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RESEARCH ARTICLE

Role for plant peroxiredoxin in cadmium chelation

Moêz Smiri[a]*, Nahida Jelali[b] and Jamel El Ghoul[a]

[a]*Department of Biology, Faculty of Sciences of Bizerta, University of Carthage, 7021 Zarzouna, Tunisia;* [b]*Laboratoire d'Adaptation des Plantes aux Stress Abiotiques, Centre de Biotechnologie, Technopole de Borj-Cedria (CBBC), B.P. 901, 2050 Hammam-Lif, Tunisia*

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Studies on heavy metal underline the role of thiols in plants and attribute tolerance to metal binding. The thiol and peroxiredoxins (Prx) contents and guaiacol peroxidase (GPOX) activity were analyzed in the cotyledons and embryo of pea (*Pisum sativum* L.) germinating seeds exposed to toxic Cd concentration. The 2-cysteine peroxiredoxin (2Cys-Prx) level as well as the non-protein thiol (–SHNP) pool increased in both tissues treated with Cd compared to the control. An oxidized dimer of 2Cys-Prx was resolved in the presence of Cd ions. The obtained results suggest that Prx constitute a main key target in Cd toxicity. Despite of the decrease in GPOX activity due to the generation of an intracellular oxidative stress, a protective action via increasing Prx expression on thiols is possible to improve the redox status.

Keywords: cadmium; germination; *Pisum sativum*; peroxiredoxin

Abbreviations: Cd, cadmium; 2Cys-Prx, 2-cysteine peroxiredoxin; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); DW, dry weight; FW, fresh weight; GPOX, guaiacol peroxidase; Prx, peroxiredoxin; ROS, reactive oxygen species; –SHNP, non-protein thiols

1. Introduction

The peroxiredoxin (Prx) contains peroxidases enzymes with broad substrate specificity whose role is the detoxification of peroxides such as hydrogen peroxide. Prx reduces also alkylhydroperoxides and small organic hydroperoxides (Lim et al. 1993; König et al. 2003; Dietz 2003a). Prx does not have redox cofactors such as metals or prosthetic groups. Their catalytic activity is based on conserved cysteine residues (Chae et al. 1994). The regeneration cycle of Prx is considered to take place by intra- or intermolecular thiol-disulfide-reactions using small thiols, such as thioredoxins, glutaredoxins, and glutathione as electron donors (Baier et al. 2004; Dietz et al. 2006).

In plants, like in many other organisms, four subgroups of Prx can be distinguished according to the number and the position of one or two conserved cysteine residues in the primary structure: 1Cys-Prx, 2Cys-Prx, type II Prx, and PrxQ, which are localized in various cell compartments (Dietz 2003a). Only the nuclear 1Cys-Prx was analyzed in barley and Arabidopsis seeds (Haslekås et al. 2003). Its function is associated with protection against desiccation-induced free-radical damage.

The redox status of protein thiols has central importance to protein structure and folding while glutathione is an important low-molecular-mass redox regulator. Glutathione can be metabolized to yield cysteine (Cys), and converted to methionine (Met), thereby supplying sulfur amino acids for protein synthesis. The reduction of sulfur is thought to occur predominantly in leaf chloroplasts where reducing power from photosynthesis is harnessed to convert sulfate to sulfide. Sulfide is then combined with the amino acid skeleton of *O*-acetyl-serine, producing Cys (Tabe & Droux 2001).

Different mechanisms of defense against cadmium stress are (1) metal binding to the cell wall, (2) reducing transport across the cell membrane and by active efflux, and (3) chelation of the metal ions and compartmentalization into the vacuole (Aina et al. 2007). Moreover, heavy metals can be chelated by organic acids, metallothioneins, and phytochelatins (Cobbett & Goldsborough 2002).

