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Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress

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Abstract

The behavior of glutathione reductase (GR, EC 1.6.4.2) activity and isoforms were analyzed in wheat (*Triticum aestivum* L.) leaves and roots exposed to a chronic treatment with a toxic cadmium (Cd) concentration. A significant growth inhibition (up to 55%) was found in leaves at 7, 14 and 21 days, whereas roots were affected (51%) only after three weeks. Wheat plants grown in the presence of 100 μM Cd showed a time-dependent accumulation of this metal, with Cd concentration being 10-fold higher in roots than in leaves. Nevertheless, lipid peroxidation was augmented in leaves in all experiments, but not in roots until 21 days. Cadmium treatment altered neither the GR activity nor the isoform pattern in the leaves. However, GR activity increased 111% and 200% in roots at 7 and 14 days, respectively, returning to control levels after 21 days. Three GR isoforms were found in roots of control and treated plants, two of which were enhanced by Cd treatment at 7 and 14 days, as assessed by activity staining on native gels. The changes in the isoform pattern modified the global kinetic properties of GR, thereby decreasing significantly (2.5-fold) the Michaelis constant (K_m) value for oxidized glutathione. Isozyme induction was not associated with an enhancement of GR mRNA and protein expression, indicating that post-translational modification could occur. Our data demonstrated that up-regulation of GR activity by the induction of distinctive isoforms occurs as a defense mechanism against Cd-generated oxidative stress in roots.

Keywords: *Triticum aestivum*; Gramineae; Wheat; Glutathione reductase; Cadmium; Oxidative stress

1. Introduction

Cadmium (Cd) is one of the most toxic pollutants found in the air, water and soil, and is non-essential for plants. It is released into the environment by traffic, metal-working industries, mining, as a by-product of mineral fertilizers, and from other sources (Nriagu and Pacyna, 1988). Cadmium can be transferred through the food chain (Wagner, 1993), and its accumulation in cereals aimed for food production, i.e. bread wheat, represents a risk for animal and human health (Chaney et al., 1999; McLaughlin et al., 1999).

Cadmium interacts with photosynthetic, respiratory and nitrogen metabolism in plants, resulting in poor growth and low biomass accumulation (Sanitá di Toppi and Gabbrielli, 1999; Pereira et al., 2002). Various detoxification processes are activated in plant cells during Cd exposure, such as complexing of the metal by phytochelatins, compartmentalization in vacuoles, immobilization at the level of cell wall, exclusion through action of plasma membrane and synthesis of stress proteins (Hall, 2002, and references therein). Cadmium is a non-redox metal unable to participate in Fenton-type reactions, but it leads to formation of reactive oxygen species (ROS), such as superoxide anion radical and hydrogen peroxide (Dietz et al., 1999; Sandalio et al., 2001; Romero-Puertas et al., 2004). It is known that cells and tissues protect themselves against oxidative damage through up-regulation of a wide variety of antioxidant products (Davies, 1986). Among them, glutathione is an abundant metabolite in plants that has diverse and important functions, including signal transduction (Gomez et al.,

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2004; Foyer and Noctor, 2005). In many reactions involving reduced glutathione (GSH), the Cys thiol group is oxidized to yield oxidized glutathione (GSSG), and the reverse reaction is catalyzed by glutathione reductase (GR, EC 1.6.4.2) using NADPH. The GSH pool maintained by GR is necessary for active protein function, and millimolar concentrations of GSH act as a key redox buffer forming a barrier between protein Cys groups and ROS. Also, phytochelatins, the principal heavy-metal-complexing peptides of plants, are derived from GSH and could contribute to limit the Cd-induced oxidative stress (Ranieri et al., 2005).

