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Cadmium induced mitochondrial redox changes in germinating pea seed

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Abstract Mitochondria play an essential role in producing the energy required for seedling growth following imbibition. Heavy metals, such as cadmium impair mitochondrial functioning in part by altering redox regulation. The activities of two protein redox systems present in mitochondria, thioredoxin (Trx) and glutaredoxin (Grx), were analysed in the cotyledons and embryo of pea (*Pisum sativum* L.) germinating seeds exposed to toxic Cd concentration. Compared to controls, Cd-treated germinating seeds showed a decrease in total soluble protein content, but an increase in –SH content. Under Cd stress conditions, Grx and glutathione reductase (GR) activities as well as glutathione (GSH) concentrations decreased both in cotyledons and the embryo. Similar results were obtained with the Trx system: Trx and NADPH-dependent thioredoxin reductase (NTR) activities were not stimulated, whereas total NAD(P) contents diminished in the embryo. However, Cd enhanced the levels of all components of the Trx system in the cotyledons. On the other hand, Cd caused a significant increase in oxidative stress parameters such as the redox ratio of coenzymes (oxidized to reduced forms) and NAD(P)H oxidase activities. These results indicate that Cd induces differential redox responses on different seed tissues. We suggest that neither Grx system nor Trx one may improve the redox status of mitochondrial thiols in the embryo of germinating pea seeds exposed to Cd toxicity, but in the cotyledons the contribution of Trx/NTR/NADPH can be established in despite the vulnerability of the coenzyme pools due to enzymatic oxidation.

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Keywords Cadmium · Germination · Mitochondria · Pea · Redox

Abbreviations

DTNB	5,5′-Dithio-*bis*-(2-nitrobenzoic acid)
DW	Dry weight
FW	Fresh weight

GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
NTR	NADPH-dependent thioredoxin reductase
Trx	Thioredoxin

Introduction

Seed germination is a crucial stage in the plant life cycle. It begins with water uptake by the dry seed during imbibition and ends when the radicle protruding through covering layers (Welbaum et al. 1998). During this process, the seed is transformed into a vigorously metabolizing system (Bewley 1997). The increase in water content inside the cells, the mobilization of reserves, the elongation and division of cells in the embryo are among the most important events, while the redox changes appear to be more closely related to the resumption of respiratory activity (Balmer et al. 2004). It is known that mitochondria synthesize redox components that modulate cellular redox homeostasis (Chen et al. 2006; Schwarzländer et al. 2009). Two major redox systems are involved in maintaining of the thiol reduced state in the plant: glutaredoxin/glutathione/glutathione reductase (EC 1.6.4.2) (Grx/GSH/GR) and thioredoxin/NADPH/NADPH-dependent thioredoxin reductase (EC 1.6.4.5) (Trx/NADPH/NTR) (Rouhier et al. 2008; Jacquot et al. 2009).

Although bacteria and animals possess only a few thioredoxin sequences, several well-characterized variants exist in photosynthetic cells. Trx m, f, x, y and cDSP32 are located in chloroplasts and the h type-located in the cytosol, endoplasmic reticulum and mitochondria. In addition mitochondria also contain a Trx of the o type (Laloi et al. 2001; Reichheld et al. 2002; Montrichard et al. 2003; Gelhaye et al. 2004; 2005; Traverso et al. 2007; Chibani et al. 2009). The oxidized form of each Trx contains a disulfide (–S–S–) bridge that is reduced to the sulfhydryl state (–SH) via either reduced ferredoxin and ferredoxin-dependent thioredoxin reductase or NADPH and NTR (Balmer et al. 2006; Jacquot et al. 2009). The functions of Trxs are currently being investigated in several plant systems, including seeds (Lozano et al. 1996; Montrichard et al. 2003; Alkhalfioui et al. 2007). Germination is accompanied by extensive change in the redox state of the major seed storage proteins and enzymes, which are present in oxidized (–S–S–) form in dry seeds and are converted to the reduced (–SH) state following imbibition. Trx appears to play a role in this transition (Yano et al. 2001; Alkhalfioui et al. 2007). Regarding gene expression analysis in response to stress, PsTrx f and PsTrx m transcripts increased in roots of pea plants subjected to abiotic treatments (Pagano et al. 2000; Traverso et al. 2008). A similar behavior was described for Trx m in the green algae *Chlamydomonas reinhardtii* showing an increase of gene expression in response to heavy metals (Lemaire et al. 1999).