ROS scavenging mechanisms are based on enzymes that can efficiently destroy superoxide radical and hydrogen peroxide. Enzymatic mechanisms include superoxide dismutase (EC 1.15.1.1), which converts hydrogen peroxide to H_2O_2, and catalase (EC 1.11.1.6), which converts H_2O_2 to water and molecular oxygen. Also enzymes cooperating in the ascorbate-glutathione cycle play an important role in H_2O_2 scavenging. This system includes enzymes such as ascorbate peroxidase (EC 1.11.1.11), monodehydroascorbate reductase (EC 1.6.5.4), dehydroascorbate reductase (1.8.5.1), and glutathione reductase

All authors contributed equally to this work.
*Corresponding author. Email: smirimoez@yahoo.fr

(EC 1.6.4.2) (Noctor & Foyer 1998). The guaiacol peroxidases are widely accepted as stress enzymes and their activation has been reported under many stressful conditions, including cadmium exposure (Radotić et al. 2000; Sobkowiak et al. 2004; Gzyl et al. 2009).

In addition to the antioxidant enzymes, plants are provided with antioxidant substances that are able to scavenge radical products. These compounds include low-molecular-weight antioxidants such as lipid-soluble α-tocopherol and water-soluble metabolites such as ascorbate and glutathione (Simontacchi et al. 2003).

The germinating seed is the first interface of material exchange between plant development cycle and environment. Delay in germination has been recorded after Cd exposure (Smiri et al. 2009). This may be correlated with several disturbances in germinative metabolism. With this aim, the main objective of this work is to evaluate the regulation of the antioxidant defense system during pea seed germination. Special focus was given to Prx, which are involved in the detoxification of alkyl hydroperoxides. In the present study, we compared seedlings grown in the presence or in the absence of external Cd. The disturbance of antioxidative enzymes and the regulation of 2Cys-Prx in *Pisum sativum* were analyzed. The study of the defense systems against Cd-toxicity during germination will provide valuable information on the evolution of antioxidant systems. GPOX is considered to be a heavy metal stress-related enzyme and is used as stress markers in Cd poisoning situation.

2. Materials and methods

2.1. Germination, cadmium treatment, and protein extraction

Seeds of pea (*P. sativum* L. Cv. Douce Provence) were disinfected with 2% of sodium hypochlorite for 10 min and then rinsed thoroughly and soaked in distilled water at 4°C for 30 min. Seeds were germinated at 25°C in the dark for 5 d over two sheets of filter paper moistened with distilled water or aqueous solution of chloride salt of 5 mM Cd. Germinating seeds were sampled for the assays. At harvest, the coat was removed and the embryonic axes and cotyledons were weighed and stored in liquid nitrogen until analysis or dried after 8 days at 70°C for dry weight determination.

Cotyledons and embryonic axes were ground in a mortar and pestle with sand and the following medium (w/v = 1/5): 50 mM Tris–HCl (pH 8.0), 0.4 M saccharose, 1 mM EDTA, 5 mM ascorbic acid, and 1 mM $MgCl_2$. The homogenate was squeezed through a double cheesecloth, centrifuged at 3000 *g* for 20 min. Mitochondria from supernatant were sedimented. The supernatant obtained was carefully decanted and used for enzyme assays, protein and non-protein thiols (–SHNP) determinations.

2.2. Identification of 2-Cysteine Peroxiredoxin

An inventory of available pea sequences related to 2Cys-Prx was made by blasting the sequences available in databases using available sequences from *A. taliana* L., *Oryza sativa* L. (TIGR) (http://www.tigr.org/tdb/mtgi), *Populus trichocarpa* Torr. & A. Gray (JGI) (http://www.jgi.doe.gov), and *P. sativum* (PlantGDB) (http://www.plantgdb.org).

2.3. Enzyme assay

Guaiacol peroxidase (GPOX; EC 1.11.1.7) activity was measured according to Fielding and Hall (1978). This method is based on monitoring the GPOX scavenging activity by using guaiacol as a hydrogen donor. The reaction mixture contained 10 mM H_2O_2 in 50 mM phosphate buffer (pH 7), 9 mM guaiacol and the enzyme extract in a total volume of 500 µl. GPOX activity was estimated by the increase in the absorbance of tetra-guaiacol at 470 nm and was expressed as micromole of guaiacol oxidized per min at 25°C.

2.4. Protein determination

Protein concentrations were evaluated by the method of Bradford (1976), using bovine serum albumin as a standard.