Previous studies have demonstrated that a modulation of GR activity occurs in response to different environmental stresses, such as cold, ozone, and greening (Madamanchi et al., 1992; Edwards et al., 1994; Biemelt et al., 1998; Anderson and Davis, 2004). Moreover, changes in isoform pattern and kinetic properties of GR have been described under these conditions in peas (Edwards et al., 1994). On the other hand, both increases and decreases in the activities of many antioxidant enzymes have been reported in Cd-treated plants (Gallego et al., 1996; Dixit et al., 2001; Sandalio et al., 2001; Pereira et al., 2002; Aravind and Prasad, 2005). Nevertheless, little is known about the behavior of GR isoforms and the expression of this antioxidant enzyme under Cd stress. Therefore, the aim of the present work was to investigate the response of GR in wheat leaves and roots exposed to a chronic treatment with a toxic Cd concentration. We determined the GR isoform pattern, protein and mRNA expression after 7, 14 and 21 days of treatment. Plant growth, Cd accumulation and lipid peroxidation were also evaluated.

2. Results

2.1. Effect of Cd treatment on plant growth

A significant growth inhibition was found in leaves of wheat plants treated with 100 μM Cd over the study period (Table 1). Fresh weight (FW) decreased 25%, 33% and 55% at 7, 14 and 21 days compared to control values, respectively. In contrast, FW remained unaltered in roots after the first two weeks of treatment, but a significant decrease was observed on day 21 (51% respect to controls) (Table 1). Except for growth inhibition, no visual symptoms of Cd toxicity, such as chlorosis, were observed in wheat leaves over time (data not shown).

2.2. Cadmium accumulation

Wheat plants grown in the presence of 100 μM Cd showed a time-dependent accumulation of this metal in leaves and roots. As shown in Table 1, a 38- and 47-fold increase in Cd content was observed in leaves after 7 and 14 days of treatment, respectively. On day 21, Cd levels were similar at those found on day 7. Cadmium accumulation was more important in roots, increasing 105- and 220-fold after 7 and 14 days, respectively. No further increment in Cd content was observed in roots after 21 days.

2.3. Effect of Cd on lipid peroxidation

In order to evaluate the oxidative stress generated by Cd, thiobarbituric acid reactive substances (TBARS) formation was determined after 7, 14 and 21 days of treatment. TBARS formation was enhanced in wheat leaves at all tested times. After 7 and 14 days, there was an increment of 51% and 92%, respectively, but a more significant increase (114%) was observed on day 21, relative to control values (Fig. 1). Surprisingly, TBARS levels were not affected in roots after 2 weeks, but a significant augmentation (68%) was found on day 21 (Fig. 1).

2.4. Effect of Cd on GR activity and isoforms

As it can be seen in Fig. 2, Cd exposure provoked no changes on GR activity in wheat leaves over the study period. However, in the roots, GR activity increased by 111%

Table 1
Changes in growth and Cd content over the study period

	Leaf			Root		
	Control	100 μM Cd	*P*	Control	100 μM Cd	*P*
Fresh weight (g)						
7 days	0.16 ± 0.01	0.12 ± 0.01	<0.01	0.10 ± 0.01	0.09 ± 0.01	n.s.
14 days	0.36 ± 0.02[#]	0.24 ± 0.01[#]	<0.001	0.12 ± 0.02	0.10 ± 0.01	n.s.
21 days	0.77 ± 0.02[#,§]	0.35 ± 0.02[#,§]	<0.001	0.35 ± 0.02[#,§]	0.17 ± 0.01[#,§]	<0.001
Cd content (μg g^{-1} FW)						
7 days	2.2 ± 0.2	83.8 ± 8.3	<0.001	5.3 ± 0.5	555.4 ± 50.2	<0.001
14 days	2.4 ± 0.2	112.6 ± 10.6[*]	<0.001	5.2 ± 0.6	1141.4 ± 100.3[#]	<0.001
21 days	2.5 ± 0.2	77.9 ± 7.3[†]	<0.001	5.6 ± 0.6	1268.5 ± 120.0[#]	<0.001

Values are expressed as mean ± SD of four independent experiments.
n.s. = not significant.
[*] $P < 0.01$
[#] $P < 0.001$ compared with 7 days.
[†] $P < 0.01$.
[§] $P < 0.001$ compared with 14 days.

