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Cadmium Affects the Glutathione/Glutaredoxin System in Germinating Pea Seeds

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Abstract The aim of this work was to investigate the effects of cadmium (Cd) on thiol and especially glutathione (GSH)-dependent reactions (glutathione content, glutaredoxin (Grx) content and activity, "glutathione" peroxidase (Gpx) activity, and glutathione reductase (GR) activity) in germinating pea seeds. Under Cd stress conditions, the overall activity as well as more specifically the expression of Grx C4 and Grx S12 increased. On the contrary, when incubated with Cd ions in vitro, the disulfide reductase activity of both isoforms was drastically inhibited. In the case of Grx C4, this correlated with the formation of protein dimers of 28 kDa as evidenced by electrophoresis analysis. Oxidative stress also affected the GSH status, since Cd treatment provoked (1) a pronounced stimulation in Gpx (a thioredoxin-dependent enzyme in plants) expression and (2) a drastic decrease in GR activity. These results are discussed in relation with the known contribution of Grx system to the thiol status during the germination of Cd-poisoned pea seeds.

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Abbreviations

Cd	Cadmium
DTT	Dithiothreitol
DW	Dry weight
FW	Fresh weight
GPX	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
HED	Hydroxyethyl disulfide
β-MET	β-Mercaptoethanol

Introduction

Seeds are easy to grow and adapt to environmental stress [1], while the germination process is very sensitive to adverse situations, in particular heavy metal pollution [2]. Cadmium (Cd) is frequently accumulated by agriculturally important crops with a significant potential to impair animal and human health [1]. The inhibitory effects of Cd on various processes in adult pea and germinating seeds have been documented by several workers [3–8]. Bioassays reflect a toxicological damage at both the biochemical and physiological levels.

Proteins are the primary effector molecules of all living systems, and therefore, virtually, any response to environmental changes, physiological, or pathological conditions will be reflected by alterations in protein activity, location, and concentration [9]. Moreover, under heavy metal stress, the cellular thiol-disulfide redox status is important for the control of protein functions [10]. Glutaredoxin/glutathione/glutathione reductase (Grx/GSH/GR) is one of the major redox systems involved in maintaining the thiol reduced state [11–16]. This reducing system is predicted to be localized in multiple subcellular compartments as there are overall 31 Grx genes in *Arabidopsis thaliana* [14, 17]. There are 14 bicysteinic Grxs (nine in the cytosol, two in chloroplasts, and three secreted) and 17 monocysteinic (12 in cytosol, four in chloroplasts, and one in mitochondria). They represent a large family of GSH-dependent oxidoreductases that catalyze the GSH-dependent reduction of disulfides or glutathione-mixed disulfide by a dithiol or a monothiol mechanism through a CxxC or CxxS active site [14, 15, 17]. Using NADPH as an electron donor, GR catalyzes the conversion of oxidized glutathione to its reduced form which functions to reduce cellular disulfide bonds, often in conjunction with Grxs. Then, they are required for a number of antioxidant and metabolic enzymes that form a disulphide as part of their catalytic cycle [15].

The most documented functions of Grxs are their involvement in the oxidative stress response [15, 16, 18], notably in plants subjected to abiotic stress, as Cd [9, 19]. The goal of this study was to evaluate the effect of Cd addition into imbibition medium of pea seeds on Grx/GSH/GR system in post-mitochondrial fractions obtained from the reserve tissues (cotyledons) and growing embryonic axes. We show here that Cd influences the content and activity of several partners of this redox regulatory network in seeds.

Materials and Methods

Germination and Cadmium Treatment

The germination conditions and Cd treatment of pea (*Pisum sativum* L. cv. douce province) seeds were as previously described [8].

Protein Extraction and Solubilization

Fresh tissues were ground with a mortar and pestle in a homogenization medium (pH 8.0) consisting of 50 mmol L^{-1} Tris–HCl, 0.4 mol L^{-1} sucrose, 1 mmol L^{-1} EDTA, 5 mmol L^{-1} ascorbic acid, and 1 mmol L^{-1} $MgCl_2$ (1:2, *w*/*v*). The homogenate was