2.5. Determination of non-protein thiols

Non-protein thiols were extracted by homogenizing the postmitochondrial fraction in 5% (w/v) sulfosalicylate (pH < 1), containing 6.3 mM diethylene triamine pentaacetic acid. After centrifugation at 10,000 × *g* for 30 min at 4°C, the supernatants were used for analysis. –SHNP was determined in the homogenates spectrophotometrically at 412 nm using 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Anderson 1985).

2.6. Electrophoresis and western-blot analysis

Western blot (Towbin et al. 1979) probed with heterologous antibodies generated against poplar proteins (dilution 1/1000). Analysis was performed after SDS-PAGE (Laemmli 1970) of pea protein obtained from cotyledons and embryonic axis of germinating pea seeds (20 µg/track), transferred to PVDF membranes and immunodetected with poplar 2Cys-Prx antibody. Blocking of nonspecific binding is achieved by placing the membrane in blocking buffer (20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.05% Tween 20 and 5% milk) for overnight. After blocking, the detection process takes place in a two-step; a dilute solution of primary antibody (dilution: 1/1000) is incubated with the membrane under gentle agitation for 2 hours. After rinsing the membrane with blocking buffer for five times to remove unbound primary antibody, the membrane is exposed to a secondary antibody for 1 h. After rinsing the

membrane with TBST (20 mM Tris–HCl (pH 7.5), 137 mM NaCl and 0.05% Tween 20), a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

2.7. Effect of Cd ions on Prx in vitro

Proteins were incubated with 5μM $CdCl_2$. Prx dimer and monomer were resolved by SDS-PAGE after incubation of protein with Cd ions in the absence or the presence of 30 mM β-mercaptoethanol (β-MET) and dithiotreitol (DTT).

2.8. Statistical analysis

The experiments were performed in duplicates. The data reported are the mean values (±SE) of six replicates. Data were subjected to one-way ANOVA. Means were separated with the Student Newman–Keuls test (Statistica 8, StatSoft Co., USA).

3. Results

3.1. Effect of Cd on Prx

To determine whether Cd-induced inhibition of embryonic axis growth during germination is associated with oxidative stress metabolism, we examined the effect of metal on peroxiredoxin. All Prx are characterized by one or two cysteine residue(s) located in a conserved structural environment of the protein and they are central for the catalylic reaction.

We identified one pea 2Cys-Prx until now (Figure 1). The protein content in Cd-treated cotyledons and embryo was ~53–76% higher than that of control ones from 5 days of exposure (Figure 2a, b). To analyze the impact of Cd on the antioxidant potential in germinating pea seeds, the Prx protein levels were quantified by western blot analysis. Using antibodies generated against poplar proteins and pea samples containing identical protein amounts (Figure 3a, b), Prx levels were analyzed. The 2Cys-Prx protein levels in cotyledons and embryonic axis increased following exposure to Cd (Figure 4a, b).

To test further the supposed thiol chelation mechanism of Cd, we analyzed the effect of Cd ions on oxidized 2Cys-Prx, where the active site thiols were converted into intramolecular disulfide (C-SS-C). The electrophoretic analysis showed that the reduced form of 2Cys-Prx (monomer; 23 KDa) can be transferred into an oxidized state in the presence of Cd (dimer; 46 KDa) (Figure 5). The reduced state was restored by the addition of either β-MET or DTT. These experiments indicate that 2Cys-Prx is able to bind Cd, presumably at the level of their active site dithiols.

3.2. Effect of Cd on antioxidant system

Changes in the activity of GPOX were found in seeds treated with Cd. GPOX activity after five-day

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at2Cys-Prx        MASVASSTTLISSPSSRVFPAKSSLSSPSVSFLRTLSSPSASASLRSGFARR-----SSL
rice2Cys-Prx      MAACCSSLATAVSSSSAKPLAGIPPAAP-----HSLSLPRAPAARPLRLSAS-----SSR
pea2cys-Prx       --MACSAPFASLLYSNPNTLFSPKFSSPRLSSLSIPNAPNSLPKLRTSLP------LSLN
pop2Cys-Prx       --MACSATSTTLISSIAAAATATTTKSMAFPISKNITLPNSFFGTRKSFQSRVPRSISLT
                      .*:       *           :         . * :       :        *