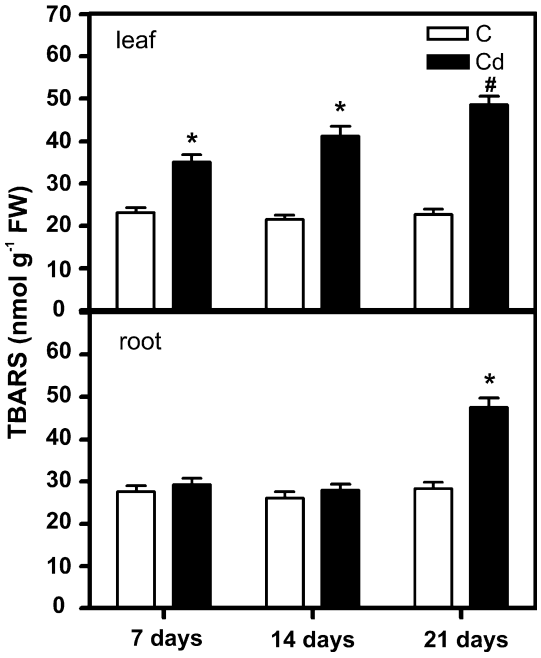

Fig. 1. TBARS formation in leaves and roots of control (C) and Cd-treated (Cd) wheat plants at 7, 14 and 21 days. Values are the mean of four independent experiments and bars indicate SE. $^{*}P < 0.01$, $^{\#}P < 0.001$ compared with control according to unpaired t test.

and 200% after 7 and 14 days, respectively. Interestingly, control values were regained after 3 weeks (Fig. 2).

We subsequently investigated the pattern of GR isoforms in leaves and roots by activity staining in non-denaturating polyacrylamide gels. Control leaves showed two GR isoforms, which were not modified by Cd treatment at any of the tested times (Fig. 3a). On the other hand, three GR isoforms were found in roots of control and treated plants. While the lower mobility isoform (GR3) remained unaffected, the other two isoforms activities (GR1 and GR2) were enhanced by Cd exposure for 7 and 14 days (Fig. 3b). This effect was totally reversed at 21 days, when the pattern of isoforms resembled that obtained in control plants. To confirm the specificity of GR activity staining, leaves and roots of 7 days Cd-treated plants were also analyzed by immunobloting. Both detection methods showed similar results (Fig. 4). In addition, no bands were observed when duplicate activity gels were stained in the absence of GSSG (data not shown).

In order to assess whether these changes in isoform pattern could modify the global kinetic properties of GR, the apparent Michaelis constant (K_m) for GSSG was determined in roots over time. As shown in Table 2, the K_m values for GSSG decreased significantly (2.5-fold respect to controls) in roots of Cd-treated plants after 7 and 14 days.

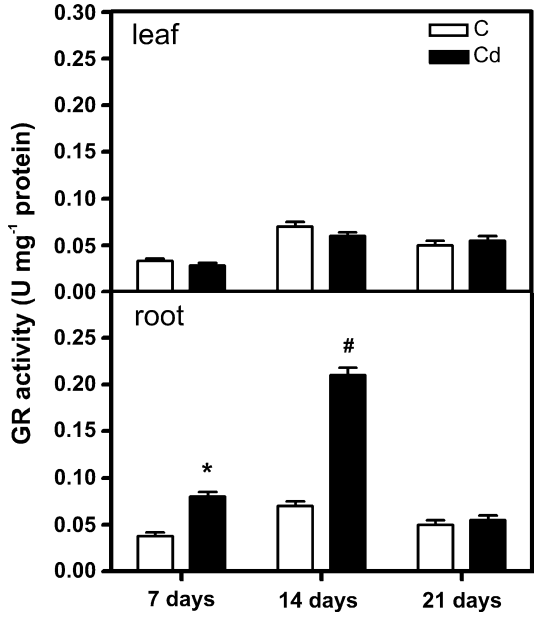

Fig. 2. GR activity in leaves and roots of control (C) and Cd-treated (Cd) wheat plants at 7, 14 and 21 days. One unit of GR is defined as the amount of enzyme necessary to oxidize 1 μmol of NADPH per min under assay conditions. Values are the mean of four independent experiments and bars indicate SE. $^{*}P < 0.001$, $^{\#}P < 0.0001$ compared with control according to unpaired t test.

