moreover, it is exposed to the use of fertilizers and pesticides which are one of the main sources of Cd contamination in the soil.

In order to study the relevance of the antioxidant systems in counteracting Cd toxicity, durum wheat plants were hydroponically grown in the presence of 0–40 μM Cd. Roots and primary leaves were taken at 3 and 7 d of treatments and analyzed for their levels of Cd, PCs, markers of oxidative stress, ASC and GSH contents and redox states, as well as for the activities of the ASC–GSH cycle enzymes. The increase in ASC biosynthesis, induced by supplying its last precursor, L-galactono-γ-lactone (GL), to seedlings was also analyzed.

Results

Cadmium effects on plant growth and phytochelatin synthesis

When durum wheat plants were grown in the presence of 10–40 μM cadmium, no necrotic or chlorotic symptoms were evident in roots or leaves, at least during the 7 d of our analysis. On the other hand, the presence of Cd affected plant growth since both root and leaf lengths were reduced in a dose-dependent manner (Fig. 1). The growth reduction involved cell proliferation, as demonstrated by the Cd-dependent inhibition of the mitotic index occurring in the root apical meristem (Table 1).

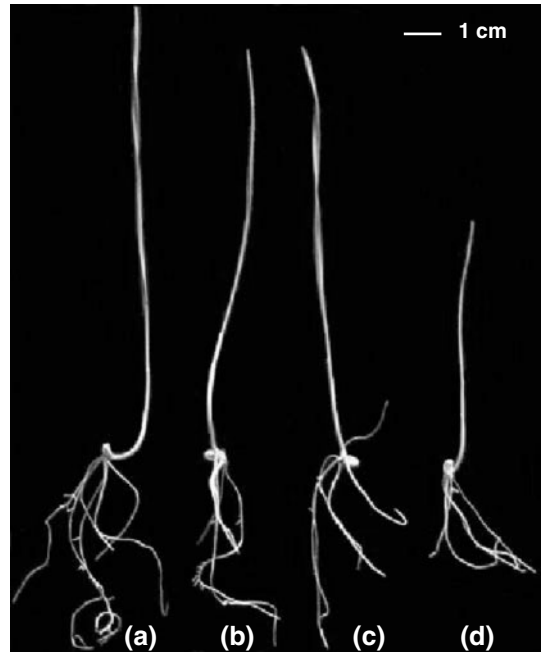

Fig. 1 Effects of cadmium on growth of wheat plants. Representative plants, grown in a hydroponic medium, in control conditions or in the presence of increasing concentrations of Cd for 7 days. (a) Control; (b) 10 μM Cd; (c) 20 μM Cd; (d) 40 μM Cd.

Table 1 Mitotic index (%) in root apex after 7 days of cadmium treatments

	Mitotic index
Control	19.36 ± 1.56a
Cd 10 μM	15.01 ± 3.3a
Cd 20 μM	8.15 ± 1.21b
Cd 40 μM	5.25 ± 1.77c

Data represent the means (± SE) of three separate experiments. In each experiment, 1,500 cells were analyzed. Different letters indicate significant differences between the values (one-way ANOVA test).

No trace of PCs was present in the roots of control plants; the synthesis of these peptides was rapidly induced in roots by Cd treatment (Fig. 2). Under a lower Cd concentration (10 μM) the synthesis of PCs reached a steady-state level after 3 d of treatment; whereas, under higher concentrations (20 and 40 μM) the synthesis of PCs increased further over time (Fig. 2). Among the different PC forms detectable in the roots, PC_3 was the predominant one, comprising 79 ± 3% and 84 ± 5% of the total PCs at 3 and 7 d, respectively. No induction of PCs occurred in the leaves, even at the highest Cd concentration used. Consistent with the synthesis of PCs, Cd was present in

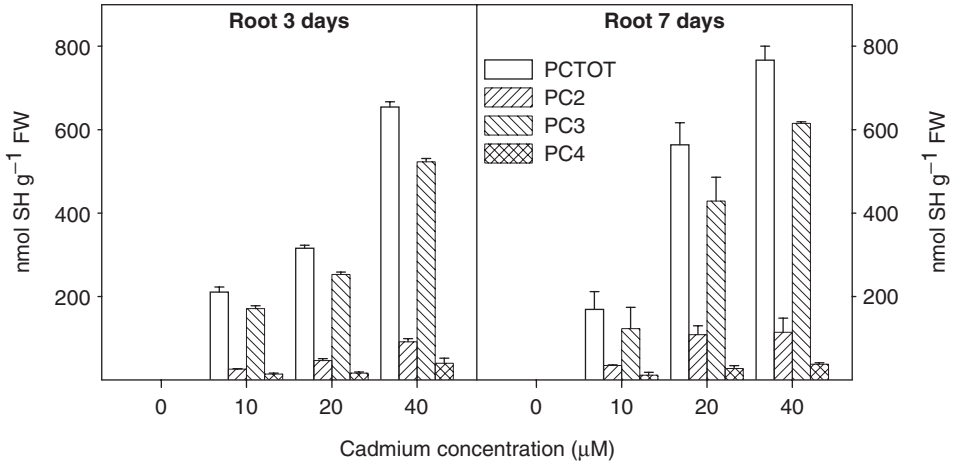

Fig. 2 Phytochelatin content in roots of plants grown in the presence of increasing Cd concentrations (0, 10, 20 and 40 μM) for 3 and 7 d. Values represent the mean (±SE) of three independent experiments. Different letters represent values which are statistically different (by one-way ANOVA test). PC_2, PC_3 and PC_4 correspond to the different forms of phytochelatins, on the bases of the polymerization levels of the $(\gamma\text{-Glu-Cys})_n$ repeat. PCTOT represents the sum of the different phytochelatins.

Table 2 Cadmium content in roots and leaves of durum wheat plants

	Root		Leaf	
	3 d	7 d	3 d	7 d
Control	43 ± 6	50 ± 7	4.3 ± 0.7	4.6 ± 0.9
Cd 10 μM	61.3 ± 3	80 ± 5	4.5 ± 0.8	5.2 ± 0.7
Cd 20 μM	71 ± 7	159 ± 12	4.0 ± 1.1	5.6 ± 0.4
Cd 40 μM	90 ± 9	267 ± 60	4.6 ± 0.4	5.2 ± 0.5

The Cd concentration is expressed as $\mu g\, g^{-1}$ DW. The values are the means of three independent experiments ±SE.

the roots where it was accumulated in a dose- and time-dependent manner (Table 2). In the leaves, no increase in Cd content was evident during the time of our analyses (Table 2), even if Cd accumulation occurred when the exposure to the heavy metal was prolonged (data not shown).