The reduced form of Trx is an excellent catalyst for the reduction of intramolecular disulfide bonds of proteins that are also reduced by GSH or Grx, the other major cellular sulfhydryl reductants (Gelhaye et al. 2003; Rouhier et al. 2008). Grx has been characterized in vitro as a specific catalyst for the reduction of protein–glutathionyl-mixed disulfides. *Arabidopsis thaliana* contains three classes of Grx encoded by at least 31 genes. There are 14 bicysteinic (9 in cytosol, 2 in chloroplasts and 3 secreted) and 17 monocysteinic (12 in cytosol, 4 in chloroplasts and 1 in mitochondria) (Rouhier et al. 2004). They contain the conserved motif Cxx[C/S], CGFS or CCx[C/S/G] in the active sites (Rouhier et al. 2002, 2004; Discola et al. 2009). 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 Grx. This thiol-disulfide interchange reaction is likely crucial for maintaining the intracellular thiol status (Gelhaye et al. 2003), notably, in mitochondria of plants subjected to abiotic stress, as Cd (Aina et al. 2007; Schwarzländer et al. 2009).

Up until now, there is plenty of information about the crucial role of redox system in the regulation of fundamental processes, but their functions in seed germination under heavy metal stress are poorly documented. Abiotic stress effects are largely suspected to be related to oxidative processes resulting in the mitochondria dysfunction in animal cells (Diotte et al. 2009) and plants (Schwarzländer et al. 2009). In this cellular compartment, the redox proteins can be involved in the regulation of –SH state (Bragadin et al. 2004; Chen et al. 2006).

Cadmium is one of the most toxic pollutants found in the air, water and soil and is regarded as a non-essential metal without any known physiological

function, although a cadmium containing carbonic anhydrase has been described in diatoms (Xu et al. 2008). This ion induces complex changes in plants at the genetic, biochemical and physiological levels leading to phytotoxicity (Gratäo et al. 2005). Among the effects, Cd alters germinative metabolism (Mihoub et al. 2005; Rahoui et al. 2008; Smiri et al. 2009). However, although toxicity has been extensively studied, its biochemical mechanism of action has not yet been well established.

Respiration is one of the important events during the early stages of seed germination (Bewley 1997). One recognized explanation for the impact of Cd on seed germination is that it induces respiratory disturbances (Chugh and Sawhney 1996). In a previous work we have demonstrated that Cd decreases the germination rate, delays the embryonic axis growth and interferes with the enzyme activities of the Krebs cycle and electron transport chain in germinating pea seeds (Smiri et al. 2009). On these grounds, it was our aim to evaluate the effect of Cd on mitochondrial redox systems (Trx and Grx) in both cotyledon and embryonic axis in an attempt to elucidate a possible mechanism of action for Cd toxicity in germinating pea seed.

Materials and methods

Chemicals

DTNB, GR, Grx, GSH, NTR, NAD(P) and Trx were from Sigma-Aldrich Chemical Company. All other chemicals were of analytical grade.

Germination conditions

Seeds of pea (*Pisum sativum* L. cv. douce province) were disinfected with 2% sodium hypochlorite for 10 min and then rinsed thoroughly and soaked in distilled water at 4°C for 30 min to obtain an initial stage (Murray et al. 1979). Seeds were germinated at 25°C in the dark over two sheets of filter paper moistened with distilled water or aqueous solution of chloride salt of 5 mM Cd. At harvest, the coat was removed and the embryonic axis and cotyledons were weighed and stored in liquid nitrogen until analysis or dried after 8 days at 70°C for dry weight determination.

Preparation of mitochondria and extraction of proteins

Mitochondria and protein extracts were obtained as previously described (Smiri et al. 2009). Before bioassays, burst mitochondria were assayed for the intactness of the outer membrane (Smiri et al. 2009). Enzyme activities, proteins, thiols, GSH and coenzymes were determined in burst mitochondria extracts (resuspension in 0.3 M KCl, 0.2% Triton X-100 with three cycles of freezing/thawing).

Protein and thiol determinations

Protein concentrations were evaluated by the method of Bradford (1976), using bovine serum albumin as a standard. Total protein thiols were assayed according to the method of Ellman (1959).

Enzyme assays

The activities of GR (Foyer and Halliwell 1976), Grx (Rouhier et al. 2002), NTR and Trx (Jacquot et al. 1994), NADPH oxidase (EC 1.6.99.6) and NADH oxidase (EC 1.6.99.3) (Ishida et al. 1987) were measured spectrophotometrically in a total volume of 500 µl at 25°C. Each of the above assays was used only in a range in which the rate of reaction was proportional to the amount of extract added. The reactions were started by adding the enzyme extracts or substrates. Control assays were carried out without substrates or extracts.

Glutathione determination

Non-protein thiols were extracted by homogenizing the burst mitochondria in 5% (w/v) sulfosalicylate (pH < 1), containing 6.3 mM diethylene triamine pentaacetic acid. After centrifugation at $10000 \times g$ for 30 min at 4°C, the supernatants were used for analysis. Total GSH was determined in the homogenates spectrophotometrically at 412 nm, using yeast-GR, 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) and NADPH (Anderson 1985).

Coenzyme extraction and concentration determination

Reduced forms (NADPH and NADH) and oxidized ones ($NADP^+$ and NAD^+) were extracted from burst

mitochondria according to the method of Zhao et al. (1987). For the coenzymes quantification, enzyme cycling assays were employed as following the procedures described by Matsumura and Miyachi (1980).