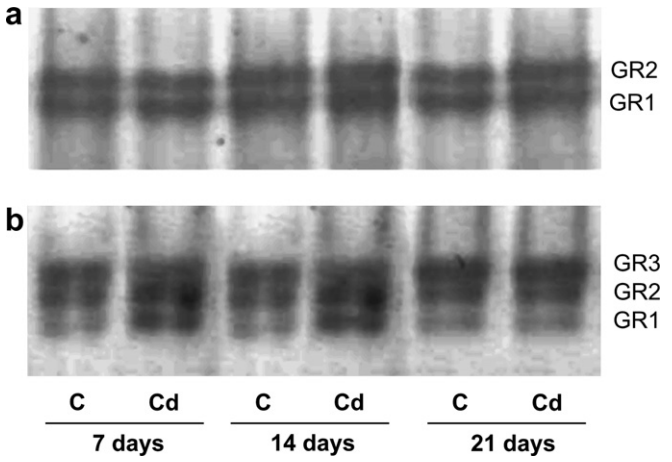

Fig. 3. Identification of GR isoforms in leaves and roots of control (C) and Cd-treated (Cd) wheat plants at 7, 14 and 21 days. Equal amounts of protein (40 μg) were separated on native PAGE gels and stained for GR activity as described in Section 4.5. (a) GR isoform pattern in leaves. (b) GR isoform pattern in roots. Results are representative of four independent experiments.

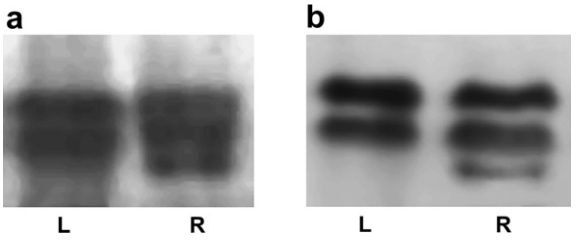

Fig. 4. GR isoform pattern analyzed by activity staining (a) or immunoblotting using the anti-GR antibodies (b). Protein extracts from leaves (L) and roots (R) of 7 days Cd-treated plants were subjected to native PAGE as described in Section 4.5 prior to the detection procedure. Results are representative of three independent experiments.

Table 2
Apparent GR K_m values for GSSG in roots of wheat plants subjected to 100 μM Cd

Time (days)	K_m (μM)	
	Control	100 μM Cd
7	32.6 ± 4.1	13.5 ± 3.1*
14	30.7 ± 3.8	11.9 ± 3.0*
21	31.9 ± 4.0	29.5 ± 3.7

Values are expressed as mean ± SD of four independent experiments.
* $P < 0.01$ compared with control according to unpaired t test.

As expected, K_m values were not significantly different on day 21.

2.5. GR mRNA and protein expression

To determine whether the aforementioned different GR isoform pattern in roots was due to de novo protein synthesis, GR mRNA and protein expression were analyzed by semi-quantitative RT-PCR and Western blotting, respectively. As shown in Fig. 5, GR transcripts levels did not show significant changes respect to controls at all tested times. On the other hand, the anti-spinach GR antibody recognized a single band of approximately 60 kDa in wheat roots, a mass consistent to that previously reported (Fig. 6a) (Lascano et al., 1998; de Lamotte et al., 2000).