Markers of oxidative stress and antioxidant defenses

In order to verify whether the presence of Cd induced oxidative stress in durum wheat tissues, lipid peroxidation, protein oxidation and hydrogen peroxide production were assayed. In the roots, the presence of Cd induced a dose-dependent increase in lipid peroxidation, which was more evident after 3 d than after 7 d of treatment (Fig. 3a). Protein oxidation also occurred in the roots. This oxidative damage reached the highest level after 3 d, with the exception of the treatment with 40 μM Cd, under which a further increase in protein oxidation was evident after 7 d (Fig. 3b).

Consistent with the absence of Cd in the leaves, no significant differences in either lipid peroxidation or protein oxidation were found in these organs (Fig. 3c, d). Under our conditions, Cd did not even affect the content of photosynthetic pigments (chl *a* and *b*, or total carotenoids) (Fig. 4).

The presence of H_2O_2 in root tissues was analyzed by using the specific fluorescent probe dihydrorhodamine 123 (DHR123; Royall and Ischiropoulos 1993). Fig. 5 shows representative images of root apex from control and treated plants, showing that the H_2O_2-dependent green fluorescence was very faint in control roots (Fig. 5a), but it increased remarkably under various Cd treatments (Fig. 5b–d). In order to confirm the specificity of the fluorescent probe for H_2O_2, roots exposed to 40 μM Cd were incubated with 1 mM ASC or $30\, U\, ml^{-1}$ catalase, two H_2O_2 scavengers, before DHR123 addition. In both cases, the fluorescent signal was weakened (Fig. 5e, f).

In the roots, Cd also induced a dose-dependent increase of the total ascorbate pool [ASC plus dehydroascorbate (DHA)] (Fig. 6a, b). This increase was mainly due to the increase in DHA, the oxidized form of ascorbate; therefore, a decrease in the ascorbate redox state (i.e the ratio between the reduced form and the total pool) was observed in the roots from the plants grown in the presence of Cd. On the other hand, no significant differences occurred in either the ascorbate pool or redox state in the leaves (Fig. 6c, d).

The glutathione pool (GSH plus GSSG) also increased in the roots under Cd exposure, in a dose-dependent manner (Fig. 7a, b). The changes in the glutathione pool were mainly due to the increase in the reduced form, i.e. GSH, since the levels of GSSG remained almost unaltered

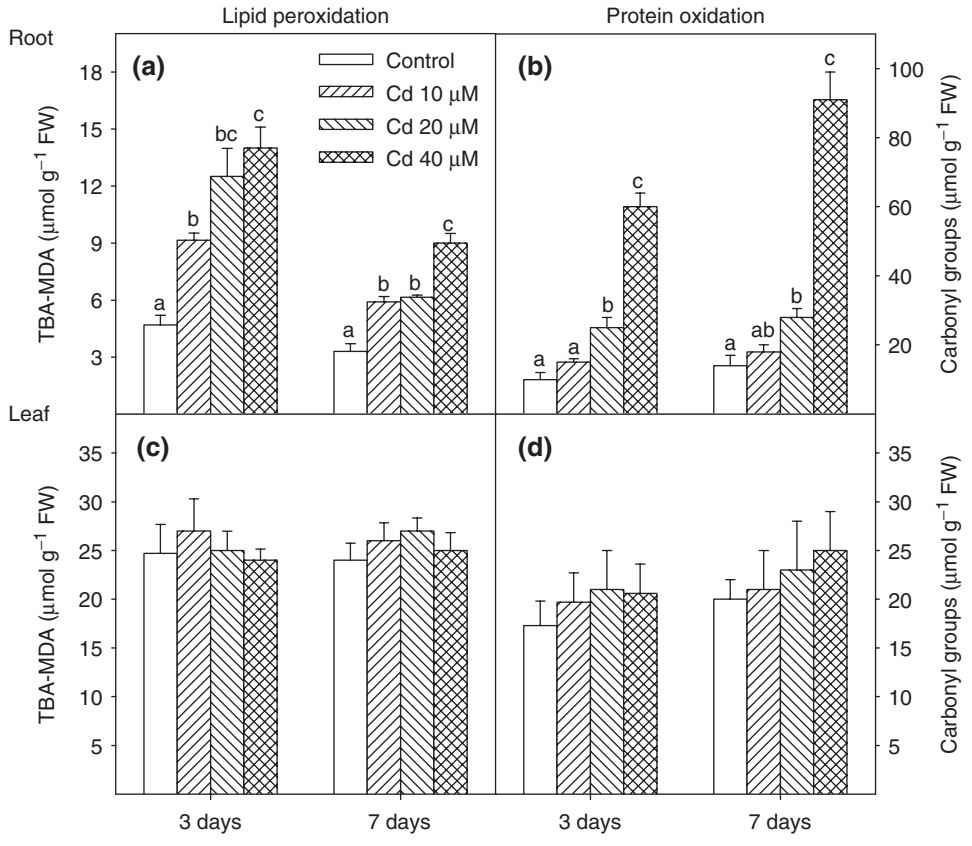

Fig. 3 Oxidative stress markers in roots and leaves of wheat plants grown in the presence of Cd. The level of lipid peroxidation in roots (a) and in leaves (c) was expressed as MDA–TBA complex; the level of protein oxidation in roots (b) and in leaves (d) was measured as total protein carbonyl group content. Data represent the means (±SE) of five separate experiments. Different letters represent values which are statistically different (by one-way ANOVA test).

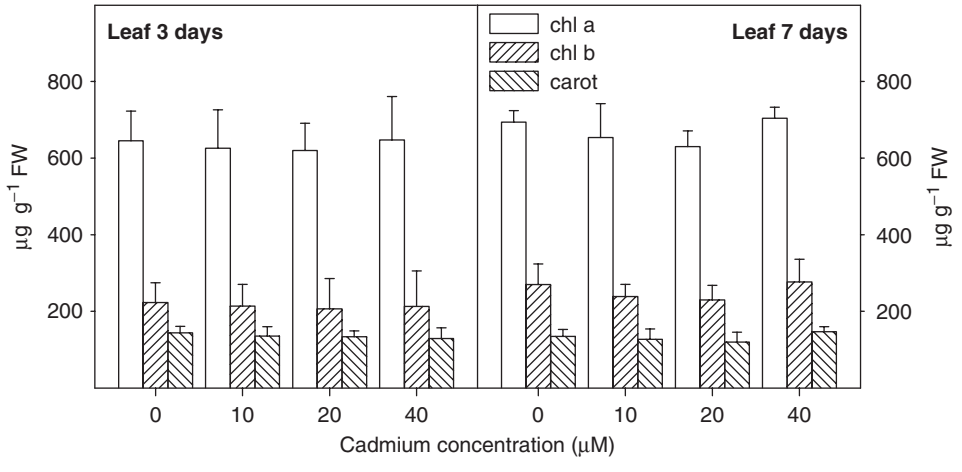

Fig. 4 Chl *a*, chl *b* and carotenoid (carot) contents in leaves of wheat plants grown in the presence of increasing Cd concentrations (0, 10, 20 and 40 μM) for 3 and 7 d. Data represent the means (±SE) of five separate experiments.

and the glutathione redox state was slightly higher under Cd treatment than in control roots. In the leaves, the glutathione pool was mainly unaffected by Cd treatment; only in the presence of 40 μM Cd did a GSH decrease occur. This decrease was particularly evident after 3 d of treatment, but it did not affect the GSH redox state (Fig. 7c, d).