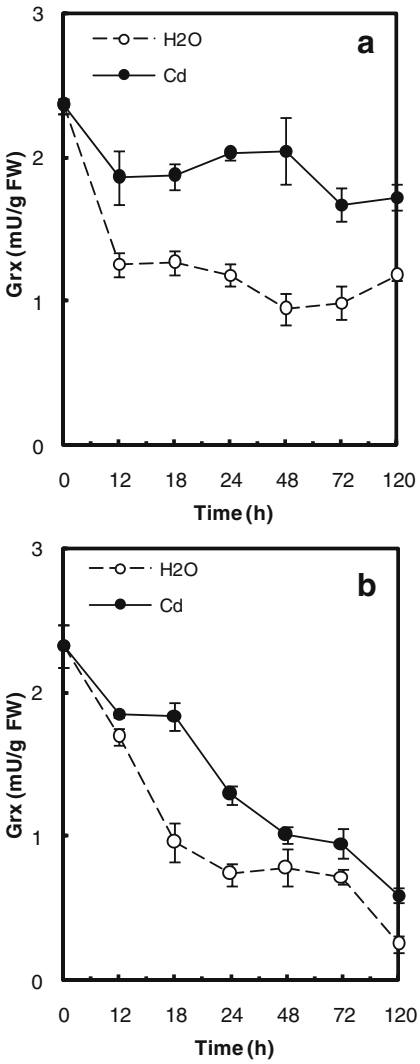

Fig. 1 Grx activity in cotyledons (**a**) and embryonic axes (**b**) of pea seeds during germination after imbibition with H_2O or 5 mmol L^{-1} Cd. Data are the mean of six independent measurements ± SE. Each measurement was performed in an extract obtained from several germinating seeds

filtered through two layers of miracloth and centrifuged at 3,000×*g* for 10 min. The resulting supernatant was centrifuged again at 20,000×*g* for 20 min. The pellet was washed with the homogenization medium and centrifuged in the same way. The mitochondrial pellet was then discarded for other uses [8]. The combination of supernatants was regarded as post-mitochondrial fraction whose quality was routinely evaluated [20] using cytochrome *c* oxidase test (data not shown). All operations were performed at 4°C.

Enzyme Activities and Thiol Determination

Grx activity was evaluated using the hydroxyethyl disulfide (HED) reduction test [17]. The activities of GR [21] and Gpx [22] were measured spectrophotometrically. Total protein thiols were assayed according to the method of Ellman [23]. Proteins were quantified using bovine serum albumin as a standard [24].

Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 15% (*w*/*v*) polyacrylamide gels by the procedure of Laemmli [25]. Proteins were then transferred to nitrocellulose sheets as in Towbin et al. [26]. Immunodetection was performed with poplar Grx C4, Grx S12, and GPX 3 antibodies.

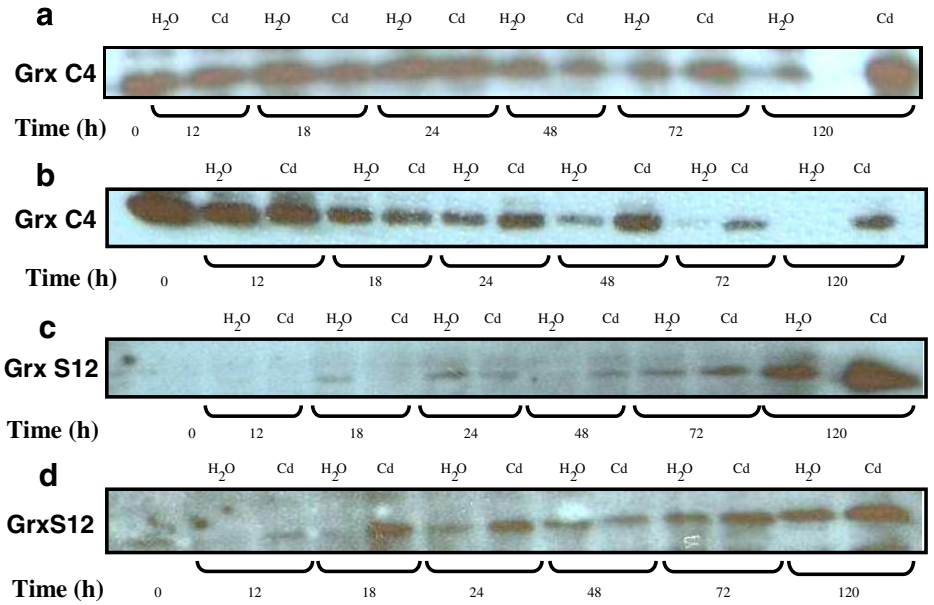

Fig. 2 Western blot analysis of Grx expression in cotyledons (**a**, **c**) and embryonic axes (**b**, **d**) of pea seeds during germination after imbibition with H_2O or 5 mmol L^{-1} Cd. Analysis were performed after SDS-PAGE of post-mitochondrial proteins obtained from several germinating seeds (20 μg per track), transferred to nitrocellulose sheet and immunodetected with poplar Grx C4 and Grx S12 antibodies. The blot is representative of two experiments