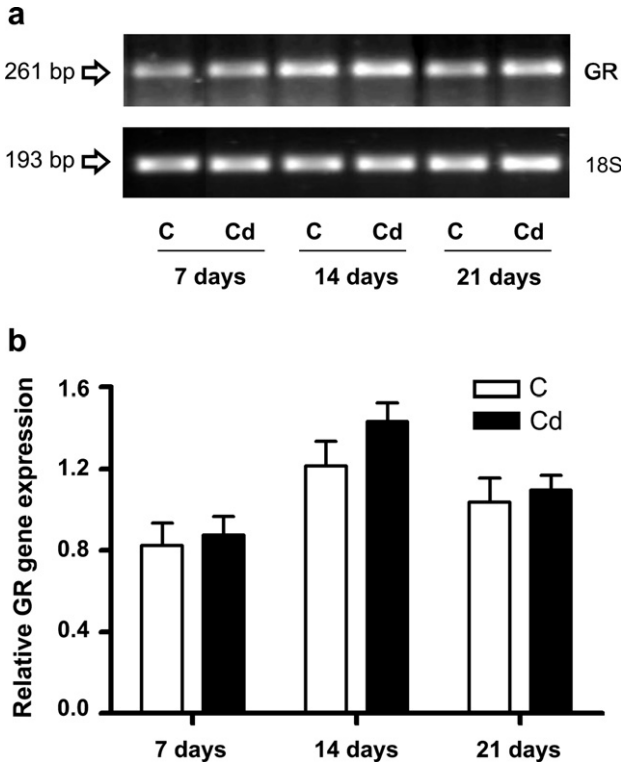

Fig. 5. GR transcript levels in roots of control (C) and Cd-treated (Cd) wheat plants at 7, 14 and 21 days. (a) GR mRNA expression was analyzed by semi-quantitative RT-PCR as described in Section 4.6. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. (b) Relative GR transcript levels expressed as the ratio of GR mRNA to 18S mRNA. Values are the mean of four independent experiments and bars indicate SE.

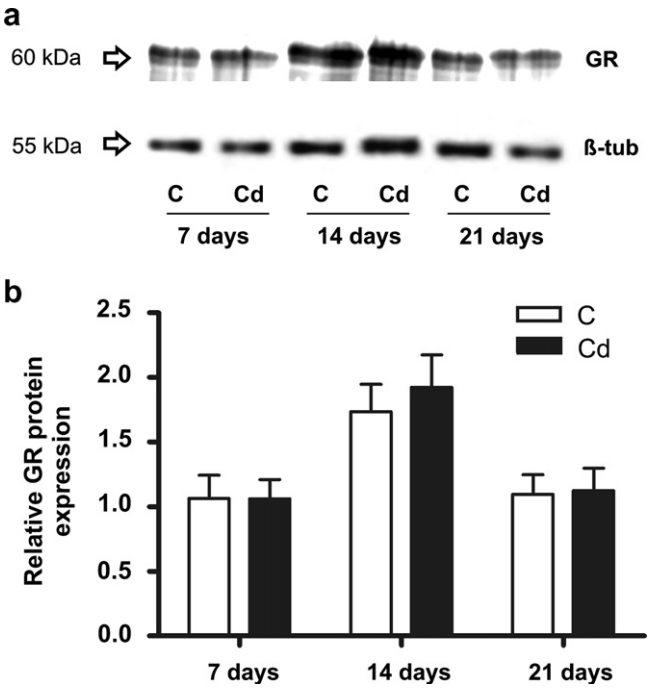

Fig. 6. GR protein expression in roots of control (C) and Cd-treated (Cd) wheat plants at 7, 14 and 21 days. (a) GR protein expression was analyzed by Western blotting as described in Section 4.7. β-Tubulin (β-tub) immunoblotting was performed as an internal control of protein loading. (b) Relative GR protein expression expressed as the ratio of GR to β-tub. Values are the mean of four independent experiments and bars indicate SE.

Densitometric analysis demonstrated that no significant differences were observed in GR protein levels between groups over the study period (Fig. 6b). These results indicated that the increased GR1 and GR2 activities in roots were not associated with augmented GR protein expression.

3. Discussion

Cadmium-dependent inhibition of growth has been reported in several plant species showing different sensitivities to this metal concentration (Lozano-Rodríguez et al., 1997; Di Cagno et al., 1999; Sandalio et al., 2001; Ortega-Villasante et al., 2005). Lozano-Rodríguez et al. (1997) have demonstrated a 70% and 30% growth inhibition in pea and maize plants treated with 50 μM Cd, respectively. In the present study, Cd produced a significant reduction in the growth of wheat plants (Table 1). This effect was more evident in leaves than in roots at 2 weeks of treatment, whereas both tissues were similarly affected (up to 55% of FW reduction) after 21 days.