Both ASC and GSH pools were larger in the leaves than in the roots, probably as a consequence of a higher

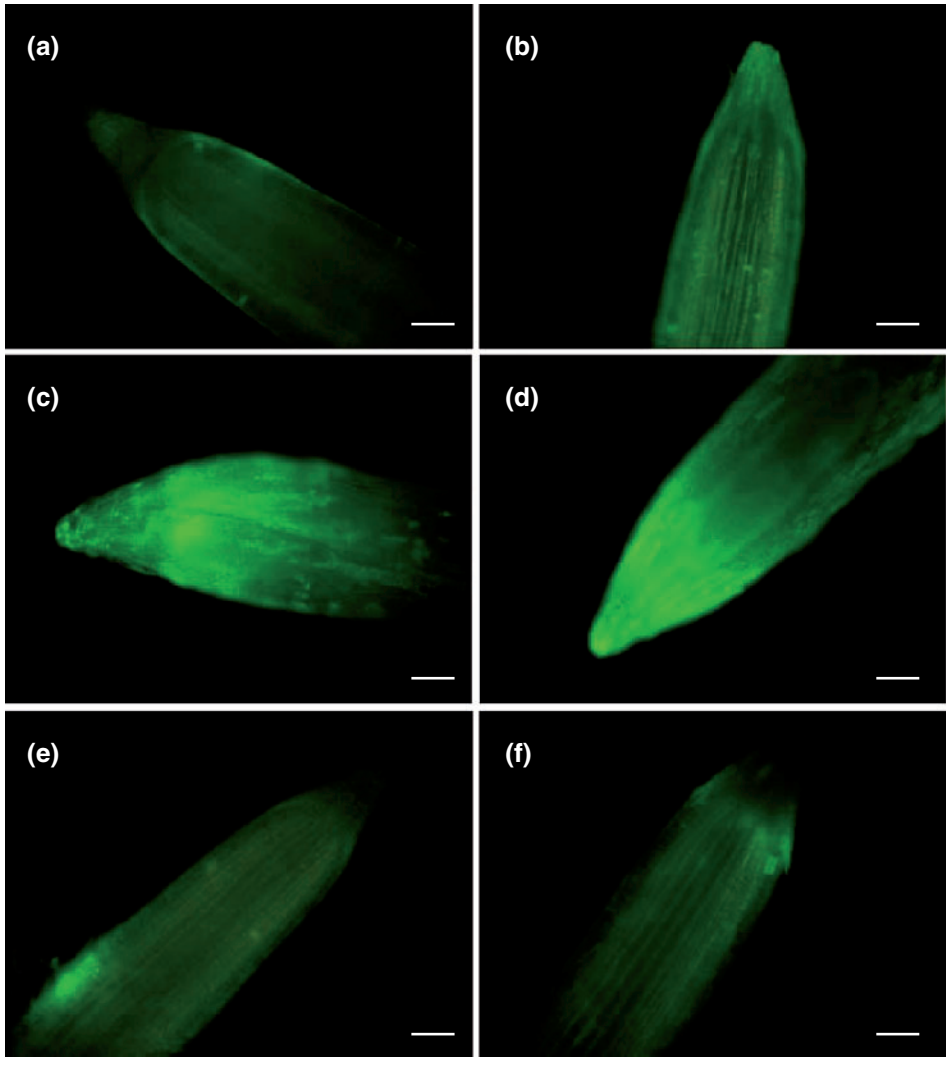

Fig. 5 Hydrogen peroxide production in roots grown in the presence of Cd. Representative images of H_2O_2 production in the apex of roots from plants after 7 d. H_2O_2 is visualized as yellow-green fluorescence due to its interaction with DHR123 (see Materials and Methods). (a) Control; (b) 10 μM Cd; (c) 20 μM Cd; (d) 40 μM Cd; (e) 40 μM Cd + 1 mM ASC; (f) 40 μM Cd + 30 U ml^{-1} catalase. In e and f, ASC and catalase were added to the medium 10 min before the addition of DHR123. Bar = 0.25 mm.

steady-state rate of ROS production in the photosynthetic tissues than in the non-photosynthetic tissues.

The effects of Cd on the enzymes of the ASC–GSH cycle were analyzed both in roots and in leaves. As shown in Fig. 8a and b, all the ASC–GSH cycle enzymes increased their activity in the roots, even if to a different extent. When plants were exposed to Cd, root cytosolic ascorbate peroxidase (APX) reached values almost 50–80% higher than the control even after 3 d of treatment (Fig. 8a). The activity of this enzyme remained higher in Cd-treated roots than in control roots for the following analyzed period (Fig. 8b). Dehydroascorbate reductase (DHAR) also increased in roots under Cd treatment. In percentage terms this increase was higher after 3 than after 7 d (Fig. 8a, b).

Monodehydroascorbate reductase (MDAR) required a more prolonged treatment in order to be affected, since a significant increase in its activity only occurred after 7 d (Fig. 8a, b). Glutathione reductase (GR) also increased under Cd treatment. The highest increase in the activities of the ASC–GSH cycle enzymes already occurred at a Cd concentration of 10 and 20 μM, with the exception of GR which showed the highest activity under 40 μM Cd.