at2Cys-Prx        SSTSRRSFAVKAQADDLPLVGNKAPDFEAEAVFDQEFIKVKLSDYIGKKYVILFFYPLDF
rice2Cys-Prx      SARASSFVARAGGVDDAPLVGNKAPDFDAEAVFDQEFINVKLSDYIGKKYVILFFYPLDF
pea2cys-Prx       RSSSSRRTFVVRASGELPLVGNSAPDFEAEAVFDQEFIKVKLSEYIGKKYVILFFYPLDF
pop2Cys-Prx       RGSHSRSTFVVKASSELPLVGNIAPDFEAEAVFDQEFINVKLSDYIGKKYVILFFYPLDF
                   .            .: ***** ****:**********:****:****************

at2Cys-Prx        TFVCPTEITAFSDRHSEFEKLNTEVLGVSVDSVFSHLAWVQTDRKSGGLGDLNYPLISDV
rice2Cys-Prx      TFVCPTEITAFSDRYDEFEKLNTEILGVSIDSVFSHLAWVQTDRKSGGLGDLKYPLISDV
pea2cys-Prx       TFVCPTEITAFSDRHAEFDAINTEILGVSVDSVFSHLAWVQSDRKSGGLGDLKYPLVSDV
pop2Cys-Prx       TFVCPTEITAFSDRHEEFEQINTEVLGVSIDSVFSHLAWVQTDRKSGGLGDLKYPLISDV
                  **************: **: :***:****:***********:**********:***:***

at2Cys-Prx        TKSISKSFGVLIHDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETMRTLQALQYIQE
rice2Cys-Prx      TKSISKSFGVLIPDQGIALRGLFIIDKEGVIQHSTINNLAIGRSVDETMRTLQALQYVQD
pea2cys-Prx       TKSISESYGVLIPDQGIALRGLFIIDKEGVIQHSTINNLGIGRSVDETKRTLQALQYVQE
pop2Cys-Prx       TKSISKSYGVLIPDQGVALRGLFIIDKEGVIQHSTINNLAIGRSVDETKRTLQALQYVQE
                  *****:*:**** ***:*********************.******** ********:*:

at2Cys-Prx        NPDEVCPAGWKPGEKSMKPDPKLSKEYFSAI
rice2Cys-Prx      NPDEVCPAGWKPGDKSMKPDPKGSKEYFAAI
pea2cys-Prx       NPDEVCPAGWKPGEKSMKPDPKGSKEYFAAV
pop2Cys-Prx       NPDEVCPAGWKPGEKSMKPDPKLSKDYFAAI
                  *************:******** **:**:*:
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Figure 1. Amino acid comparison of 2Cys-Prx. The sequences are from *A. thaliana* (at), at2-CysPrxA (At3g11630.1), *O. sativa* (rice), rice2-cysPrx (12002.m08438), *Pisum sativum* (garden pea), pea2-Cys Prx (Q93X25-1), and *P. trichocarpa* (pop), pop2-cysPrx (eugene3.00160660). Asterisks indicate conserved amino acids in all sequences, dots indicate amino acids with similar biochemical properties, and dashes represent gaps introduced to optimize the alignment. The available sequences are from *A. taliana, O. sativa* (TIGR) (http://www.tigr.org/tdb/mtgi), *P. trichocarpa* (JGI) (http://www.jgi.doe.gov), and *P. sativum* (PlantGDB) (http://www.plantgdb.org).