Statistics

The experiments were repeated at least twice. The data presented here are from representative experiments. The mean values ± SE are reported in the tables and figures of this article. The significance of differences was determined at the 0.05 level of probability.

Results

Changes in protein and thiol contents in response to cadmium treatment

The data presented in Fig. 1 show that a 5 mM Cd treatment significantly affects the concentration of

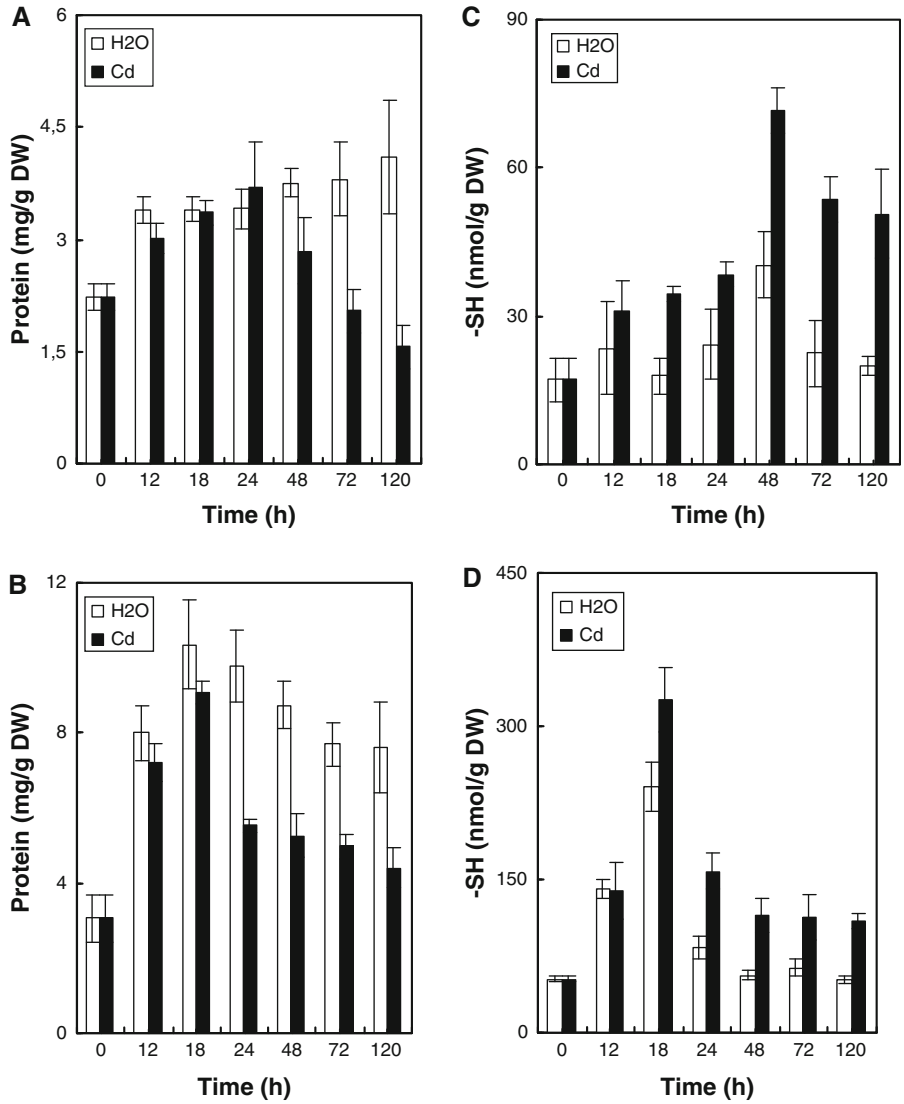

Fig. 1 Mitochondrial protein and –SH content in cotyledons (**a**, **c**) and embryonic axes (**b**, **d**) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds

mitochondrial soluble proteins in both cotyledons and embryo of germinating pea seed. After the beginning of imbibition with H_2O an increase in protein content was observed, while less than 38 and 58% of control protein contents were present in cotyledons (Fig. 1a) and embryo (Fig. 1b), respectively, after 5 days of Cd exposure. On the other hand, protein thiol content enhanced markedly in the mitochondria of Cd-treated seeds, as compared to the controls (twofold) (Fig. 1c, d). The following results led us to examine the relationships between mitochondrial thiol management and some enzymatic redox thiol-dependent systems in reserve tissues and growing embryonic axes during germination of Cd-poisoned pea seeds.

Effect of Cd on Grx system

When compared to the respective controls, cotyledons and embryo of Cd-treated germinating seeds showed a drastic inhibition of mitochondrial Grx and GR activities (Fig. 2), as well as a severe diminution of GSH content (Fig. 3). These data clearly show that as consequence of Cd stress there was no protective effect of Grx system on thiols in mitochondria.