Effect of Cd Ions on Grx in Vitro

Proteins were incubated with appropriate amounts of $CdCl_2$ corresponding to real metal contents in tissues of Cd-treated germinating seeds [8]. Disulfide reductase activity of Grx [17] was performed after an incubation period of 30, 120, and 240 min at 4°C. Grx dimer and monomer were resolved by SDS-PAGE after incubation of protein with Cd ions in the absence or in the presence of 30 mmol L^{-1} β-mercaptoethanol (β-MET) and dithiothreitol (DTT).

Glutathione Assay

Non-protein thiols were extracted by homogenizing the post-mitochondrial fraction in 5% *w*/*v* sulfosalicylate (pH<1) containing 6.3 mmol L^{-1} diethylene triamine pentaacetic acid. After centrifugation at 10,000×*g* for 30 min at 4°C, the supernatants were used for total GSH determination [27].

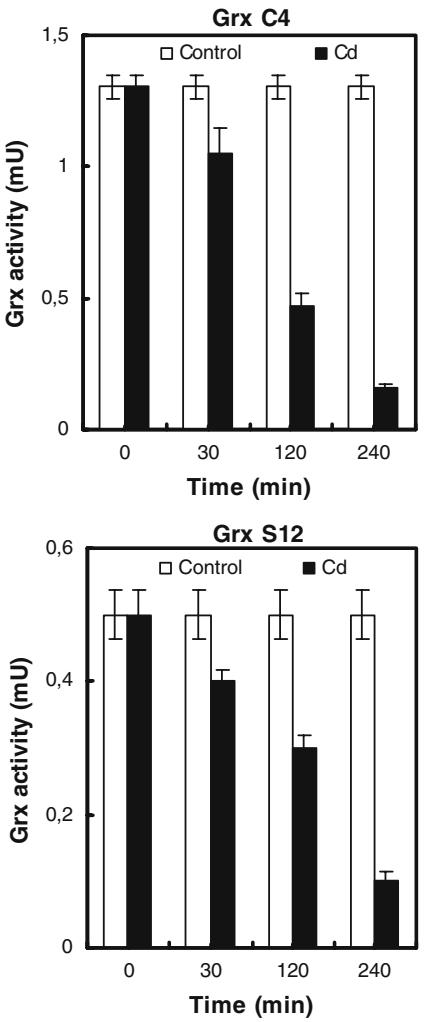

Fig. 3 Effect of Cd ions on Grx activity in vitro. Poplar proteins (5 μg) were mixed with 5 μg Cd or equal volume of H_2O (*Control*) and incubated for different times before the enzyme assay. Disulfide reductase activity was performed as HED reduction test at 25°C. The test was performed in 500 μL of 100 mmol L^{-1} Tris–HCl, pH 8, 2 mmol L^{-1} EDTA, 150 μmol L^{-1} NADPH (Sigma, St. Louis, MO, USA), 1 mmol L^{-1} glutathione, 1 U GR (Sigma), and 7 mmol L^{-1} HED (Acros, Noisy-le-grand, France). The Grx isoforms were added after 2 min of incubation, allowing disulfide bridge formation between glutathione and HED. The oxidation of NADPH was measured at 340 nm. Data are the mean of six independent measurements ± SE

Results and Discussion

The Grx system plays a fundamental role in controlling the redox status in animals and plants subjected to abiotic stress as Cd [19, 28]. Exposure of pea seeds to cadmium during germination results in the increase of the overall Grx activity: about 47% and 136% from