In the leaves, the activity of the ASC–GSH cycle enzymes was also affected by Cd treatment (Fig. 8c, d); although, during the time of our analysis, an increase in Cd did not occur in the upper parts of wheat plants. An increase in APX, MDAR and DHAR in the leaves from Cd-treated plants was already evident after 3 d of treatment and with the lowest Cd concentration. On the other hand,

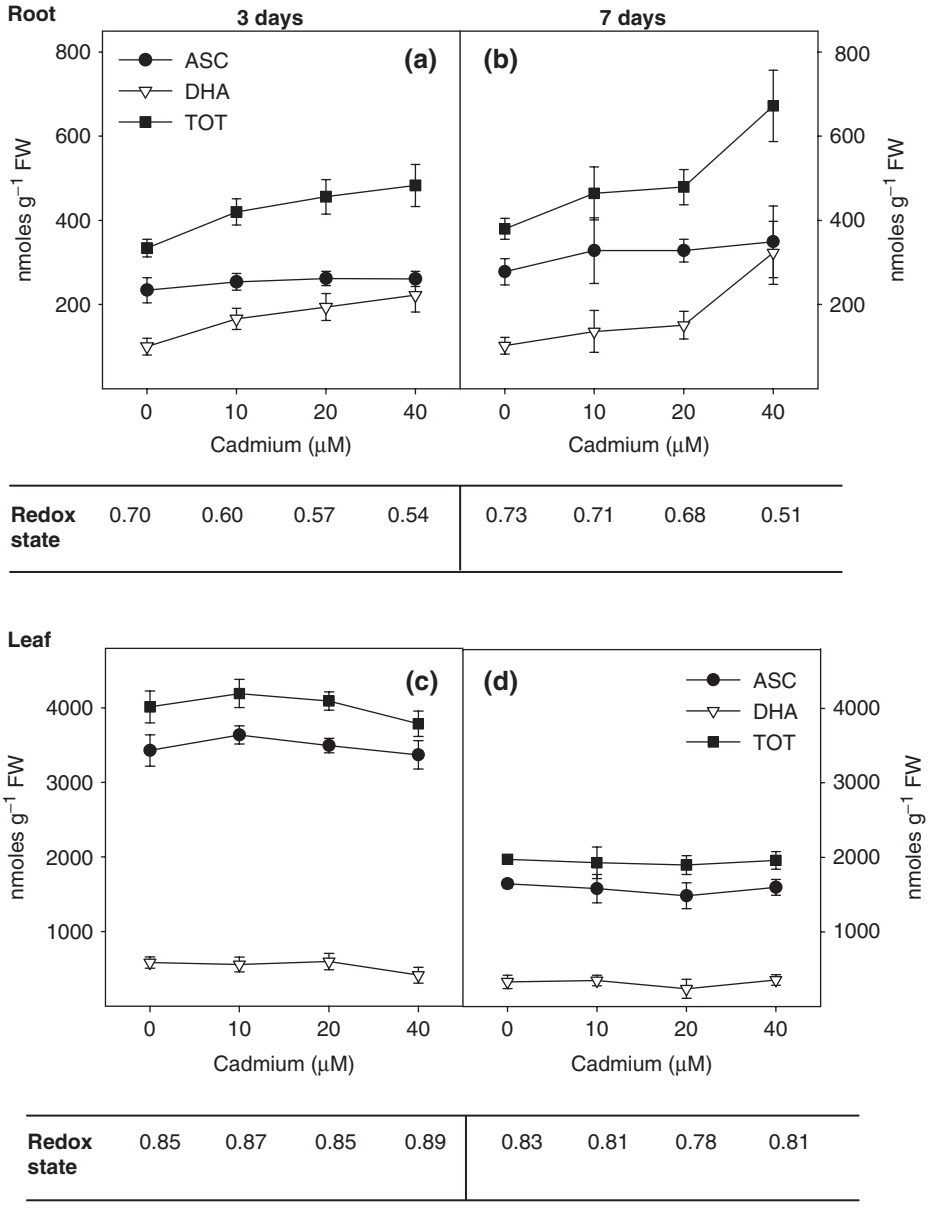

Fig. 6 Ascorbate pool and redox state in roots and leaves of plants grown in the presence of increasing Cd concentration. ASC, DHA and the total pool were determined in roots (a, b) and leaves (c, d) from wheat plants after 3 and 7 d of Cd treatment, as reported in Materials and Methods. Values represent the means (±SE) of five experiments. The redox state was calculated as the ASC/ASC + DHA ratio.

GR activity was transiently inhibited by Cd after 3 d of treatment (Fig. 8c, d).

The effects of cadmium in plants with an enhanced level of ascorbate

In order to verify whether an increase in ASC content actually helped wheat plants to cope with Cd toxicity, the ASC content was experimentally increased in the plants by supplying its last precursor, GL, in the hydroponic medium. This procedure has been widely utilized for increasing the ASC content in both hydroponically grown plants and cell cultures (Arrigoni et al. 1997, de Pinto et al. 1999, Pallanca and Smirnoff 2000, Potters et al. 2000). The ASC-enriched plants were exposed to 20 μM Cd treatment. In these plants, Cd uptake was also restricted to roots and was similar to that observed in the plants grown in the absence of GL (Table 3). Fig. 9a shows that when 5 mM GL was supplied to the hydroponic medium, the ASC content increased almost five times in durum wheat roots in both control and Cd-treated plants. Under our experimental conditions, this increase remained almost constant over time (Fig. 9a). The increase in ASC content affected the GSH pool, which