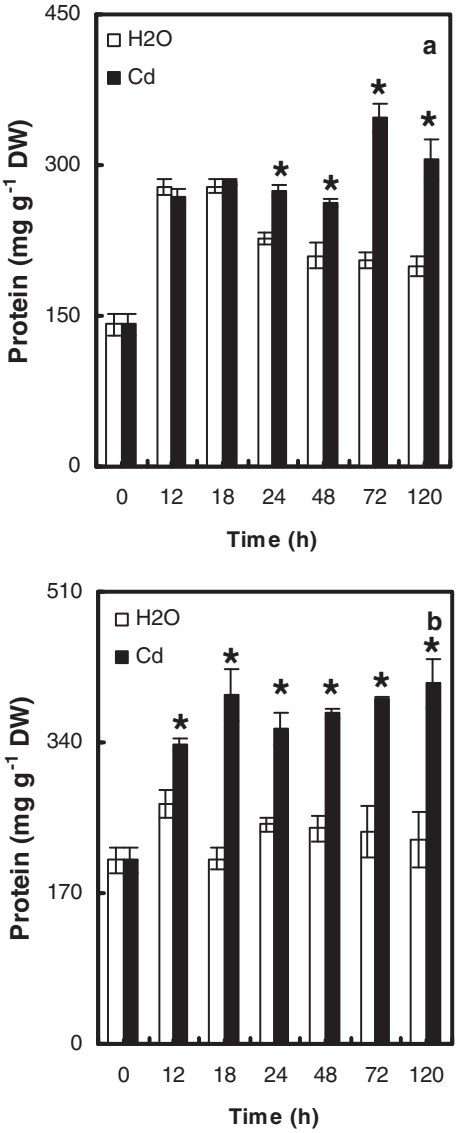

Figure 2. Protein content in cotyledons (a) and embryonic axes (b) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds. Asterisks indicate the significant levels at $p < 0.05$.

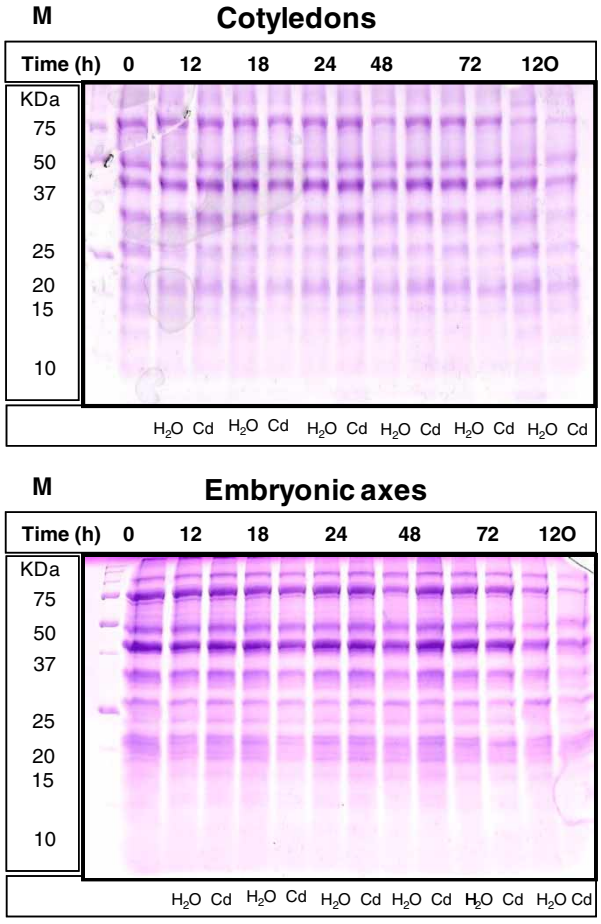

Figure 3. SDS-PAGE. Twenty microgram proteins from cotyledons and embryonic axes of pea seeds imbibed for 0–120 h with H_2O or 5 mM Cd were loaded per lane. Experiments were performed in duplicate.

treatment period decreased by 43 and 83% in cotyledons and embryonic axes, respectively (Figure 6). –SHNP pool was significantly increased in Cd-treated seeds, as compared to the controls (Figure 7).

4. Discussion

Environmental stresses often lead to great yield losses under various agricultural production systems. Among diverse abiotic stresses, Cd is a deleterious problem affecting the productivity and the quality of economically valuable crops. A wide range of cellular responses occur when plants are exposed to a variety of environmental stresses such as Cd toxicity (Szollosi et al. 2009). Studies on Cd accumulation and their effects on plants revealed that they are strongly phytotoxic, causing growth inhibition (Zhang et al. 2010). Cd toxicity in both animals and plants is due to the pro-oxidative effect (Rauser & Meuwly 1995; Stochs & Bagchi 1995). The critical targets of Cd binding are the thiol groups of proteins (Smiri et al. 2010). Prxs are special type of peroxidases recently identified that contain a conserved cysteine at the active site. Since the activity was supported by thiols, the protein was named thiolspecific antioxidant (Chae et al. 1994). Although Prxs have come to light, recently, they are now appear as a growing protein family which are considered to be involved in the protection against oxidative stress. They are present in organisms from all kingdoms and exist in multiple isoforms (Rouhier et al. 2001). These proteins are homodimers and each subunit has two conserved cysteines.