Effect of Cd on Trx system

The actions of cadmium treatment on Trx and NTR activities, as well as total NAD(P) contents measured

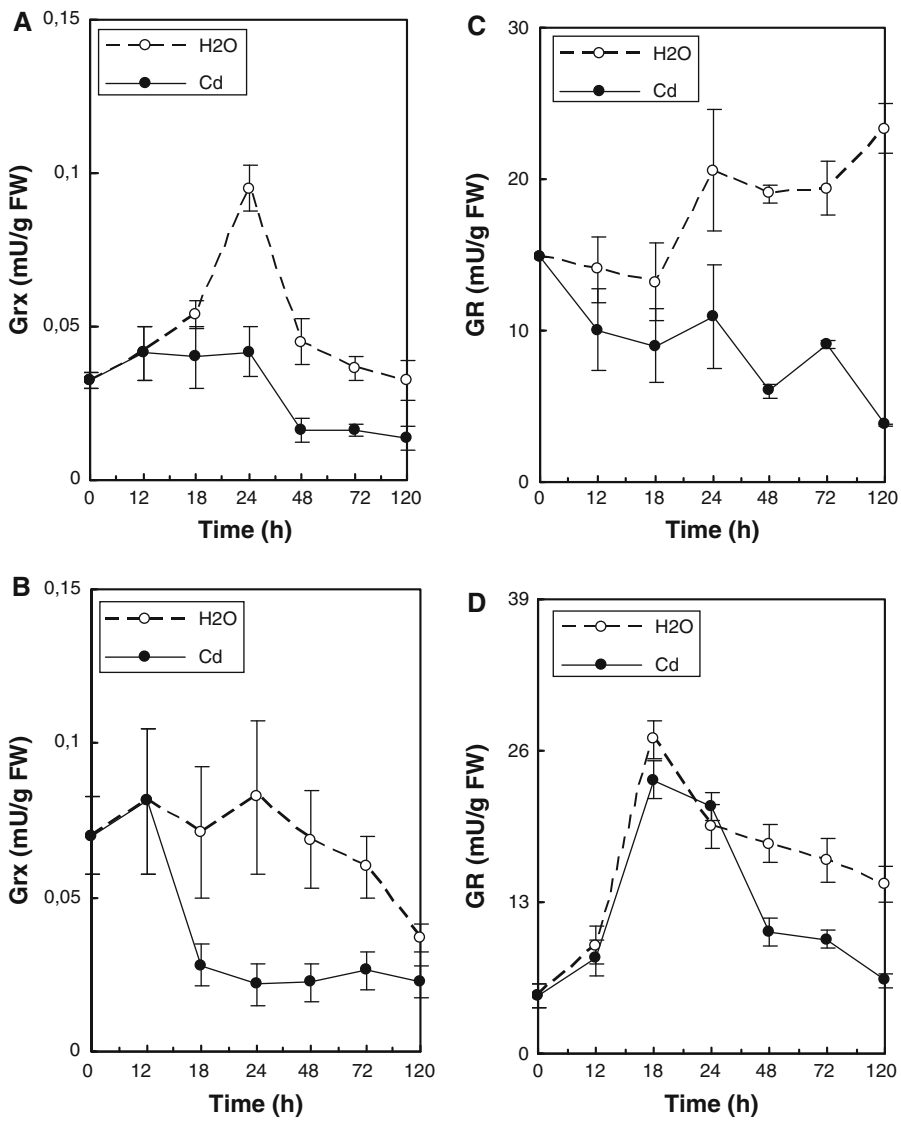

Fig. 2 Mitochondrial Grx and GR activities in cotyledons (**a**, **c**) and embryonic axes (**b**, **d**) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds

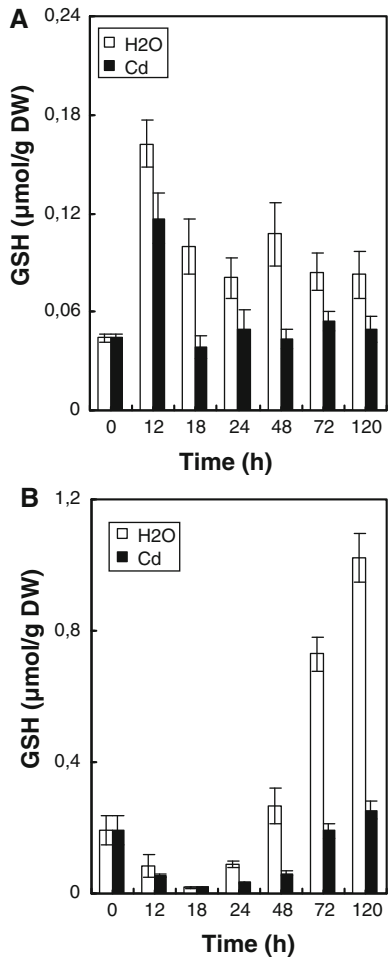

Fig. 3 Content of total mitochondrial glutathione in cotyledons (**a**) and embryonic axes (**b**) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds