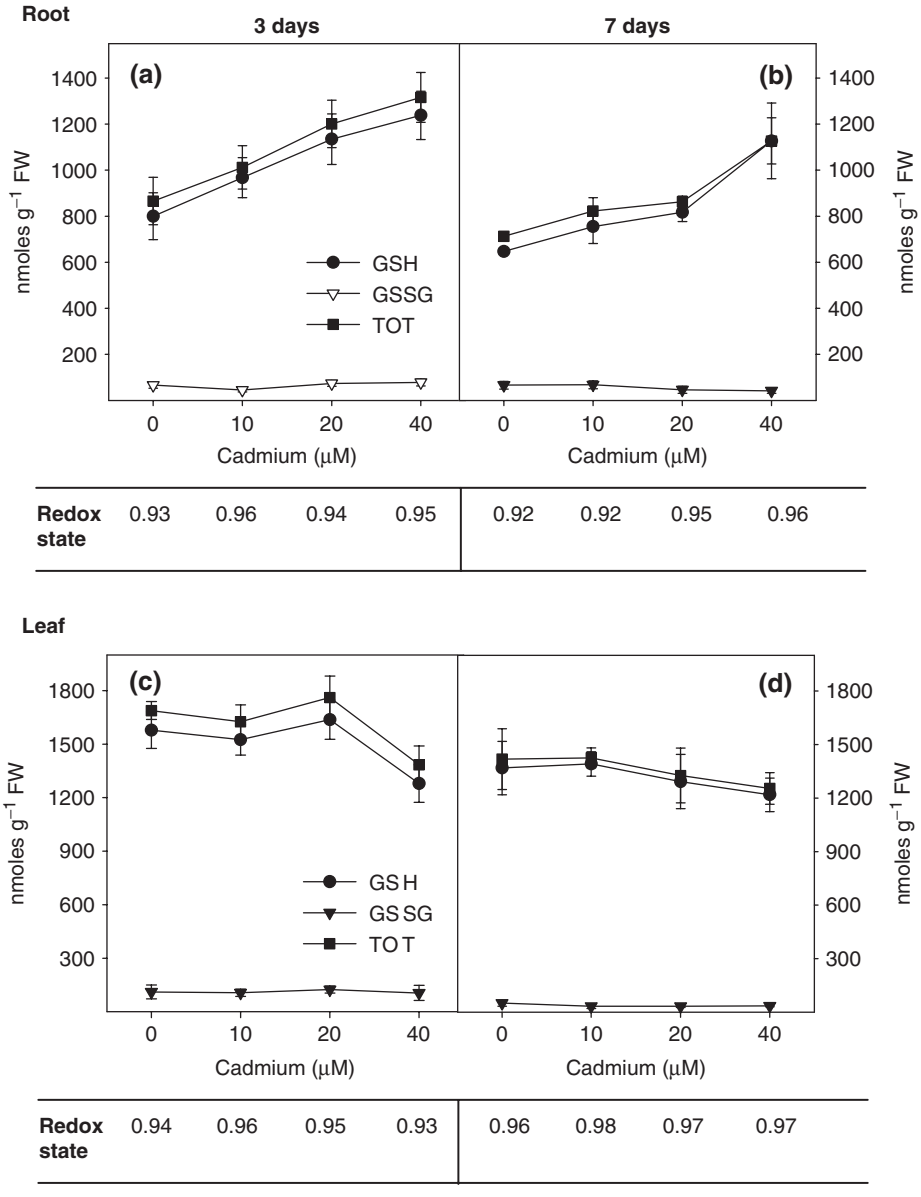

Fig. 7 Glutathione pool and redox state in the roots and leaves of plants grown in the presence of increasing Cd concentrations. GSH, GSSG and the total pool were determined in the roots (a, b) and leaves (c, d) from wheat plants after 3 (a, c) and 7 (b, d) days of Cd treatment, as reported in Materials and Methods. Values represent the means (±SE) of five experiments. The redox state was calculated as the GSH/GSH + GSSG ratio.

also increased slightly in the GL-treated plants (Fig. 9b). The presence of higher levels of these antioxidants had a protective effect on both lipid peroxidation and protein oxidation, since in GL-treated roots the levels of these two oxidative stress markers remained very similar to the control values, in spite of the presence of Cd (Fig. 10). A significant decrease in PC synthesis was evident in the plants treated with GL, in spite of Cd uptake being similar to that of plants growing in the absence of GL (Table 3) The increased level of ASC and GSH also alleviated the inhibitory effect of Cd on plant growth (data not shown).

Discussion

The toxicity of Cd for plants has been widely documented (Prasad 1995). Although different plants have a different capability to minimize the damaging effects of Cd, the inhibition of growth is a commonly observed event. (Dixit et al. 2001, Vitória et al. 2001, Stolt et al. 2003, Sharma et al. 2004, Ranieri et al. 2005). The results reported here also indicate that Cd negatively affects the growth of durum wheat plants. It has been suggested that this growth reduction is due to the competition for the same uptake

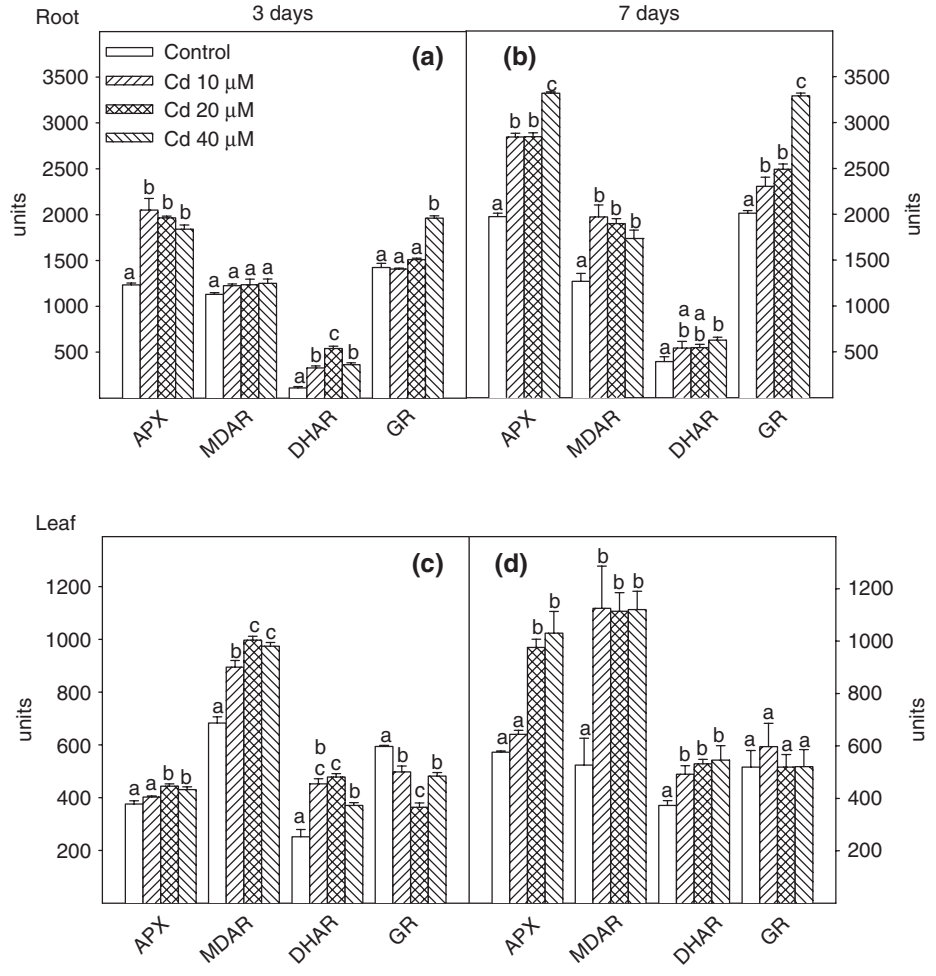

Fig. 8 Effect of Cd on the ascorbate–glutathione cycle enzymes. Changes in the activities of ascorbate–glutathione cycle enzymes in the roots (a, b) and in leaves (c, d) from wheat plants after 3 and 7 d of Cd treatment. Activities are expressed in units. APX, 1 unit = 1 nmol ASC oxidized $min^{-1} mg^{-1}$ protein; MDAR, 1 unit = 1 nmol NADH oxidized $min^{-1} mg^{-1}$ protein; DHAR, 1 unit = 1 nmol DHA reduced $min^{-1} mg^{-1}$ protein; GR, 1 unit = 1 nmol NADPH oxidized $min^{-1} mg^{-1}$ protein. Data represent the means (±SE) of five experiments. Different letters represent values which are statistically different (by one-way ANOVA test).

