Rapid alteration of cellular redox homeostasis upon exposure to cadmium and mercury in alfalfa seedlings

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Summary

• Here, the kinetics of oxidative stress responses of alfalfa (*Medicago sativa*) seedlings to cadmium (Cd) and mercury (Hg) (0, 3, 10 and 30 μM) exposure, expanding from a few minutes to 24 h, were studied.
• Intracellular oxidative stress was analysed using 2′,7′-dichlorofluorescin diacetate and extracellular hydrogen peroxide (H_2O_2) production was studied with Amplex Red. Growth inhibition, concentrations of ascorbate, glutathione (GSH), homoglutathione (hGSH), Cd and Hg, ascorbate peroxidase (APX) activity, and expression of genes related to GSH metabolism were also determined.
• Both Cd and Hg increased cellular reactive oxygen species (ROS) production and extracellular H_2O_2 formation, but in different ways. The increase was mild and slow with Cd, but more rapid and transient with Hg. Hg treatments also caused a higher cell death rate, significant oxidation of hGSH, as well as increased APX activity and transient overexpression of *glutathione reductase 2*, *glutamylcysteinyl synthetase*, and *homoglutathione synthetase* genes. However, Cd caused minor alterations. Hg accumulation was one order of magnitude higher than Cd accumulation.
• The different kinetics of early physiological responses *in vivo* to Cd and Hg might be relevant to the characterization of their mechanisms of toxicity. Thus, high accumulation of Hg might explain the metabolism poisoning observed in Hg-treated seedlings.

Key words: cadmium, GSH|hGSH, fluorescence, *Medicago sativa* (alfalfa), mercury, microscopy, oxidative stress.

Introduction

Accumulation of toxic metals, such as cadmium (Cd) and mercury (Hg), as a result of several anthropogenic activities poses an important risk to many environments (Gratão *et al.*, 2005). Plants take up metals (Gratão *et al.*, 2005), and this ability is exploited to clean up metal-polluted environments, using technologies known as phytoremediation (Lynch & Moffat, 2005). To improve the implementation of such technologies, a big effort is under way to understand the cellular mechanisms of tolerance to toxic metals (Clemens *et al.*, 2002). Once metals are taken up, many cellular structures and metabolic processes are affected, and the accumulation of reactive oxygen species (ROS) is one of the characteristic symptoms (Gratão *et al.*, 2005).

Formation of ROS, such as $O_2^{\cdot-}$, $^{\cdot}OH$ and H_2O_2, is intrinsic to the metabolism of aerobic organisms. Plants have evolved a complex antioxidant network to maintain ROS at a

steady-state level when they are subjected to hazardous environmental conditions (Mittler *et al.*, 2004). When the redox cellular status is compromised, metabolites such as ascorbate (AA) and glutathione (GSH), and antioxidant enzymes such as superoxide dismutases (SODs), ascorbate peroxidases (APXs) and catalases (CATs), help to scavenge excess ROS (Foyer & Noctor, 2005). For a long time, $O_2^{\bullet-}$ and H_2O_2 were considered to be dangerous species, but this notion is rapidly changing in the light of new regulatory functions in cell signalling cascades described recently (Pastori & Foyer, 2002). An imbalance between ROS production and scavenging leads to cellular damage, as observed in several plant species (Lozano-Rodríguez *et al.*, 1997; Cho & Park, 2000).

The balances between GSH and oxidized GSH (GSSG), and/or their homologues reduced/oxidized homoglutathione (hGSH/hGSSGh), as well as AA and its oxidized forms (monodehydroascorbate and dehydroascorbate), are crucial for the activity of ROS-scavenging enzymes (Noctor *et al.*, 2002). Heavy metals may cause alterations in the pools of these redox metabolites and in the activity of ROS-scavenging enzymes, although the responses found are in some instances rather contradictory: Xiang & Oliver (1998) reported a depletion of GSH, with a subsequent rise in GSSG with Cd treatments. However, Freeman *et al.* (2004) observed a higher GSH concentration, which was explained on the basis of a higher GSH demand for the synthesis of phytochelatins, of which it is a precursor. Indeed, it was suggested that heavy metal resistance is dependent on GSH concentration and the capacity to synthesize new thiols (Xiang *et al.*, 2001). Transgenic plants overaccumulating GSH (Xiang *et al.*, 2001; Li *et al.*, 2006), or with increased GSH recycling capacity via the overexpression of glutathione reductase (GR; Pilon-Smits *et al.*, 2000), exhibited enhanced tolerance to heavy metals. In contrast, the response to heavy metals of enzymes involved in attenuation of ROS (SOD, APX, CAT or GR) greatly depends on the species, plant age and culture conditions (Schützendübel & Polle, 2002; Gratão *et al.*, 2005). Nevertheless, in most cases oxidative stress symptoms generally appear when treatments are long enough to produce extensive cell damage, most probably causing general metabolism failure. Thus, to study early physiological responses, Sanitá di Toppi & Gabbrielli (1999) encouraged the use of realistic metal doses (usually found in metal-contaminated sites) or the treatment of plants for short periods.