in mitochondria of cotyledon and embryo tissues were rather different. As shown in Fig. 4, heavy metal stress stimulated the capacities of Trx and NTR in the cotyledons (Fig. 4a, c, respectively), but not in the embryo (Fig. 4b, d). Moreover, the impact of cadmium poisoning on the amounts of total nicotinamide (oxidized + reduced forms) differed depending on the tissue considered: increase in cotyledon (Fig. 5a, c) versus decrease in embryo (Fig. 5b, d) for both NADP and NAD, as compared to respective controls. To assess the possible involvement of coenzyme forms in the mitochondrial response to Cd stress, the calculation of NADP balance was performed as the percentage of NADP from total nicotinamide (NADP + NAD) (Table 1). It seems that both NADP and NAD can be implicated in redox reaction of mitochondria, since no significant modification was recorded in the tissues under stress conditions as compared to the controls (Table 1).

Enzymatic oxidation of NAD(P)H after Cd exposure

The effects of cadmium treatment on redox ratio of coenzymes (oxidized to reduced forms) and NAD(P)H oxidase activities are shown in Table 2 and Fig. 6. Cadmium caused a significant consumption of reduced nicotinamide, as evidenced by the increase in $NAD(P)^+$/NAD(P)H ratio (Table 2), probably by the stimulation of enzymatic oxidation via NAD(P)H oxidase activities (Fig. 6).

Discussion

Certain heavy metals, such as Cu or Fe, can be toxic through their participation in Fenton-type reactions producing reactive oxygen species, which are known to be extremely harmful for living cells (Stochs and Bagchi 1995). However, Cd is a non-redox metal unable to take part in these kind of reactions. Nevertheless, it has been clearly demonstrated that Cd induces changes in the antioxidative status in plants (Chaoui et al. 1997, 2004; Chaoui and El Ferjani 2005; Gratão et al. 2005). Moreover, previous study has demonstrated that Cd inhibited several mitochondrial enzyme activities and, consequently, delayed the resumption of respiration in germinating pea seeds (Smiri et al. 2009). Here, we demonstrated that the alterations in mitochondrial redox status can also interfere with the deleterious effects of cadmium stress.

The plant mitochondrial proteome contains subunits of respiratory complexes and supercomplexes (Millar et al. 2005) which are quite inhibited by Cd in germinating pea seeds (Smiri et al. 2009) probably via proteolytic degradation or by a failure in synthesis, as evidenced by the diminution of protein content. However, the thiol status of soluble proteins was not affected by Cd in the same manner. In fact, Cd stress seems to increase the reduced –SH pool, at least under the experimental conditions described here. The