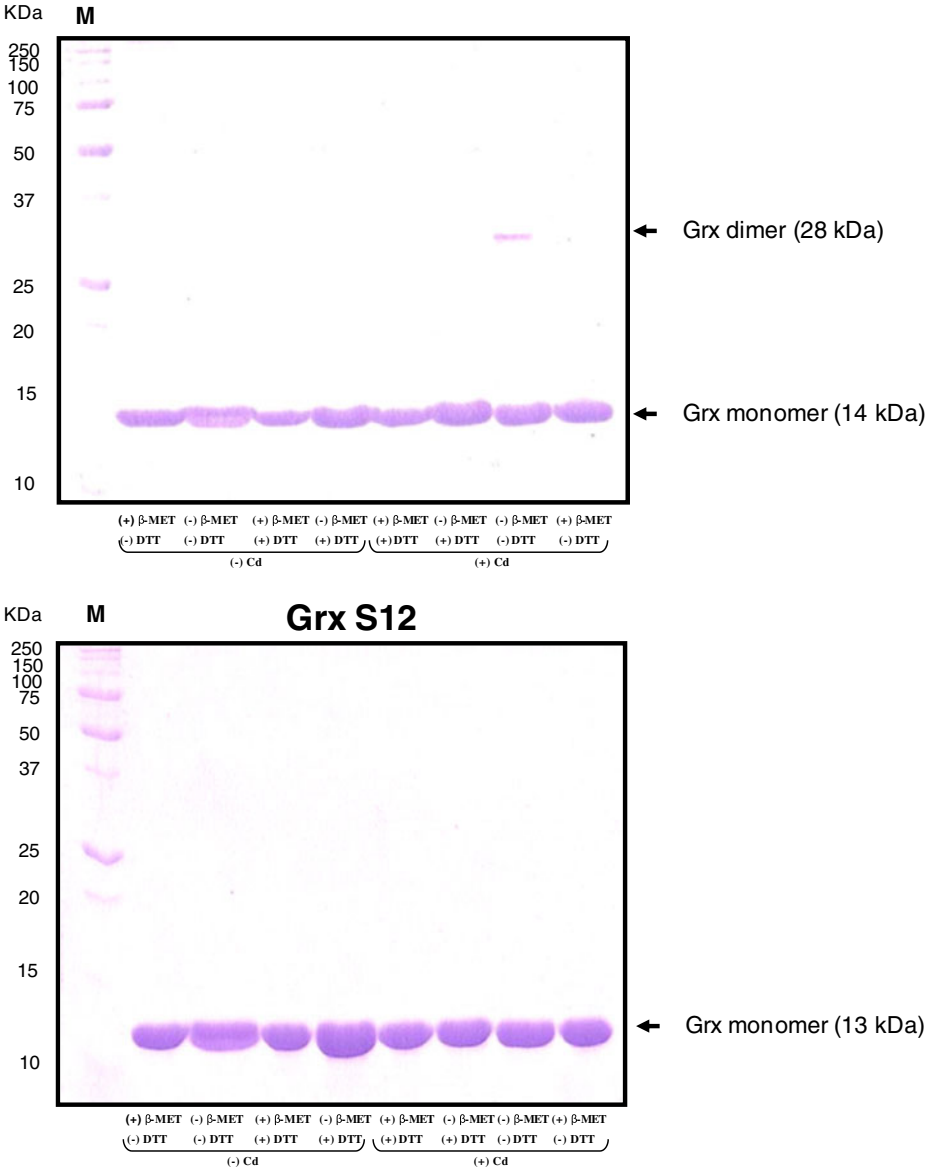

Fig. 4 Effect of Cd binding on Grx. Poplar proteins (5 μg per lane) were mixed with 5 μg Cd, 30 mmol L^{-1} β-MET, and DTT (+) or equal volume of H_2O (−) and incubated for 240 min before being subjected to 15% SDS-PAGE. *M*, molecular weight markers are indicated in kilodaltons. Grx dimer and mononer were stained with Coomassie bleue. Experiments were performed in duplicate

controls in cotyledons and embryonic axes, respectively, after 5 days of treatment (Fig. 1). Moreover, the expression of some isoforms, namely, Grx C4 and Grx S12, was induced following the exposure to Cd (Fig. 2).

However, heavy metals are known to bind thiols with high affinity [29, 30]. Although Cd has been implicated as a pro-oxidant, it differs from other metal ions like Cu and Fe because it does not participate in redox cycling [31]. This can implicate a direct binding of Cd ion to critical cellular components such as proteins with vicinal dithiols that are expected to be particularly sensitive to inactivation. Consequently, the active site of reduced Grxs is a possible target of Cd fixation. To test further the supposed mechanism of inactivation, we have analyzed the effect of pre-treatment with Cd ions on the activity of (a) bicysteinic Grx C4 and (b) monocysteinic Grx S12