Direct binding of Cd^{2+} to critical cellular components may be part of the mechanisms of its toxicity, and Prxs are expected to be particularly sensitive to inactivation by Cd^{2+} via oxidation of active site to C-SS-C (Figure 5). It has been shown that proteins of the thiol-disulfide oxidoreductase family, all of which contain CysXXCys sequences, are sensitive to Cd^{2+} (Rollin-Genetet et al. 2004; Smiri et al. 2010). This implies a direct binding of Cd to critical cellular

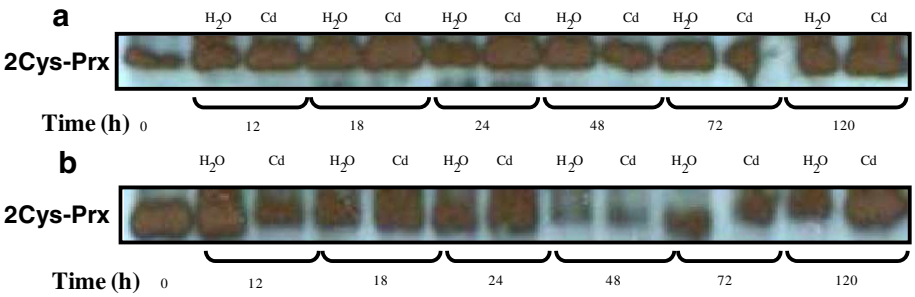

Figure 4. Western analysis of 2Cys-Prx expression in cotyledons (a) and embryonic axes (b) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Analyses were performed after SDS-PAGE of extracts obtained from several germinating seeds (20 μg protein/track), transferred to nitrocellulose sheet and immunodetected with poplar 2Cys-Prx antibody. The blot is representative of two experiments.

components as the mechanism of toxicity, and proteins with vicinal disulfides are expected to be particularly sensitive to inactivation by Cd. As suggested previously (Chrestensen et al. 2000), the likely mechanism of cadmium-mediated inactivation/inhibition of the redox proteins is in coordination with the vicinal thiol groups. We postulate a role for plant Prx in Cd^{2+} chelation. The high degree of Prx expression observed in cotyledons and embryonic axes is consistent with a possible role of Prx as a Cd^{2+} sink (Figure 5).

Barranco-Medina et al. (2007) have been suggested that pea Prx has a particular function under cold and heavy metals stress conditions. The pea peroxiredoxin homolog PsPrxII F, has a molecular mass of 18.75 kDa, and, at positions 59 and 84, carries the two catalytic cysteinyl residues which are characteristic for this particular Prx subgroup. Activity of site-directed mutagenized C84S-variant lacking the so-called resolving Cys dropped to about 12% of WT Prx while C59S lost its peroxidatic activity completely. Likewise, WT PsPrxII F and C84S-variant but not C59S protected plasmid DNA against strand breakage in a mixed function oxidation assay. WT PrxII F and the variant proteins aggregated to high mass oligomers. Upon oxidation with hydrogen peroxide PsPrxII F focused in a series of spots of distinct pI but similar molecular masses in two-dimensional gels indicating different oxidation states of the protein. Partial oxidation was also detected in leaf extracts and isolated mitochondria.

The redox status of sulfhydryl groups is important to cellular functions such as the synthesis and folding of proteins and the regulation of the structure and activity of enzymes, receptors, and transcription factors. Cadmium is known to exhibit high affinity for thiol groups and may therefore severely disturb many cellular functions. We have demonstrated that the control of redox balance and oxidative damage is one of the primary functions of Prx. When exposed to