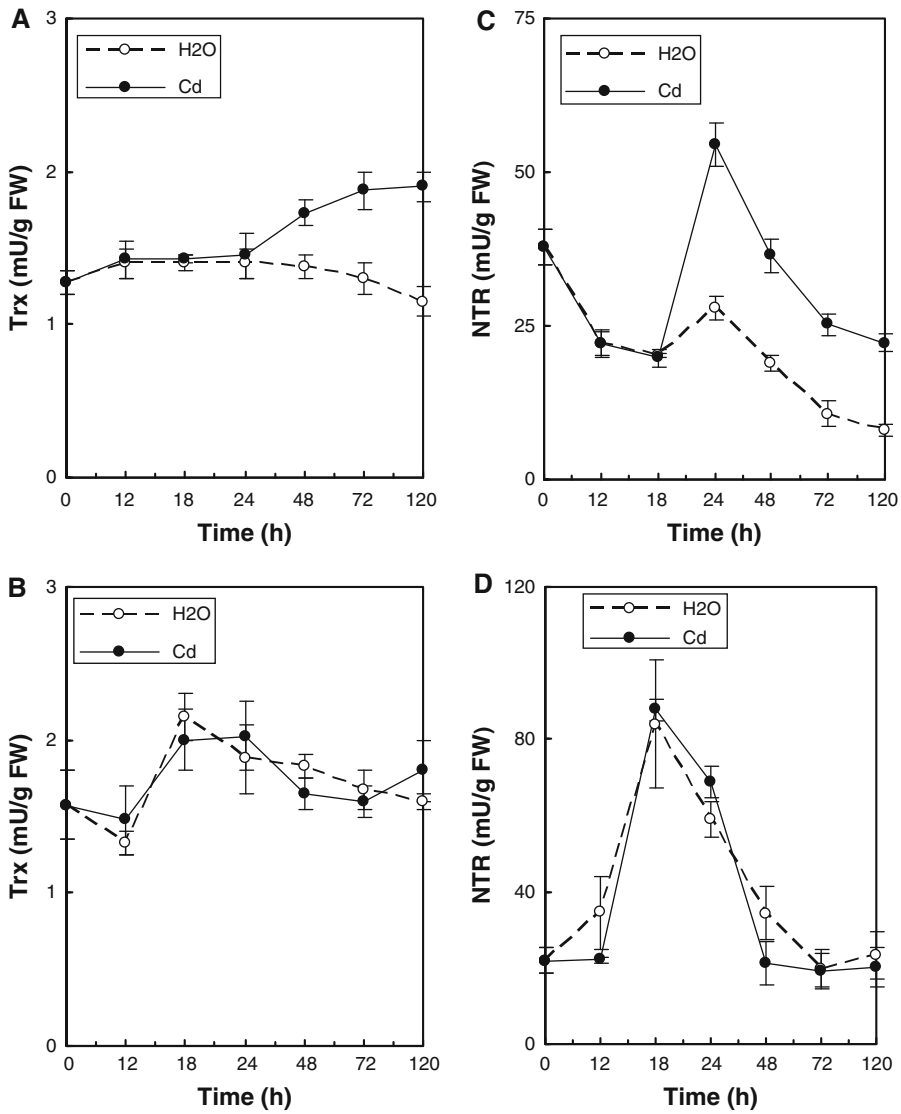

Fig. 4 Mitochondrial Trx and NTR activities in cotyledons (**a**, **c**) and embryonic axes (**b**, **d**) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds

mitochondrial Grx system appears to play a fundamental role in controlling redox status in animals and plants subjected to abiotic stress as Cd (Diotte et al. 2009; Schwarzländer et al. 2009). Nevertheless, cytosol/mitochondrion exchanges should be considered as an important aspect of the redox state of mitochondria in Cd treated plants. During the recent years evidence has accumulated on the existence of a regulatory network that modulates gene expression in response to Cd; one of the main response observed after analyses of transcriptome profile of Cd treated *A. thaliana* is the induction of genes involved in GSH metabolism (Weber et al. 2006). Although restricting GSH biosynthesis, mostly in the cytosol, is sufficient for normal development (Pasternak et al. 2008), plants activate the sulphur assimilation pathway to provide an enhanced supply of GSH for phytochelatin biosynthesis. It is well established that defence against Cd is mostly based on GSH: Cd/GSH complex, phytochelatin precursor (Cobbett and Goldsborough 2002), reductant in the chloroplastic ascorbate-GSH cycle (Chaoui et al. 1997; Chaoui and El Ferjani 2005). Anyway, in germinating pea seeds, no thiol protective effect versus Cd stress was seen with the mitochondrial Grx system. In fact, Grx and GR activities and GSH levels were drastically depressed in both cotyledons and the embryo. In the latter, similar results were found concerning Trx system, since Trx and