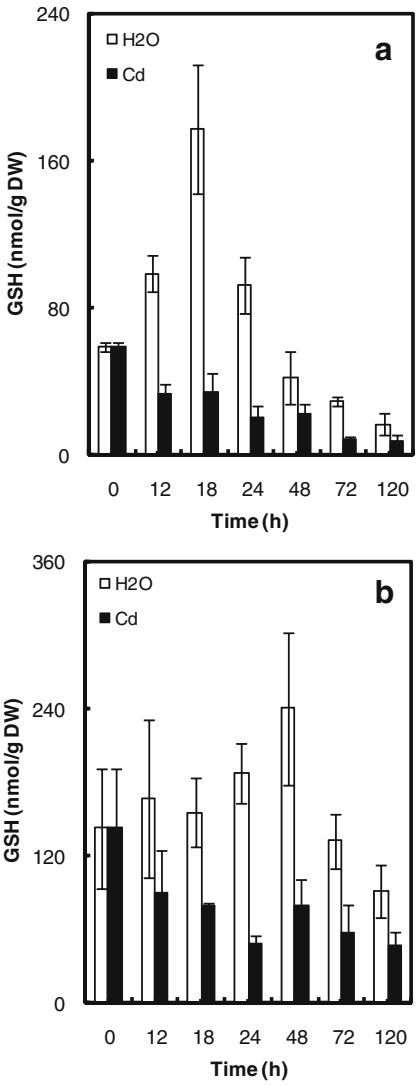

Fig. 5 Content of total glutathione in cotyledons (**a**) and embryonic axes (**b**) of pea seeds during germination after imbibition with H_2O or 5 mmol L^{-1} Cd. Data are the mean of six independent measurements ± SE. Each measurement was performed in an extract obtained from several germinating seeds

where the active site contains one thiol (Cys) and one hydroxyl (Ser) [15, 17]. The disulfide reductase activity, performed as HED reduction test, of both isoforms was markedly diminished in a time-dependent manner (Fig. 3). The inhibition of the wild-type Grx C4 activity may be due, at least in part, to the formation of dimer. In fact, the electrophoretic analysis showed that the reduced form of Grx C4 (monomer; 14 kDa) can be converted to an oxidized state in the presence of cadmium (dimer; 28 kDa) (Fig. 4). The reduced state was restored by the addition of either β-MET or DTT. These experiments indicate that Grxs are able to bind Cd, presumably at the level of their active site thiols. However, Grx S12 remained in the monomeric state (13 kDa) even in the presence of the heavy metal (Fig. 4).

Thus, in spite of the negative effect of a direct interaction between Cd ion and Grx, it seems that heavy metal stress might enhance the protein expression and,

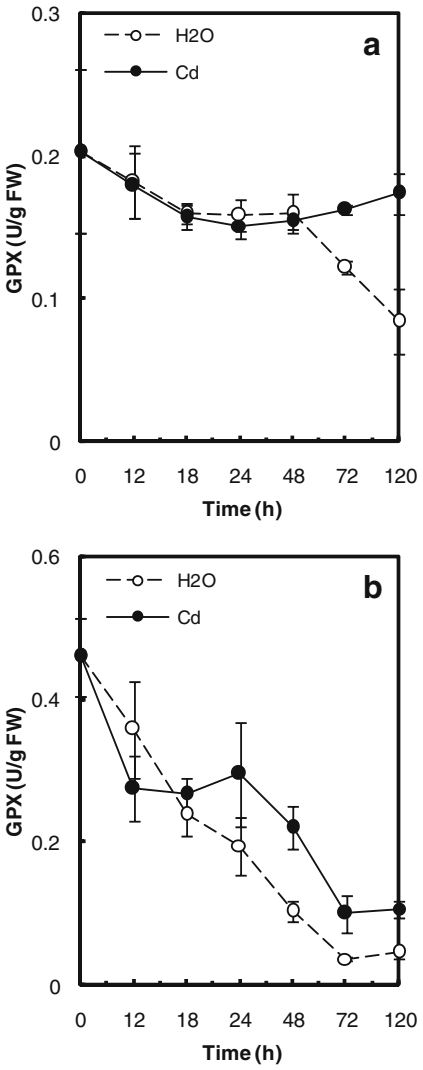

Fig. 6 GPX activity in cotyledons (**a**) and embryonic axes (**b**) of pea seeds during germination after imbibition with H_2O or 5 mmol L^{-1} Cd. Data are the mean of six independent measurements ± SE. Each measurement was performed in an extract obtained from several germinating seeds