The formation of TBARS is an indicator of free-radical generation in the tissues, and it may be used as a reliable index of lipid peroxidation in biological systems (Heath and Packer, 1968). Our data demonstrated that, as a result of Cd treatment, TBARS levels were enhanced in wheat

leaves at all tested times indicating that oxidative stress occurred, whereas in roots this response was only observed after 21 days (Fig. 1). Despite the apparent greater sensitivity of leaves, Cd concentration was about 10-fold higher in roots (Table 1). Taking into account the fact that Cd is exclusively absorbed by roots in this model, we conclude that this ion has a low degree of transport to the aerial part of the plant. This is consistent with previous reports that have revealed comparable Cd accumulation ratios in pea (Lozano-Rodríguez et al., 1997; Dixit et al., 2001; Sandalio et al., 2001). Cadmium retention in roots might be due to cross-linking of Cd to carboxyl groups of the cell wall and to an interaction with thiol residues of soluble proteins, explaining why Cd is mostly found in the cell wall and in soluble fractions (Lozano-Rodríguez et al., 1997). In addition, the level of free Cd ions in roots may remain low since most of these ions are compartmentalized in vacuoles or form Cd-phytochelatin complexes (Vögeli-Lange and Wagner, 1990; Sun et al., 2005). This behavior accounts for the lesser extent of oxidative stress observed in roots, and is one of the several strategies of tolerance to heavy metals (Hall, 2002).

Glutathione reductase is a key enzyme of the ascorbate-glutathione cycle that protects cells against oxidative damage maintaining a high GSH/GSSG ratio (Foyer and Noctor, 2005). This enzyme has shown differential responses under cadmium stress. Dixit et al. (2001) have demonstrated in pea that GR was more activated in roots than in leaves when plants were treated with 4 and 40 μM Cd during 7 days. Moreover, GR activity did not show significant changes in pea leaf extracts after 28 days of Cd treatment (Sandalio et al., 2001). Pereira et al. (2002) studied roots and leaves of *Crotolaria juncea* with 2 mM Cd and found an important increase of GR activity in leaves. On the other hand, Cd treatment has decreased the activity of this enzyme in *Helianthus annuus* and *Ceratophyllum demersum* (Gallego et al., 1996; Aravind and Prasad, 2005). In the present study, wheat leaves did not show any change in their GR activity over time, whereas roots presented a remarkable increase after 7 and 14 days of treatment (Fig. 2). These data clearly support the idea that GR activity is not only related to different organs and plant species but also to Cd concentration and/or a given period of exposure.

The pattern of GR isoforms was subsequently analyzed and a change in root profile was found in Cd-treated plants (Fig. 3). This kind of response has been previously reported for other abiotic stresses. Edwards et al. (1994) observed an alteration in the pattern of GR isozymes in pea leaves subjected to cold, ozone and greening treatments. A shift in the intensities of GR bands were described in mesocotyls but not in roots of maize seedlings during acclimation to chilling (Anderson et al., 1995). It has been also reported that total and specific GR activities changed in parallel to the isoform pattern during wheat grain development (de Lamotte et al., 2000). In this way, the modification of the GR isoform population, whose individual members have different characteristics, could bring about a net change in the properties of the total GR activity, such as the lowering of the K_m for GSSG described herein (Table 2). Moreover, the appearance of GR isoforms with a higher affinity for one of the enzyme substrates may result in an increased recycling of glutathione during periods of oxidative stress. This response may be a strategy to protect roots against Cd-induced oxidative damage, at least during the first two weeks. Longer treatments (3 weeks) produced enhanced TBARS formation and poor growth respect to control plants, indicating that the antioxidant capacity of this tissue was overwhelmed. It is noteworthy that the oxidative injury in roots correlated with the reversion of the changes in the enzyme activity as well as the GR isoform pattern and K_m value for GSSG.

Total GR transcripts were also determined in roots by semi-quantitative RT-PCR, showing that Cd did not affect the level of GR mRNA at all tested times (Fig. 5). Accordingly, the specific protein content in root extracts, as assessed by Western blotting, did not vary with the Cd treatment (Fig. 6). The augmented GR activity together with the changes in the isoform pattern, in the absence of a parallel enhancement of GR mRNA and protein expression, indicates that a post-translational activation of this enzyme could occur. These results are in agreement with those reported in Cd-stressed peas by Romero-Puertas et al. (2006), who have found a twofold increase in the peroxisomal GR activity without augmented protein content, suggesting a post-translational modification of this isozyme. Similarly, increased GR activities did not correlate with enhanced protein expression in pea plants subjected to cold and greening, but with the exception of ozone treatment (Edwards et al., 1994). In contrast, Baek and Skinner (2003) demonstrated increased expression levels of GR transcripts during cold acclimation in wheat plants.