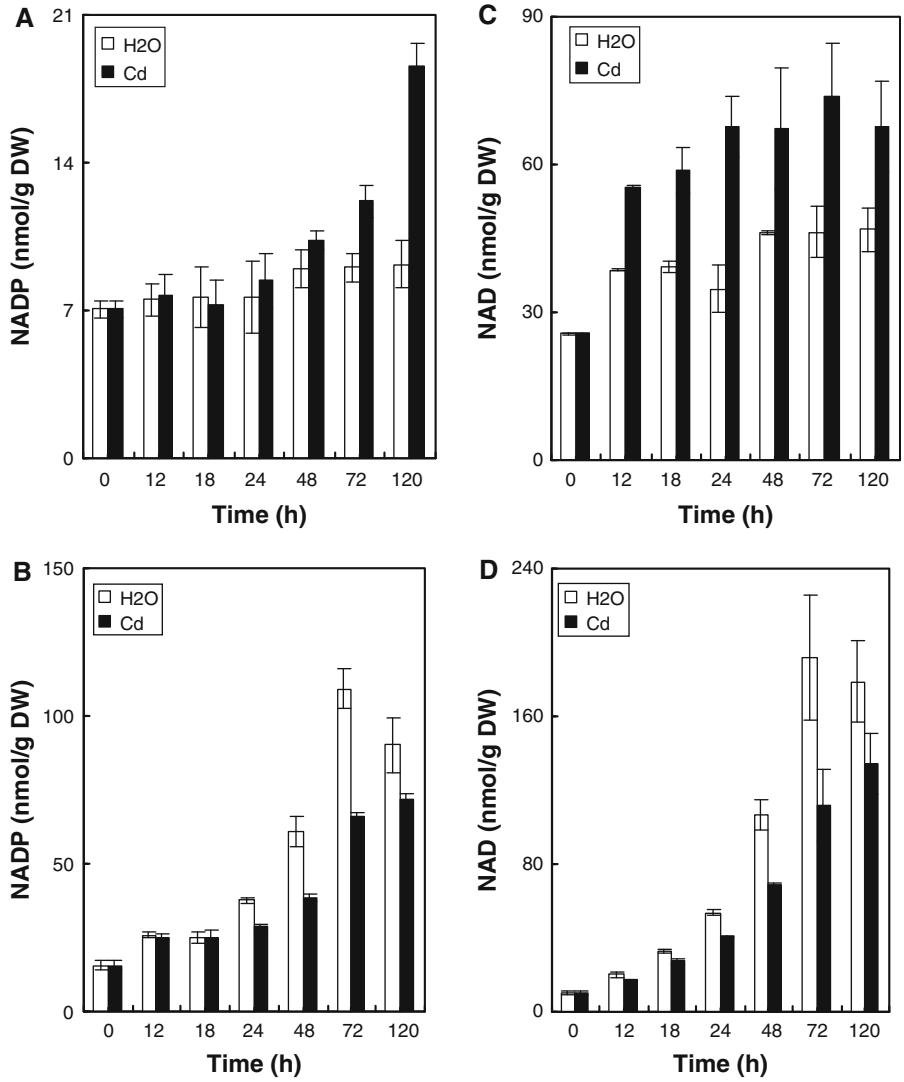

Fig. 5 Content of total mitochondrial nicotinamide (reduced + oxidized forms) in cotyledons (**a**, **c**) and embryonic axes (**b**, **d**) of pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds

NTR activities were not modified, while total NAD(P) contents diminished in the Cd treatment. Mitochondria contain Trx, a regulatory disulfide protein, and an associated flavoenzyme, NTR, which provide a link to NADPH in the organelle (Balmer et al. 2004; Reichheld et al. 2005). Unlike animal and yeast counterparts, the function of Trx in plant mitochondria is largely unknown. Metals are also good inhibitor of NTR in rat liver mitochondria (Bragadin et al. 2004), perhaps via an oxidation phenomenon, since the human mitochondrial Trx can be oxidized in response to inducers of oxidative stress; yet the functional consequences of the oxidation have not been determined (Chen et al. 2006). Moreover, the tolerance to heavy metal toxicity depends on the reducing power that can be supplied via secondary NAD(P)H recycling dehydrogenase activities (Mattioni et al. 1997) which are markedly inhibited in embryonic axes of Cd-poisoned pea seeds (Smiri et al. 2009).

Thus, we suggest that neither Grx system nor Trx one may improve the redox status of mitochondrial thiols in the embryo of germinating pea seed exposed

Table 1 Effect of Cd on mitochondrial NADP balance in germinating pea seeds

Time (h) Tissues	Treatments	NADP balance (NADP:NADP +NAD)						
		0	12	18	24	48	72	120
Cotyledons	H_2O	21 ± 1	16 ± 1	16 ± 3	17 ± 4	16 ± 1	16 ± 1	16 ± 1
	Cd		12 ± 1	11 ± 1	11 ± 1	13 ± 1	14 ± 1	21 ± 1
Embryonic axes	H_2O	60 ± 5	56 ± 1	43 ± 2	41 ± 1	36 ± 2	36 ± 2	33 ± 3
	Cd		59 ± 2	48 ± 4	41 ± 1	35 ± 1	37 ± 1	34 ± 1

NADP balance was evaluated as the percentage of NADP from total coenzymes (NADP + NAD)

Data are the means ± SE of 12 individual measurements. Each measurement was performed in an extract obtained from several germinating seeds. For each tissue, no significance of differences between control and Cd treatment values was recorded at the 0.05 level of probability

Table 2 Effect of Cd on redox ratio of mitochondrial coenzymes in germinating pea seeds

Time (h) Tissues	Redox ratio	Treatments	0	12	18	24	48	72	120
Cotyledons	NADP + /NADPH	H_2O	16 ± 1	11 ± 1	11 ± 1	9 ± 1	6 ± 1	6 ± 1	6 ± 1
		Cd		11 ± 1	12 ± 2	9 ± 1	10 ± 1*	14 ± 1*	20 ± 1*
	NAD + /NADH	H_2O	111 ± 1	60 ± 3	51 ± 2	35 ± 3	39 ± 1	35 ± 3	28 ± 2
		Cd		72 ± 3	73 ± 4*	75 ± 7*	65 ± 6*	56 ± 5*	44 ± 4*
Embryonic axes	NADP + /NADPH	H_2O	11 ± 1	10 ± 1	8 ± 1	8 ± 1	8 ± 1	7 ± 1	5 ± 1
		Cd		10 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1	8 ± 1*
	NAD + /NADH	H_2O	3 ± 1	4 ± 1	8 ± 1	11 ± 1	11 ± 1	11 ± 2	9 ± 1
		Cd		7 ± 1	17 ± 1*	33 ± 1*	60 ± 7*	28 ± 4*	19 ± 2*

Data are the means ± SE of 12 individual measurements. Each measurement was performed in an extract obtained from several germinating seeds. Values followed by an asterisk are significantly different from the respective controls at the 0.05 level of probability

to Cd toxicity, but in the cotyledons the contribution of Trx/NTR/NADPH can be established in despite the vulnerability of the coenzyme pools due to enzymatic oxidation. In fact, the levels of all components of Trx system were elevated in Cd-poisoned cotyledons. A stimulation in gene expression of some Trx variants in response to heavy metals (Lemaire et al. 1999) as well as to other abiotic stress has been reported (Pagano et al. 2000). Concerning the coenzyme forms, it appears that both NADP and NAD can be implicated in the mitochondrial response to Cd stress, since Cd provoked no significant change in NADP balance (determined as the percentage of NADP from total coenzymes: NADP + NAD). In general, the NAD(P) balance is kept constant in plant mitochondria under stress conditions (Muto et al. 1981; Kasimova et al. 2006).