The mechanisms underlying the metal-induced oxidative stress responses remain to be elucidated (Hall, 2002; Gratão *et al.*, 2005). Little is known about the early effects of metal treatments, mainly because of the difficulty of detecting ROS accumulation or oxidative damage at the cellular level. One approach has been to use tobacco (*Nicotiana tabacum*) BY-2 cells subjected to several doses of Cd^{2+}. The onset of oxidative stress was analysed in these cells using highly sensitive fluorescent probes that react with H_2O_2, such as oxidative quenching of pyranine (Olmos *et al.*, 2003) or reaction with Amplex Red (Garnier *et al.*, 2006). In these studies, it was shown that a plasma membrane-bound NADPH oxidase might be involved in the mechanism of ROS generation under Cd exposure. This analysis was carried out after preincubating the BY-2 cells with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI). However, very high concentrations of Cd^{2+} (above 1 mM), far in excess of the concentrations found in polluted soils, were used.

Recently, we have successfully used the fluorescent probe 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) to visualize oxidative stress in intact alfalfa (*Medicago sativa*) seedlings treated with moderate concentrations of Cd or Hg, from 3 to 30 μM (Ortega-Villasante *et al.*, 2005). In addition, we estimated the cellular concentrations of GSH and hGSH *in vivo* using monochlorobimane (MCB), which forms a GSH|hGSH-MCB fluorescent adduct, and the amount of cellular death using propidium iodide (PI). In summary, our previous results showed that plants treated with Cd and Hg exhibited several stress responses: depletion of GSH|hGSH, enhancement of oxidative stress, and an increase in the amount of cell death. Interestingly, at the same doses Hg produced stronger toxic effects than Cd, with greater increases in cell necrosis and GSH|hGSH depletion being observed. These effects were detected after 6 h of exposure, and in some cases were similar to the effects found after 24 h of treatment (Ortega-Villasante *et al.*, 2005). These results encouraged us to explore the detection of very early stress responses by shortening the metal exposure time to a few minutes.

Tolerance to environmental stresses should depend on those factors that act at early stages in a signal transduction network. The aim of the present work was to assess the kinetics of short-term responses in alfalfa seedlings to Cd and Hg from minutes to 24 h. The kinetic analysis included measurement of the content of antioxidant metabolites (GSH/GSSG, hGSH/hGSSGh and AA), activities of ROS-scavenging enzymes (SOD and APX), the transcription of genes related to GSH|hGSH metabolism (γ-glutamylcysteine synthetase (*ECS*), glutathione synthetase (*GS*), homoglutathione synthetase (*hGS*), glutathione reductase 1 (*GR1*), glutathione reductase 2 (*GR2*), glutathione peroxidase (*GPX*), and phytochelatin synthase (*PCS*)), and metal tissue accumulation.

Materials and Methods

Chemicals

All dyes were purchased from Molecular Probes (Carlsbad, CA, USA), and stocks were prepared for single use. Stocks of 100 mM MCB, 100 mM H_2DCFDA, and 20 mM Amplex Red in DMSO were stored at –20 C. PI (1 mg ml⁻¹) was stored at –20 C in deionized water. DPI (20 mM) was stored at room temperature in DMSO. A stock solution of 100 mM L-buthionine sulphoximine (BSO; Sigma, St Louis, MO, USA) was prepared in deionized water and stored at –20 C. $CdSO_4$ and $HgCl_2$ were analytical grade, purchased from Merck (Darmstadt, Germany).