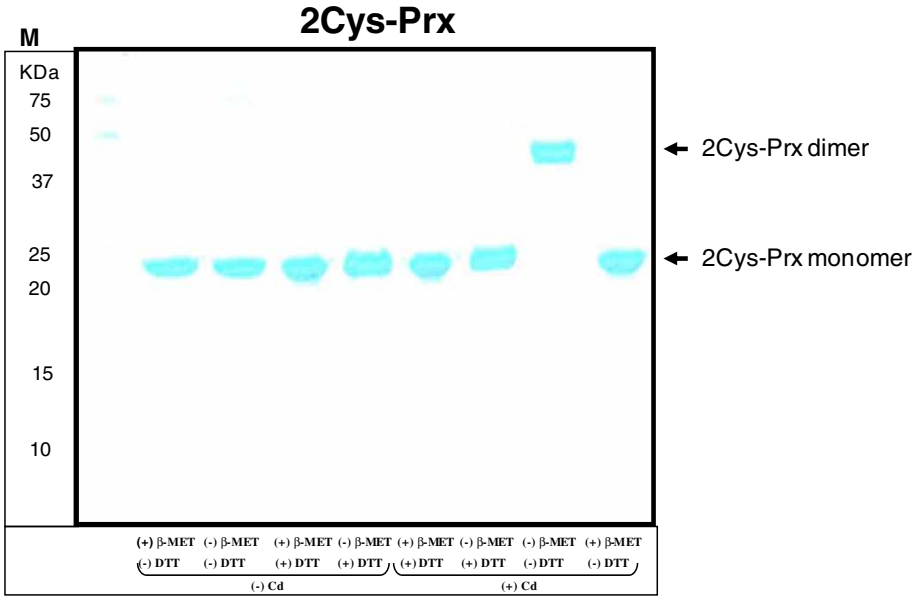

Figure 5. Effect of Cd binding on 2Cys-Prx. Poplar proteins (5 μg per lane) were mixed with 5 μg Cd, 30 mM β-MET and DTT (+) or equal volume of H_2O(−) and incubated for 240 min before being subjected to 15% SDS-PAGE. M, molecular weight markers are indicated in KDa. 2Cys-Prx dimer and monomer were stained with Coomassie Blue. Experiments were performed in duplicate.

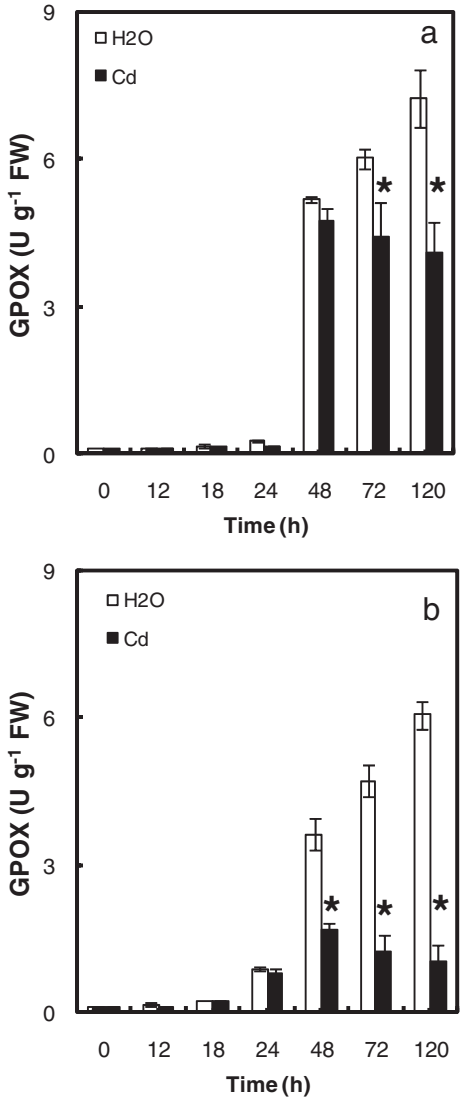

Figure 6. GPOX activity in cotyledons (a) and embryonic axes (b) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Data are the mean of six independent measurements ±SE. Each measurement was performed in an extract obtained from several germinating seeds. Asterisks indicate the significant levels at $p < 0.05$.