Table 3 Cd uptake and phytochelatin synthesis in GL-treated plants

	Cadmium ($\mu g\, g^{-1}$)	Phytochelatin (nmol SH g^{-1} FW)		
		PC_2	PC_3	PC_4
Control	50 ± 7a	n.d.	n.d.	n.d.
C + GL	45 ± 8a	n.d.	n.d.	n.d.
Cd (20 μM)	159 ± 12b	120 ± 30a	487 ± 71a	37 ± 10
Cd (20 μM) + GL	154 ± 19b	11 ± 3b	50 ± 12b	n.d.

Cd concentration was measured in durum wheat roots, after 7 d of treatment, and is expressed as $\mu g\, g^{-1}$ DW; phytochelatin concentration is expressed as nmolSH g^{-1} FW. The values are the means of three independent experiments ±SE. PC_2, PC_3 and PC_4 correspond to the different forms of phytochelatins, on the bases of the polymerization levels of the $(\gamma\text{-Glu-Cys})_n$ repeat; n.d. = not detectable. Different letters indicate significant differences between the values within the same analyzed parameter (one-way ANOVA test).

systems between Cd and other divalent ions required for plant development (Alcantara et al. 1994, Lozano-Rodríguez et al. 1997, Rivetta et al. 1997). Our results suggest that Cd also affects plant growth by inhibiting cell division in a dose-dependent manner. During the time of our analyses, the Cd level only increases in roots. The retention of heavy metals in roots is an early defense mechanism aimed at limiting their toxicity and at

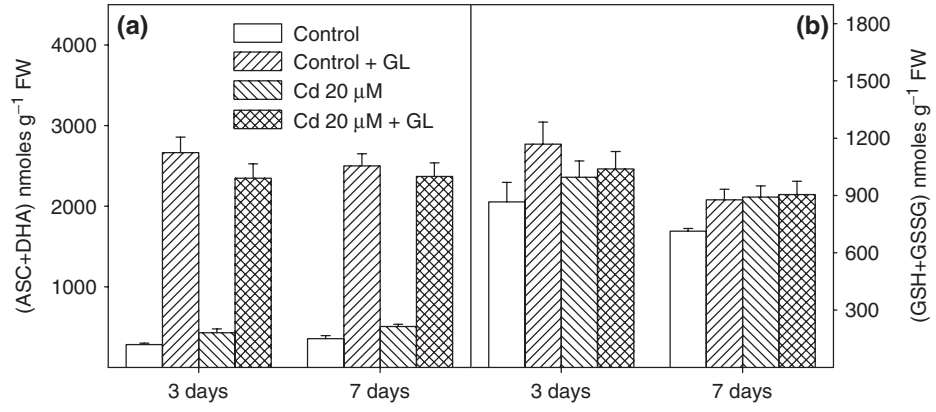

Fig. 9 Effects of L-galactono-γ-lactone treatment on ascorbate (a) and glutathione (b) pools of durum wheat roots. Plants were grown in the presence of 20 μM Cd for 3 and 7 d. Where indicated, 5 mM GL was added as reported in Materials and Methods. The bars show total ascorbate contents, ASC + DHA (mean values of three experiments ± SE).

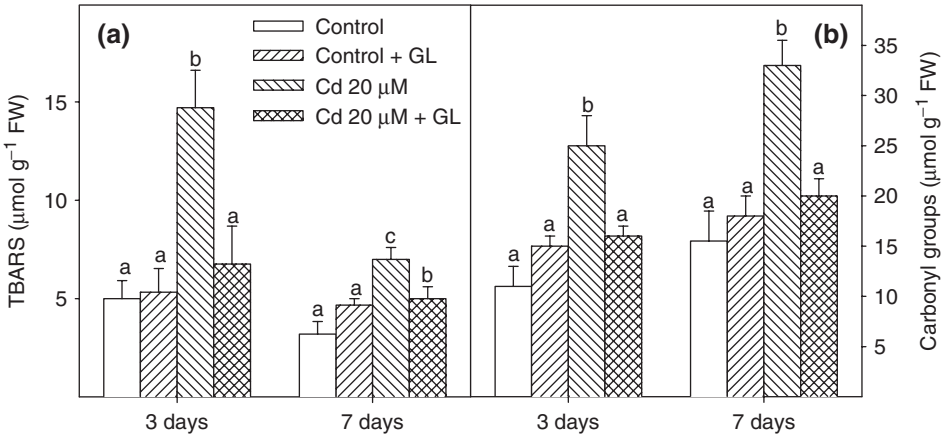

Fig. 10 Effect of the increased ascorbate biosynthesis on oxidative stress markers. Lipid peroxidation (a) and protein oxidation (b) were analyzed in roots from plants grown in the presence of 5 mM GL, 20 μM Cd or 20 μM Cd plus 5 mM GL for 3 and 7 d. Data represent the means (±SE) of three experiments. Different letters represent values which are statistically different (by one-way ANOVA test).

allowing leaves to prepare themselves to cope with a predictable oxidative stress (see below). Our results are in agreement with previous data underlining that Cd first accumulates in the roots, where its presence correlates with its external concentration (Ueki and Citovsky 2001). Only a more prolonged Cd exposure allows the heavy metal to be significantly transported to the upper organs of plants (Leita et al. 1991, Ueki and Citovsky 2001, Romero-Puertas et al. 2004, Ranieri et al. 2005).

Cd cannot directly generate ROS, since it does not catalyze Fenton-like reactions in biological systems (Salin 1988). However, when Cd is accumulated in the roots of durum wheat plants, ROS overproduction occurs, as is evident from the analysis of H_2O_2. This Cd-dependent H_2O_2 production is in agreement with the results obtained with other species subjected to different Cd treatments (Balestrasse et al. 2001, Cho and Sohn 2004, Romero-Puertas 2004, Ortega-Villasante et al. 2005). The moderate amount of H_2O_2 detected in control roots is required for normal cell differentiation and elongation (Potikha et al. 1999, Córdoba-Pedregosa et al. 2003); whereas, under Cd treatment, ROS overproduction could be responsible for oxidative damage. Consistently, Cd-dependent lipid peroxidation and protein oxidation, chosen as markers of oxidative stress, increase in the roots, where Cd is accumulated, but not in the leaves, where an increase in the heavy metal content is not yet evident. In roots, the oxidative damage is more evident after 3 d of treatments than after 7 d. This is probably due to the lag required to activate the defense responses able to counteract Cd toxicity.