Plant material and hydroponic system

Seeds of alfalfa (*Medicago sativa* L. var. Aragon) were cultured after Ortega-Villasante *et al.* (2005). Briefly, seedlings were grown for 24 h on 1.5% (weight/volume (w/v)) agarose in complete darkness at 28 C, and then were transferred to a Fåhraeus-slide hydroponic system, where they were kept immersed in a Murashige–Skoog (MS) nutrient solution. After 24 h of acclimatization in conditions of 16 : 8 h light:dark at 28 : 18 C, seedlings were treated with Cd, Hg, or BSO (see each experiment), and were collected at different times for a maximum of 24 h. Before analyses, seedling length was measured and the relative growth inhibition was calculated.

Extracellular H_2O_2

To analyse immediate H_2O_2 release upon Cd or Hg treatment, an assay was designed, based on Garnier *et al.* (2006), which allows the detection of extracellular H_2O_2 in root segments. Amplex Red is oxidized to the fluorescent derivative resorufin, in a reaction catalysed by peroxidases. Root segments of untreated seedlings (1 cm) were cut, equilibrated in MS medium titrated with 2 mM 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6.0 in the dark for 1 h, and placed individually in a 96-well microtitre plate filled with the same medium (200 μl) supplemented with different concentrations of Cd, Hg or BSO, as described in the corresponding experiments. To assess the involvement of NADPH oxidase in the oxidative burst, root segments were preincubated for 15 min in the presence of 5 μM DPI. Amplex Red was supplied to a final concentration of 50 μM immediately before the measurement of fluorescence, which was quantified in a titre plate reader (Spectrafluor Tecan; Salzburg, Austria) at excitation wavelength (λ_{exc}) = 542 nm and emission wavelength (λ_{em}) = 590 nm. Data were collected every 5 min for 90 min.

Dye loading and confocal laser scanning microscopy

The amounts of cellular oxidative stress (staining with H_2DCFDA), GSH|hGSH (fluorescent adduct with MCB), and necrosis (labelling with PI) were determined using a confocal microscope (TCS SP2; Leica, Wetzlar, Germany) as described by Ortega-Villasante *et al.* (2005). Immediate ROS production resulting from Cd and Hg exposure was analysed by preincubating untreated seedlings with 10 μM H_2DCFDA for 15 min, and subsequently exposing them to 30 μM Cd or Hg. Fluorescence was recorded every 5 min for up to 120 min, and at the end of the observations cell death was monitored after counterstaining with 25 μM PI. In parallel, a second batch of seedlings was labelled with MCB after 120 min of exposure to the metals. Microscopy images are representative observations from at least five independent experiments. Fluorescence was quantified as average pixel density in the defined regions of interest, and corrected against the background signal with the microscope software, according to the manufacturer's recommendations.

Analysis by high-performance liquid chromatography–electrospray–time of flight mass spectrometry (HPLC-ESI-TOFMS) of GSH, GSSG and AA

GSH/GSSG, hGSH/hGSSGh and AA were determined by HPLC coupled to ESI-TOFMS as described by Rellán-Álvarez *et al.* (2006b).

APX and SOD activities in nondenaturing gels and western blots

APX (EC 1.11.1.11) and SOD (EC 1.15.1.1) activities were detected after nondenaturing gel electrophoresis, and APX was immunodetected by western blot following the procedures described by Rellán-Álvarez *et al.* (2006a).