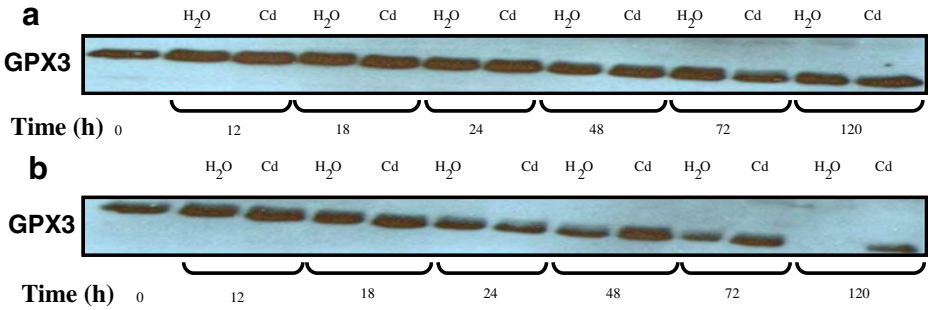

Fig. 7 Western analysis of GPX expression in cotyledons (**a**) and embryonic axes (**b**) of pea seeds during germination after imbibition with H_2O or 5 mmol L^{-1} Cd. Analysis were performed after SDS-PAGE of post mitochondrial proteins obtained from several germinating seeds (20 μg per track), transferred to nitrocellulose sheet and immunodetected with poplar GPX3 antibody. The blot is representative of two experiments

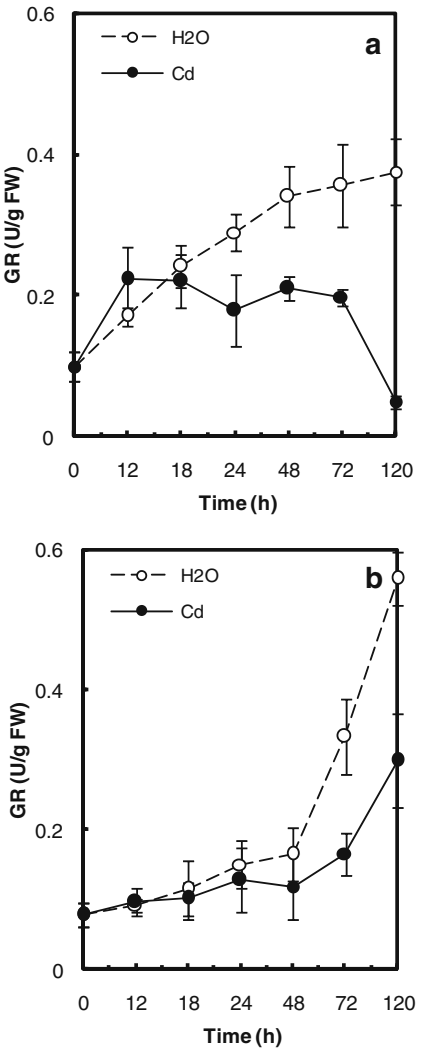

Fig. 8 GR activity in cotyledons (**a**) and embryonic axes (**b**) of pea seeds during germination after imbibition with H_2O or 5 mmol L^{-1} Cd. Data are the mean of six independent measurements ± SE. Each measurement was performed in an extract obtained from several germinating seeds

consequently, the absolute activity. The stimulatory effect should compensate the vulnerability of Grx to dimerization or any other mechanism of chemical inactivation imposed by Cd.

Under normal conditions, the intra-cellular redox state is predominately reducing, but processes like oxidative stress can shift the redox balance toward an oxidizing state [15, 16, 18, 32]. In adult pea, we have demonstrated that Cd causes an oxidative stress [4, 5]. In this study, we bring evidence for relationships between the GSH-dependent redox status, Grx, and Cd treatment during pea seed germination.

The level of GSH decreased at least twofold in Cd-treated germinating seeds as compared to controls (Fig. 5). Peroxidation is one of the expected consequences of oxidative stress in pea [4, 5]. Cadmium treatment stimulated the enzymatic activity of GPX