3.1. Concluding remarks

In conclusion, our data clearly demonstrated that Cd differentially altered GR activity and isoforms in leaves and roots of wheat plants. The changes in the isoform pattern did not occur as a consequence of an enhancement of GR mRNA and protein expression, indicating that post-translational modification might take place. The up-regulation of GR activity by the induction of distinctive active isoforms provides additional defense against Cd-generated oxidative stress in roots. Further studies are required to elucidate the molecular mechanisms that control these changes under Cd stress.

4. Experimental

4.1. Plant material and treatments

Wheat (*Triticum aestivum* L. cv. buck poncho) seeds supplied by the Instituto Nacional de Semillas (INASE,

Argentina) were surface sterilized with 5% v/v NaOCl for 15 min and then washed with distilled H_2O several times. The seeds were germinated on plastic pods filled with vermiculite and irrigated with Hoagland's nutrient solution (Hoagland and Arnon, 1950) devoid of cadmium (control) or containing 100 μM of $CdCl_2$ since the day of sowing. Plants were grown in a controlled climate room at 24 ± 2 °C and 50% relative humidity, with a photoperiod of 16 h and a light intensity of 175 μmol m^{-2} s^{-1}. Plants were harvested at 7, 14 and 21 days after treatment, and leaves and roots were separated and used for the determinations.

4.2. Plant growth and total Cd content

Fresh weight (FW) of the roots and fully expanded apical leaves was taken immediately after harvesting. The concentration of Cd in leaves and roots was determined by atomic absorption using a Perkin–Elmer AAnalyst 300 spectrophotometer (Dixit et al., 2001). Briefly, immediately after harvesting, leaves and roots were extensively washed in distilled H_2O; then, samples were oven-dried, ground to powder and digested in a HNO_3–$HClO_4$ (3:1, v:v) mixture before Cd content assay.

4.3. Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh control and treated leaves (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500*g* for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% TCA containing 0.5% (w/v) TBA and 100 μl 4% butylated hydroxytoluene (BHT) in ethanol was added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000*g* for 15 min and the absorbance was measured at 532 nm. Value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

4.4. Glutathione reductase preparation and assay

Leaves and roots extracts were prepared from 0.5 g of tissue, homogenized under ice-cold condition in 1.5 ml of extraction buffer containing 100 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, and 1% (w/v) PVP. The homogenates were centrifuged at 10,000*g* for 30 min at 4 °C, and the supernatant fraction was supplied with 10 mM dithiothreitol (DTT) and used for the assay. Glutathione reductase activity was determined at 25 °C following oxidation of NADPH at 340 nm in 1-ml of reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH and 0.5 mM GSSG (Rao et al., 1996). The Michaelis constant (K_m) of GR for oxidized glutathione (GSSG), was determined in crude protein extracts in the presence of a saturating concentration of NADPH (0.2 mM). Limiting GSSG concentrations varied between 5 μM and 50 μM. The K_m values were calculated using Lineweaver-Burk plots.

4.5. Native PAGE and GR activity staining

Protein extracts were subjected to discontinuous PAGE under non-denaturing and non-reducing conditions as described by Laemmli (1970), except that SDS was omitted. Equal amounts of protein (40 μg) were separated in 10% polyacrilamide gels (4% stacking) during 3 h at 4°C with a constant current of 30 mA per gel using Mini-PROTEAN 3 Electrophoresis System (BioRad, Hertz, UK). Glutathione reductase activity staining was performed by incubating gels in a solution containing 250 mM Tris–HCl buffer (pH 7.5), 3 mM EDTA, 3.4 mM GSSG, 0.5 mM NADPH, 0.2 mg ml^{-1} of 2,6 dichlorophenolindophenol (DCPIP) and 0.2 mg ml^{-1} of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Rao et al., 1996). Duplicate control gels were stained in the absence of GSSG.