Moreover, Cd treatment caused a decrease in the reduced forms of nicotinamide, as evidenced by the enhancement of redox ratio (presented as $NAD(P)^+$/NAD(P)H). This suggests the comsuption of NAD(P)H via NTR activity in Cd-treated cotyledons. However, Cd also stimulated $NAD(P)^+$/NAD(P)H ratio in embryonic axes where the disulfide reductase capacity of Trx was not modified by stress. Thus, a second assumption is that the increase in redox ratio of nicotinamide would be a consequence of an oxidation reaction imposed by Cd. In fact, NAD(P)H oxidase activities were strongly stimulated after Cd exposure. Similar results have been obtained with other tissues in adult pea (Chaoui et al. 2004; Chaoui and El Ferjani 2005).

In conclusion, these results indicate that cadmium induces differential redox responses on different seed tissues, which include increase in the protein thiol level, induction of Trx system, at least in cotyledon, and inhibition of the Grx one. The generation of an intracellular oxidative stress after Cd treatment can result in alteration in coenzyme pattern.

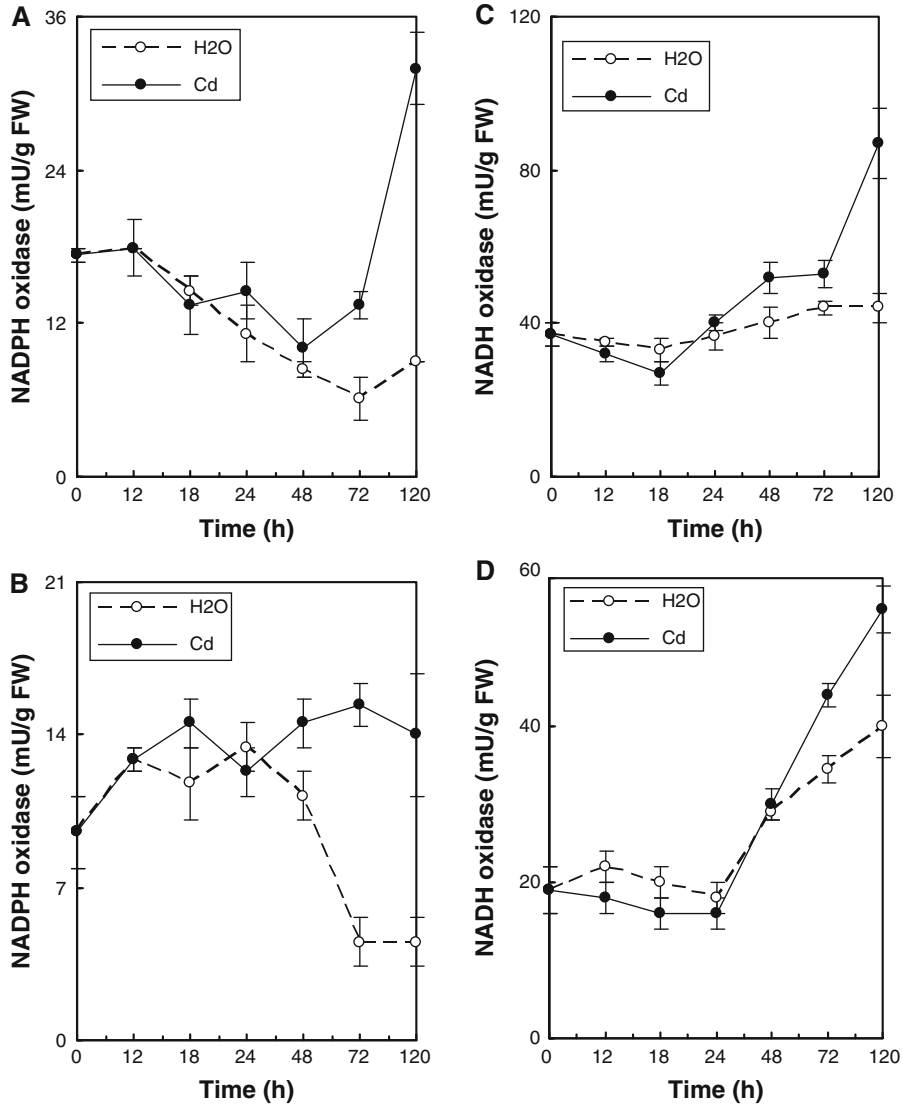

Fig. 6 Mitochondrial NADPH oxidase (**a**, cotyledons; **b**, embryonic axes) and NADH oxidase (**c**, cotyledons; **d**, embryonic axes) activities in pea seeds during germination after imbibition with H_2O or 5 mM Cd. Values are the averages of six individual measurements (±SE). Each measurement was performed in an extract obtained from several germinating seeds

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