RNA extraction and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted using a LiCl precipitation procedure (Sambrook & Russell, 2001), with minor modifications, treated with RNAse-free DNAse (Roche, Basel, Switzerland) and stored until use at –80 C. Total RNA (1 μg) was used to synthesize the complementary DNA strand (cDNA). The reverse transcription reaction was performed with random hexamers, using the First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's recommendations. PCR was carried out with 1 μl of single-stranded cDNA in a final volume of 25 μl, containing the following mixture: PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM of both forward and reverse specific primers and 1 U *Taq* polymerase (GE Healthcare, Uppsala, Sweden). The programme used in the thermocycler was: initial denaturation (94 C, 3 min); several cycles of denaturation (94 C, 30 s), annealing (56 C, 30 s) and extension (72 C, 1 min); and a final extension step (72 C, 5 min). The total number of cycles was determined empirically for each set of primers, as shown in Table 1. Since few sequences of *M. sativa* are available in databases, heterologous oligonucleotide primers (Table 1) were designed based on the sequences of *Medicago truncatula* (available at www.medicago.org and www.tigr.org). After agarose electrophoresis of the amplified PCR products, band intensity from ethidium bromide staining was analysed with Kodak 1D IMAGE ANALYSIS SOFTWARE version 3.6 (Eastman-Kodak, Rochester, NY, USA). Transcript abundance was normalized against the amplification of 18S rRNA (QuantumRNA Universal 18S; Ambion, Austin, TX, USA). The identity of the expected cDNA fragments amplified by PCR using the above-mentioned oligonucleotide primers was confirmed by automated sequencing, and their accession numbers are given in Table 1.

Table 1 Primers designed for *Medicago truncatula* to amplify several cDNA fragments

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Cycles	Accession number
ECS	gctgccttttgtttttgatgac	gcagctcacaagagagataacc	756	34	AM407888
GR1	aacgtatcacacttcttctcc	caactgtgtcacctcaacttc	610	35	AM407889
GR2	tacttcactttccctctcttgttc	ctgaaactaaaatgtgtttg	635	36	AM407890
GPX	tggttccatggcttctttc	caatatccttacagtaaaatc	261	38	AM407891
PCS	ctagtctataccggagaaccc	ccatctccaaggacctttcc	240	35	AM407892
hGS	aagaaggagaaattctxcgag	aatgaagggccgacctgg	803	40	AM411122
GS	aagaaggagaaattctxcgag	ggaggctcaaatggtaatgtg	755	36	AM411123

Also shown is the length of the fragments amplified, the optimal number of cycles to obtain a linear response of amplification and the accession number of the sequenced fragments.
ECS, γ-glutamylcysteinyl synthetase; *hGS*, homoglutathione synthetase; *GPX*, glutathione peroxidase; *GR1*, glutathione reductase 1; *GR2*, glutathione reductase 2; *GS*, glutathione synthetase; *PCS*, phytochelatin synthase.

Cd and Hg tissue concentrations

Whole seedlings were collected, washed twice in deionized water, rinsed briefly in 10 mM sodium ethylenediaminetetraacetic acid (Na_2EDTA), weighed and dried for 72 h at 60 C. Dried plant material was submerged in 2 ml of the acidic oxidative mixture (HNO_3:H_2O_2:H_2O; 0.6 : 0.4 : 1; v/v), and autoclave digested (120 C, 1.5 atmospheres, 30 min). Once cooled to room temperature, the digests were filtered through a polyvinylidenefluoride (PVDF) filter and diluted in water to 5 ml. The Cd concentration was determined by graphite furnace atomic absorption spectrophotometry (AAnalyst 800; Perkin Elmer, Walthum, MA, USA). Hg was analysed using a flux injection absorption spectrometer (1100B; Perkin Elmer), equipped with the cold-vapour generator FIAS (400: Perkin Elmer).

Statistical analysis

Data shown are mean ± standard deviation (SD) for at least five independent experiments. Mean differences were compared using Duncan's multiple range test with SPSS for Windows (Release 13.0; SPSS Inc., Chicago, IL, USA).

Results

Immediate induction of oxidative stress

To study the earliest oxidative stress responses of alfalfa seedlings to Cd and Hg, we monitored the production of extracellular H_2O_2 and intracellular ROS using fluorescence probes immediately after addition of the toxic metals. In alfalfa root segments, Cd and Hg triggered the formation of extracellular H_2O_2. The accumulation was concentration dependent and much higher with Hg than with Cd (Fig. 1). Thus, fluorescence slightly increased with 3 μM Hg (20%), whereas 10 and 30 μM Hg increased fluorescence by > 40% and 55%, respectively (Fig. 1). In contrast, even 10 and 30 μM

Cd produced only a slight continuous increase in fluorescence, showing a maximum 20% increase with respect to the controls (Fig. 1). Production of extracellular H_2O_2 was also analysed in untreated roots challenged with BSO, a potent inhibitor of GSH|hGSH synthesis, which is also known to enhance oxidative stress. The addition of BSO produced similar fluorescence values to those obtained for 30 μM Cd (Fig. 1).