4.6. Isolation of RNA and RT-PCR analysis

Total RNA was extracted from soybean roots using Trizol reagent (Invitrogen, CA, USA) as described by the manufacturer. The amount of RNA isolated was determined by measuring the absorbance at 260 nm. Four micrograms of total RNA were treated with RNase-free DNase I (Promega, CA, USA) according to the manufacture's instructions and then 1.0 μg was reverse transcribed into cDNA in a 20 μl reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3.0 mM $MgCl_2$, 1 mM dNTPs, 0.2 μg random hexamer, 20 U of RNase OUT (Invitrogen, CA, USA) and 200 U of M-MLV Superscript II RT (Invitrogen, CA, USA) for 10 min at 25 °C, 50 min at 42 °C and 15 min at 70 °C. A primer pair specific to *Triticum aestivum* GR cDNA (sense primer, 5′-ACCTGTTGCTCTGATGGAGG-3′; antisense primer, 5′-TCGGTATCAGCATCAACCAC-3′) was designed using the wheat gene sequence in the TIGR database (accession no. **TC250996**; http://www.tigr.org/tdb/tgi/tagi/). A reaction mixture (25 μl) containing cDNA preparation (5 μl), 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, primers (0.2 μM each) and 0.625 U Taq DNA polymerase (Invitrogen, CA, USA) was used. The PCR profile was set as follows: 94 °C for 1 min, then 29 cycles at 94 °C denaturing for 0.5 min, 54 °C annealing for 1 min, and 72 °C extension for 1 min, and then a final step of 72 °C for 7 min. Also, primers for wheat 18S ribosomal cDNA (sense primer, 5′-GGCTACCACATCCAAGGAA-3′; antisense primer, 5′-CTATTGGAGCTGGAATTACCG-3′) were used as an internal control for the amount of RNA and RT efficiency. Samples were kept at 94 °C for 1 min and then

subjected to thermocycling (19 cycles of 0.5 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min). Linear amplification for semi-quantitative RT-PCR was obtained using an optimized number of PCR cycles for each primer set. The amplified transcripts were visualized on 1.5% agarose gels with the use of ethidium bromide. Specific products of the expected size (261 bp for GR and 193 bp for 18S) were observed and their identities were confirmed by automatic sequencing. Ethidium bromide stained gels were photographed (Fotodyne Incorporated, WI, USA) and analyzed using Gel-Pro Analyzer 3.1 software (Media Cybernetics, MD, USA). The ratio of GR mRNA to 18S mRNA was quantified.

4.7. Western-blot analysis for GR

Homogenates obtained for GR activity assays were also analyzed by Western immunoblot technique. Forty micrograms of protein from root homogenates were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis using a 10% acrylamide resolving gel (Mini-PROTEAN 3 Electrophoresis System, BioRad, Hertz, UK), according to Laemmli (1970). Separated proteins were then transferred to nitrocellulose membranes and non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS (pH 7.4) for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with rabbit antibodies raised against commercial chloroplastic spinach GR diluted 1:1000 in Tris–NaCl buffer plus 1% non-fat milk. These antibodies have shown to cross-react with chloroplastic GR of wheat (Lascano et al., 1998). Immune complexes were detected using peroxidase-conjugated goat anti-rabbit immunoglobulin G and visualized with the enhanced chemiluminescence western blotting procedure (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were reprobed with anti-β-tubulin (Sigma, MO, USA) as a loading control according to Krajewski et al. (1996). The films were photographed (Fotodyne Incorporated, WI, USA) and analyzed using Gel-Pro Analyzer 3.1 software (Media Cybernetics, MD, USA). The ratio of GR to β-tubulin protein expression was quantified.

4.8. Protein determination

Protein concentration was evaluated by the method of Bradford (1976) using bovine serum albumin as a standard.

4.9. Statistics

Continuous variables are expressed as mean ± SD. One-way analysis of variance with the post hoc Tukey test was used for intra-group comparisons to assess changes in growth parameters and Cd content over time. The unpaired *t* test was used for inter-group comparisons. A probability value <0.05 was considered statistically significant.

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