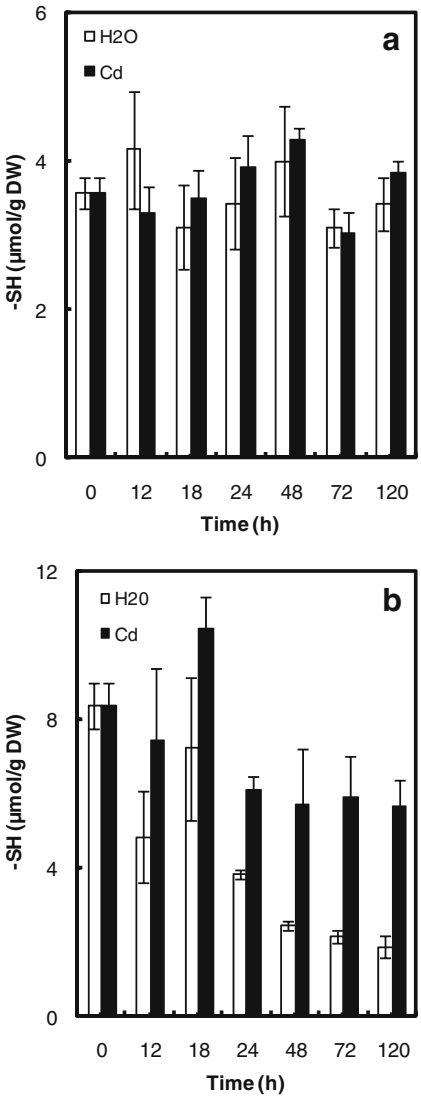

Fig. 9 Content of total protein thiol in cotyledons (**a**) and embryonic axes (**b**) of pea seeds during germination after imbibition with H_2O or 5 mmol L^{-1} Cd. Data are the mean of six independent measurements ± SE. Each measurement was performed in an extract obtained from several germinating seeds

after 48 h of exposure (Fig. 6). GPX 3 isoform was immunodetected with high expression levels in dry seed tissues and was markedly decreased during H_2O imbibition but remained significantly high at least in the Cd-treated embryos (Fig. 7b). Moreover, Cd stress provoked a drastic decrease in GR activity: about 87% and 47% from cotyledon and embryo controls after 5 days, respectively (Fig. 8).

The formation of disulfides and mixed disulfides between GSH and other thiols such as protein can be expected to occur under oxidative stress conditions [15]. A similar phenomenon might be also responsible for the decline in the GSH status in Cd-treated germinating pea seeds, since protein–glutathionyl–mixed disulfides formation is a mechanism of regulation and implicates Grx as a key player in this cellular process [15].

We can exclude a possible role for peroxidases in the GSH depletion (Figs. 6 and 7). Indeed plant peroxidases have a thiol-oxidase function [33], but so-called plant "glutathione" peroxidases are in fact thioredoxin- and not glutathione-dependent enzymes [22]. Anyway, under Cd stress, an increase in the GPX activity has been reported during germination [9]. Moreover, the resulting loss in the activity of enzymes such as GR (Fig. 8) leads to an increase in the generation of an intracellular oxidative stress. These observations are in agreement with the results obtained by Noriega et al. [34] and Yannarelli et al. [35]. It has been also reported that redox perturbations, especially in GSH levels, act as signal in stimulating stress response [10, 36–40].

On the other hand, the protein thiols seem to be protected against the Cd-imposed oxidation. In fact, SH-protein content remained statistically unchanged in the cotyledons and enhanced markedly in the embryos of Cd-treated seeds, as compared to the respective controls (Fig. 9). This can attest to the possibility of the cellular thiol response of germinating pea seed against Cd toxicity.

Conclusion

All data led us to underlie the relationships between thiol management and some Grx thiol-dependent responses during the germination of Cd-treated pea seeds. Despite the loss in GSH pool and GR activity due to the generation of an intracellular oxidative stress, a protective action of high Grx expression and activity on thiols is possible to improve the redox status. However, Grxs are often involved in concert with thioredoxins whose gene expression and activity can be also regulated by heavy metals. For these reasons together, we have started an investigation to elucidate how the thioredoxin system might contribute to control the redox homeostasis in germinating pea seeds exposed to cadmium.