Fluorescence changes were compared in seedlings treated with 30 μM Cd or Hg in the absence or presence of DPI, an inhibitor of NADPH oxidase activity. As shown in Fig. 1, H_2O_2 formation was low in control samples, and no significant changes were observed with DPI. In root segments treated with 30 μM Cd, fluorescence increased slightly, whereas 30 μM Hg greatly increased extracellular H_2O_2, in agreement with the results shown in Fig. 1. Preincubation with DPI decreased H_2O_2 concentrations below those of controls in roots treated with 30 μM Cd, but hardly affected the H_2O_2 produced by Hg (Fig. 1), suggesting different mechanisms of cellular toxicity for the two metals.

The greater induction effect of Hg at very short times of exposure was in contrast to our previous results obtained at 6 and 24 h (Ortega-Villasante *et al.*, 2005), where Hg produced weaker oxidative stress induction, even below that of controls, as visualized in epidermal cells by the fluorescence of oxidized H_2DCFDA. To verify the oxidative stress responses (Fig. 1), new analyses of cellular ROS production *in vivo* were performed by monitoring oxidized H_2DCFDA fluorescence from a few minutes to 120 min. The images obtained showing the immediate oxidative stress responses of *M. sativa* epidermal root cells to 30 μM Cd or Hg are shown in Fig. 2 and the corresponding films (Supplementary Material). In controls, green fluorescence caused by H_2DCFDA oxidation was restricted to a few cells that might have suffered mechanical damage as a result of manipulation (Fig. 2, Supplementary Material Film S1). However, seedlings exposed to 30 μM Cd exhibited a fluorescence increase that persisted until the observation finished (120 min; Fig. 2b, Supplementary Material Film S2). By contrast, the 30 μM Hg treatment led to a quick,
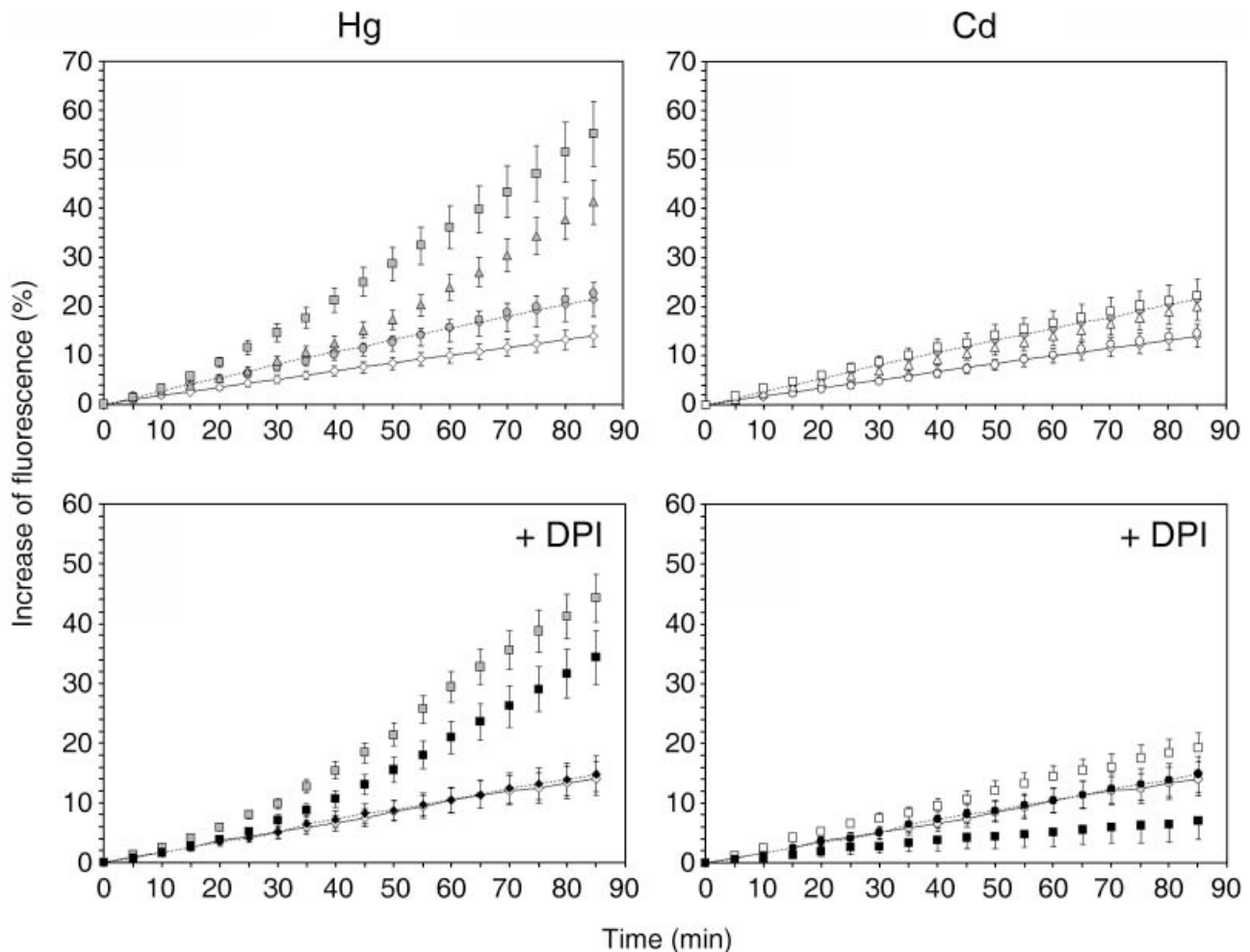
Fig. 1 Kinetics of extracellular hydrogen peroxide (H_2O_2) production in *Medicago sativa* root segments exposed to different cadmium (Cd) and mercury (Hg) concentrations, and the effect of the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI). The **c**oncentrations (μM) of the two metals used were 0 (empty rhombus), 3 (circle), 10 (triangle) and 30 (square). Also, 1 mM L-buthionine sulphoximine (BSO) (grey rhombus) was assessed to determine the effect of reductions in the cellular glutathione (GSH) pool. The involvement of NADPH oxidase in the oxidative burst triggered by both toxic metals (30 μM) was evaluated by adding 5 μM of the inhibitor DPI to the corresponding metal doses (closed symbols). The fluorescence increase shown is relative to time 0 and error bars represent the maximum standard error.