Among the strategies aimed at Cd detoxification, in the experiments presented here the pivotal role of PCs should be pointed out. A positive correlation between exposure time, concentration of Cd and number of γ-Glu-Cys

repeat units in PCs has been reported (Grill et al. 1987, Vögeli-Lange and Wagner 1996). The observed preponderance of PC_3 may be ascribed to the prompt formation of a cytosolic 'low molecular weight' complex, produced by Cd bound to PC_3 (Vögeli-Lange and Wagner 1990, Vögeli-Lange and Wagner 1996), and by the formation of a vacuolar 'high molecular weight' complex, in which PC_3 is usually well represented (Rauser and Meuwly 1995). In the case of PC_3, the sulfydryl : Cd molar ratio is indeed more advantageous than for PC_2; thus PC_3 retains a detoxification capacity higher than that of PC_2 (Rauser and Meuwly 1995). PC synthesis does not induce GSH depletion in the roots; in contrast, a net increase in GSH occurs in parallel with Cd accumulation in this organ. In spite of neither Cd nor PCs being detectable in durum wheat leaves, a decrease in GSH occurs in these organs when plants are grown in the presence of 40 μM Cd. Further analyses are required to ascertain whether this decrease is caused by GSH translocation from leaves to roots, in order to make a larger amount of GSH available for PC synthesis, or by changes in thiol metabolism in the leaves.

The increase in ASC and GSH, observed in durum wheat roots as a consequence of Cd exposure, is not in agreement with the behavior observed in pea roots subjected to similar Cd concentrations (Rodríguez-Serrano et al. 2006), where decreases in both the antioxidant metabolites and some ROS-scavenging enzymes (catalase, superoxide dismutase and class III peroxidase) were observed. However, it is worth noting that several differences in the effects of Cd on antioxidant metabolism have been reported in the literature for different species and also for the same species under treatment with different Cd concentrations or when different organs were analyzed (see Rodríguez-Serrano et al. 2006, and references therein). The behavior of the redox states of ASC and GSH pools might also support the more relevant involvement of ASC than of GSH in the redox reactions triggered by Cd, since ASC oxidation only increases under Cd exposure, whereas the GSH redox state remains unaltered. However, it is also possible that the increase in GSSG under Cd exposure is masked by other events (GSSG degradation or utilization for other biosynthetic routes).

It has been reported that one of the pathways through which Cd induces an increase in ROS and oxidative stress in plants is the inhibition of antioxidant enzymes or the depletion of antioxidant molecules (Shaw 1995, Gallego et al. 1996, Schickler and Caspi 1999, Chien et al. 2001). Our results clearly indicate that, in durum wheat, this is not the case. In fact, apart from the ASC and GSH pools, the enzymes of the ASC–GSH cycle involved in ROS scavenging (May et al. 1998, Noctor and Foyer 1998) increase their activities in the roots of Cd-treated plants. Moreover, in the same cultivar of durum wheat, the activity of catalase, another pivotal enzyme for ROS removal (Vandenabeele et al. 2004), also increases under similar treatment (Berardino 2003). The amount of Cd required to induce this antioxidant increase seems to be rather low, since the greatest effect is reached after 3 d of treatment and with the lowest Cd concentrations, with the exception of GR (the increase of which was more pronounced under 7 d of treatment with 40 μM Cd). In the absence of an antioxidant depletion, ROS might be actively produced by specific peroxidases and/or NADPH oxidases (Bollwell et al. 2002). Consistently, Cd has been reported to activate these ROS-producing enzymes in different plant species (Piqueras et al. 1999, Romero-Puertas et al. 2004, Ranieri et al. 2005, Rodríguez-Serrano et al. 2006).

It is worth noting that Cd also induces an increase in the ASC redox enzymes in leaves, although no symptoms of oxidative stress (increase in lipid peroxidation or protein oxidation, decrease in the redox state of ASC and GSH) are present in these organs. The rise in ASC redox enzymes was already evident after 3 d of treatment, and with the lowest concentration of Cd used. This increase resembles the activation of a systemic response aimed at preparing durum wheat plants to counteract the predictable oxidative stress caused by the successive translocation of the heavy metal from roots to leaves. It has been reported that APX is systemically induced in *Arabidopsis thaliana* when a single leaf, or part of it, undergoes high light stress (Mullineaux and Karpinski 2002). A low amount of Cd has also been reported to induce systemic alteration of post-trascriptional gene silencing, a mechanism for the regulation of gene expression activated in tobacco plants by virus infection. This indicates that Cd is able to activate the onset of a signal that is transferred from the site of production to other parts of the plant (Ueki and Citovsky 2001). Our results also suggest that the ASC–GSH cycle could be involved in the systemic responses activated by Cd.

The results obtained in durum wheat plants treated with GL, the last ASC precursor, also provide new evidence of the relevance of ASC metabolism in the protection against heavy metals. Under our experimental conditions, GL treatment is able completely to reverse the effects of oxidative stress induced by Cd. Consistently, the inhibition of the Cd-dependent H_2O_2 production, by increasing ASC biosynthesis, has been recently reported (Zhao et al. 2005). On the other hand, the increase in GSH biosynthesis, induced by γ-glutamylcysteine synthetase overexpression, has only a marginal effect on Cd tolerance (Arisia et al. 2000). Also, *A. thaliana* mutants lacking γ-glutamylcysteine synthase or plants treated with L-buthionine-[S,R] sulfoximine, a specific inhibitor of GSH biosynthesis, are more sensitive to Cd than wild-type or control plants, respectively, as a consequence of the GSH depletion (Cobbet et al. 1998, Arisia et al. 2000). This is consistent with the

requirement for GSH for PC biosynthesis, in addition to protecting tissues against ROS. GL treatment also induces a slight increase in the GSH pool. A similar increase has also been reported to occur in tobacco cells treated with GL, and it might be due to a type of GSH protection by the presence of an increased amount of another antioxidant metabolite (de Pinto et al. 1999). The inhibition of the Cd-dependent synthesis of PCs is more intriguing. Our results are not able to clarify how GL (or the consequent improved cellular antioxidant capability) impairs the activation of PC biosynthesis. On the basis of the data presented here, some hypotheses can be put forward. The increased levels of metabolites with antioxidant properties induced by GL treatment, could better guarantee the cellular redox homeostasis and prevent the oxidative damage (lipid peroxidation and protein oxidation) induced by Cd. Under this condition, the signal responsible for triggering PC synthesis might not be present in the Cd-exposed tissues. GL could also inhibit PC biosynthesis by its capability to chelate Cd, as is the case for other divalent ions (Topper and Stetten 1952, Doherty 1953). Therefore, GL could substitute for PCs in protecting cellular metabolism with a similar mechanism

This possibility highlights the interest in obtaining plants in which the ASC biosynthetic route has been enhanced, both for improving ROS scavenger systems and for removing heavy metals from metabolic reactions.