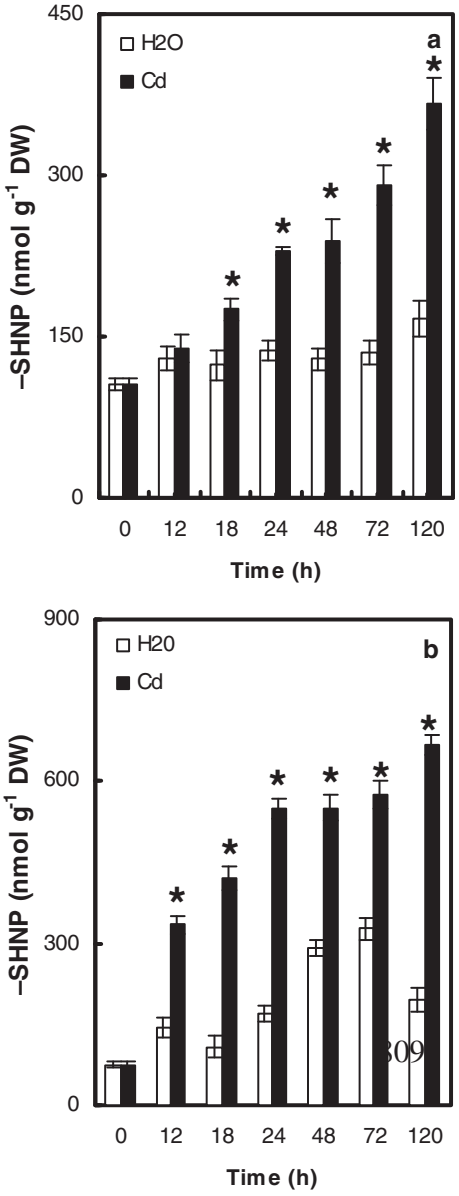

Figure 7. SHNP content in cotyledons (a) and embryonic axes (b) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds. Asterisks indicate the significant levels at $p < 0.05$.

cadmium, Prx was susceptible to structural alterations. Binding of cadmium occurs to Cys of Prx.

Cd is not active redox but contributes indirectly to oxidative stress by affecting the cellular thiol redox balance (Stochs & Bagchi 1995). As Cd generates an oxidative stress, we have highlighted the disturbance of some enzymes involved directly or indirectly in the antioxidative cellular mechanisms (Smeets et al. 2009; Sobrino-Plata et al. 2009; Szollosi et al. 2009; Zhang et al. 2010). All analyzed GPOX, as well as –SHNP, were present in dry pea seeds which allowed the antioxidative machinery to be active as soon as the enzymes were reactivated by seed imbibitions (Figures 6 and 7). Cd-induced alteration in the activity of antioxidant enzymes can increase the oxidative stress leading to the oxidative modification/inactivation of proteins. Our results showed that exposure to Cd resulted in an important decrease in GPOX activity in both tissues of germinating pea seeds (Figure 6). These observations are in agreement with those obtained by Sandalio et al. (2001) who demonstrated that, the antioxidant enzymes activity of both catalase and guaiacol peroxidase was depressed in pea leaves with increasing concentrations of Cd. The effects of heavy metals on the GPOX activity and their involvement in the defense mechanisms of plant tissue against metal-induced damages have been widely reported, but remain controversial. Presented results demonstrated that in pea seeds there was no direct correlation between the tolerance to cadmium and the total activity of antioxidant enzymes. On the other hand, we cannot rule out an involvement of GPOX isoenzymes in the tolerance

mechanisms. Our results imply that under Cd stress different defense strategies operate in the germinating pea seeds, including high accumulation of Prx and enhanced activity of GPOX, which together enable short-term survival of sensitive cells in cadmium-supplemented medium.

In addition to the antioxidant enzyme, concentrations of total non-protein thiols in cotyledons and embryonic axes were significantly elevated in the presence of Cd (Figure 7). In *Phaseolus vulgaris* and *Helianthus annuus*, it has been shown that Cd induces changes of the antioxidant status either by increasing the superoxide radical production and lipid peroxidation, or by making alterations in the enzymatic and nonenzymatic antioxidants (Gallego et al. 1996). Results indicated that Prx has an important effect on the antioxidant system, in particular, –SHNP pools. The stimulations of Prx expression seem inefficient to improve the redox status. The negative changes in the antioxidant systems can explain, at least in part, the delay in seed germination.

In conclusion, the treatment of pea seedlings with $CdCl_2$ induced an oxidative stress situation in cotyledons as well as in embryonic axes. We suggest that Prx has a role in sulfhydryl homeostasis. Quantification of these proteins provides new insight for understanding the molecular bases of response to Cd toxicity in seeds during germination.

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