abrupt rise in oxidative stress, followed by sharp fluorescence quenching (Fig. 2c, Supplementary Material Film S3). PI counterstaining at the end of the observation revealed few necrotic cells in 30 μM Cd-treated seedlings (Fig. 2), but many more in 30 μM Hg-treated seedlings (Fig. 2). Quantification of fluorescence intensity confirmed the different phytotoxic behaviours of the two metals: a continuous increase with 30 μM Cd (Fig. 2), and a sharp and transient increase with 30 μM Hg (Fig. 2). An interesting observation of the oxidative stress induction process was the scattered spatial pattern of oxidized H_2DCFDA staining. Epidermal cells became progressively and heterogeneously affected as metal exposure was extended, probably as a result of physiological status changes.

The characterization of early cellular responses upon exposure to toxic elements was completed with the analysis of GSH|hGSH at the cellular level using MCB staining after

120 min of exposure to 30 μM Cd or Hg. Subtle differences between the two treatments were found; root epidermal cells of seedlings exposed to 30 μM Cd showed similar MCB-GSH|hGSH fluorescence to controls (Supplementary Material Figs S1a,b). However, with 30 μM Hg there was a significant depletion of the cellular pool of GSH|hGSH (Supplementary Material Figs S1c,d). Counterstaining with PI showed more cell death in Hg-treated roots, as evidenced by the relative intensity of PI fluorescence against the background fluorescence of MCB (Supplementary Material Fig. S1d).