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References

1. Wagner GJ (1993) Accumulation of cadmium in crop plants and its consequences to human health. Adv Agron 51:173–212

2. Ernst WHO (1998) Effects of heavy metals in plants at the cellular and organismic level ecotoxicology. In: Gerrit S, Bernd M (eds) Bioaccumulation and biological effects of chemicals. Wiley, Heidelberg, pp 587–620
3. Chugh LK, Sawhney SK (1996) Effect of cadmium on germination, amylases and rate of respiration of germinating pea seeds. Environ Pollut 92:1–5
4. Chaoui A, Jarrar B, El Ferjani E (2004) Effects of cadmium and copper on peroxidase, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. J Plant Physiol 161:1225–1234
5. Chaoui A, El Ferjani E (2005) Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedling. C R Biol 328:23–31
6. Mihoub A, Chaoui A, El Ferjani E (2005) Biochemical changes associated with cadmium and copper stress in germinating pea seeds (*Pisum sativum* L.). C R Biol 328:33–41
7. Rahoui S, Chaoui A, El Ferjani E (2008) Differential sensitivity to cadmium in germinating seeds of three cultivars of faba bean (*Vicia faba* L.). Acta Physiol Plant 30:451–456
8. Smiri M, Chaoui A, El Ferjani E (2009) Respiratory metabolism in the embryonic axis of germinating pea seed exposed to cadmium. J Plant Physiol 166:259–269
9. Aina R, Labra M, Fumagalli P, Vannini C, Marsoni M, Cucchi U, Bracale M, Sgorbati S, Citterio S (2007) Thiol-peptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots. Environ Exp Bot 59:381–392
10. Cobbet CS (2000) Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr Opin Plant Biol 3:211–216
11. Lee KO, Lee JR, Yoo JY (2002) GSH-dependent peroxidase activity of the rice (*Oryza sativa*) glutaredoxin, a thioltransferase. Biochem Biophys Res Commun 296:1152–1156
12. Gelhaye E, Rouhier N, Jacquot JP (2003) Evidence for a subgroup of thioredoxin h that requires GSH/Grx for its reduction. FEBS Lett 555:443–448
13. Fernandes AP, Holmgren A (2004) Glutaredoxins: glutathione dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid Redox Signal 6:63–74
14. Rouhier N, Gelhaye E, Jacquot JP (2004) Plant glutaredoxins: still mysterious reducing systems. Cell Mol Life Sci 61:1266–1277
15. Rouhier N, Stéphane DL, Jacquot JP (2008) The role of glutathione in photosynthetic organisms, emerging functions for glutaredoxins and glutathionylation. Ann Rev Plant Biol 59:143–166
16. Rouhier N, Couturier J, Johnson MK, Jacquot JP (2010) Glutaredoxins: roles in iron homeostasis. Trends Biochem Sci 35:43–52
17. Rouhier N, Gelhaye E, Jacquot JP (2002) Exploring the active site of plant glutaredoxin by site-directed mutagenesis. FEBS Lett 511:145–149
18. Lemaire SD (2004) The glutaredoxin family in oxygenic photosynthetic organisms. Photosynth Res 79:305–318
19. Schwarzländer M, Fricker MD, Sweetlove LJ (2009) Monitoring the in vivo redox state of plant mitochondria: effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge. Biochim Biophys Acta 1787:468–475
20. Douce R, Christensen EL, Bonner RWJR (1972) Preparation of intact plant mitochondria. Biochim Biophys Acta 275:148–160
21. Foyer CH, Halliwell B (1976) The presence of gluthathione and gluthathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133:21–25
22. Navrot N, Collin V, Gualberto J, Gelhaye E, Hirasawa M, Rey P, Knaff DB, Issakidis E, Jacquot JP, Rouhier N (2006) Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol 142:1364–1379
23. Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77
24. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254
25. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
26. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
27. Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Meth Enzymol 113:548–555
28. Diotte NM, Xiong Y, Gao J, Chua BHL, Ho YS (2009) Attenuation of doxorubicin-induced cardiac injury by mitochondrial glutaredoxin 2. Biochim Biophys Acta 1793:427–438
29. Vallee BL, Ulmer DD (1972) Biochemical effects of mercury, cadmium, and lead. Annu Rev Biochem 41:91–128
30. Van Assche F, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ 13:195–206

31. Stochs SJ, Bagchi D (1995) Oxidative mechanism in the toxicity of metal ions. Free Radic Biol Med 18:321–336
32. Isakov N, Witte S, Altman A (2000) PICOT-HD: a highly conserved protein domain that is often associated with thioredoxin and glutaredoxin modules. Trends Biochem Sci 25:537–539
33. Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid Redox Signal 11:861–905
34. Noriega GO, Balestrasse KB, Batlle A, Tomaro ML (2007) Cadmium induced oxidative stress in soybean plants also by the accumulation of δ-aminolevulinic acid. Biometals 20:841–851
35. Yannarelli GG, Fernández-Alvarez AJ, Santa-Cruz DM, Tomaro ML (2007) Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. Phytochemistry 68:505–512
36. May MJ, Vernoux T, Leaver C, Van Montagu M, Inzé D (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. J Exp Bot 49:649–667
37. Yano H, Wong JH, Cho MJ, Buchnan BB (2001) Redox changes accompanying the degradation of seed storage proteins in germinating rice. Plant Cell Physiol 42:879–883
38. Foyer CH, Noctor G (2003) Redox sensing and signaling associated with reactive oxygen in chloroplast, peroxisomes and mitochondria. Physiol Plant 119:355–364
39. Wojtyla Ł, Garnczarska M, Zalewski T, Bednarski W, Ratajczak L, Jurga S (2006) A comparative study of water distribution, free radical production and activation of antioxidative metabolism in germinating pea seeds. J Plant Physiol 163:1207–1220
40. Posmyk MM, Kontek R, Janas KM (2009) Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotoxicol Environ Saf 72:596–602