Materials and Methods

Plant growth conditions and treatments

Wheat seeds (*Triticum durum* cv Creso) were germinated in controlled conditions (continuous darkness; 22°C, 55% relative humidity) for 4 d, after which homogeneous plantlets were transferred to hydroponic pots as described in Di Cagno et al. (1999). Three different sets of 10 pots, each containing four plantlets, were supplemented with 10, 20 and 40 μM $CdCl_2$, respectively; a fourth series of 10 pots, without added Cd, was used as a control. Hydroponic pots were maintained in a growth chamber with a 14 h photoperiod at a light intensity of 360 $\mu mol\, m^{-2}\, s^{-1}$ at 25 ± 1°C. Where indicated, 5 mM GL (Sigma-Aldrich, Milan, Italy) was added to the nutrient solution. Plants were collected after 3 and 7 d; primary roots (2 cm from the apex) and primary leaves were collected and immediately utilized for the analyses.

Mitotic index

Root apices (1–2 mm) were cut from plants after 7 d of Cd exposure, fixed in Carnoy solution (3 : 1 ethanol : glacial acetic acid) and rinsed in distilled water. Fixed tissues were hydrolyzed with 1 N HCl in three steps: 15 min at room temperature, 5 min at 60°C and 20 min at room temperature, rinsed with distilled water, then stained with Feulgen stain (0.5 g of basic fuchsin, 0.5 g of sodium metabisulfite, 100 ml of 1 N of HCl, 100 ml of distilled water). Apices were crushed onto glass in the presence of 45% acetic acid, and cells were observed under the microscope. The mitotic index was expressed as the percentage of dividing cells. Almost 1,500 cells from three different root apices were analyzed for each treatment.

Determination of cadmium, phytochelatins and oxidative stress markers

Cd was measured on the whole root system and primary leaf after 3 and 7 d of treatment. Plant tissues were dried at 80°C for 48 h and the dried material was used for Cd determination according to Angelova et al. (2004). Briefly, a sample (1 g) of dried material was added to 1 ml of HNO_3 and put in a sand bath to evaporate. It was subsequently transferred into a furnace (400°C). The procedure was repeated until the ash was white. It was finally dissolved in 2 ml of 20% HCl, transferred into a scaled 10 ml flask and brought to volume with double-distilled water.

The quantitative analysis was performed using a graphite furnace atomic absorption spectrophotometer (Analyst 800-THGA-800 Perkin Elmer) (Storelli et al. 2005). Three reagent blanks were similarly processed and used to derive detection limits of the analytical procedure (0.02 $ng\, g^{-1}$). Each sample was analyzed in duplicate and the replicate samples indicated that the error did not exceed 7%. The recovery tests, which involved the addition of a known amount of inorganic cadmium before digestion, yielded 96 ± 4% on average.

PCs were analysed in apical roots and leaves following Ranieri et al. (2005).

Lipid peroxidation was measured as malondialdehyde (MDA) content determined by thiobarbituric acid (TBA) reaction as reported by Guidi et al. (1999). The amount of MDA–TBA complex was calculated using an extinction coefficient of 155 $mM^{-1}\, cm^{-1}$.

Protein oxidation was determined by reaction with 2,4-dinitrophenylhydrazine (DNPH; Fluka-Schweiz, Braunschweig, Germany) according to Di Cagno et al. (2001). Carbonyl content was calculated using an extinction coefficient of 22 $mM^{-1}\, cm^{-1}$.

H_2O_2 determination

DHR123 (Sigma-Aldrich, Milan, Italy) was used as a probe for H_2O_2 assay (Royall and Ischiropoulos 1993). Wheat plantlets were placed in a glass tube with the roots soaked in a solution containing DHR123 (20 μM) for 5 min. The roots were then delicately rinsed in distilled water, cut at 3 cm from the apex and observed under a fluorescent microscope (DMLS, Leica) with an excitation filter of 450–490 nm and a barrier filter of 510 nm. Where indicated, roots were incubated with 1 mM ASC or 30 $U\, ml^{-1}$ purified catalase (Sigma-Aldrich, Milan, Italy) prior to the DHR123 treatment, in order to verify that the fluorescent signal was actually due to H_2O_2.

Assay of ascorbate, glutathione and ascorbate–glutathione cycle enzymes

For ASC and GSH determination, 0.3 g of apical roots or leaves were homogenized with cold 5% metaphosphoric acid at 4°C at a 1 : 6 ratio (w/v) in order to obtain deproteinized extracts. After centrifugation at 20,000 × *g* for 15 min, the supernatants were collected and used for the analysis of ASC and GSH levels and the redox state according to de Pinto et al. (1999).

For the enzyme activity determinations, 0.5 g of apical roots or leaves were ground to a fine powder in a mortar in the presence of liquid nitrogen and mixed with an extraction buffer, consisting of 50 mM Tris–HCl (pH 7.5), 0.05% cysteine and 0.1% bovine serum albumin (BSA), in a 1 : 3 ratio (w/v). Homogenates were

centrifuged for 15 min at 20,000×*g*, and the supernatants were used for enzymatic determinations.

Cytosolic APX (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11), DHAR (glutathione:dehydroascorbate oxidoreductase, EC 1.8.5.1) and MDAR (NADH:monodehydroascorbate radical oxidoreductase, EC 1.6.5.4) were assayed as described in De Gara et al. (2003). GR (NADPH:glutathione disulfide oxidoreductase, EC 1.6.4.2) was assayed using the method of Osswald et al. (1992). Enzyme activities were measured using a Beckman (Fullerton-CA) DU 7000 spectrophotometer.

Protein was measured according to Bradford (1976) using BSA as a standard.

Statistics

The reported values are the average of 3–5 independent experiments ± SE as indicated in the figure legends. The differences between treatments were analyzed by one-way analysis of variance (ANOVA).

Acknowledgments

The authors thank Dr. Emanuela Vurro, University of Parma, for her help in phytochelatin analysis.

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(Received November 19, 2007; Accepted January 19, 2008)