Kinetic analyses of physiological responses to Cd and Hg

To further study physiological responses to Cd and Hg that might be related to the onset of oxidative stress during the
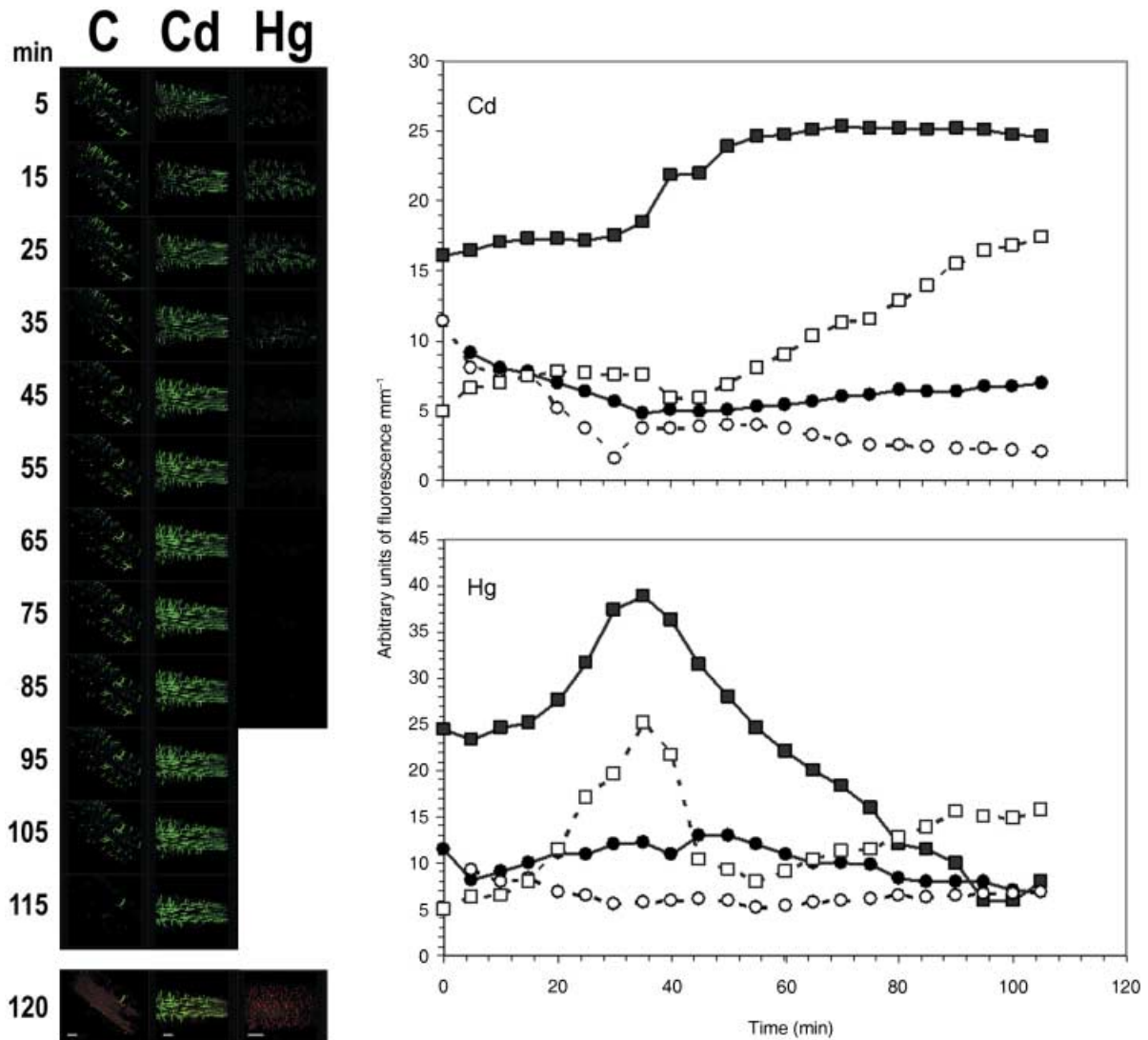
Fig. 2 Confocal imaging of *Medicago sativa* root cells exposed to heavy metals and stained with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA). Time–courses are shown for control roots (C), 30 μM cadmium (Cd) and 30 μM mercury (Hg). Images were collected at 10-min intervals, after a 5-min lag phase. A final image was obtained after 120 min with propidium iodide (PI) counterstaining to show necrotic cells. Bar, 200 μm. Analysis of changes with time of fluorescence intensity, measured using H_2DCFDA, in root epidermal cells of *M. sativa* exposed to control (MS growth media; circles) and 30 μM of Cd or Hg (squares). The analysis was performed in parallel with two different roots for each treatment (open or closed symbols). Results are representative of the trend observed in five independent experiments.

short time interval already described, a new experiment was designed. Alfalfa seedlings were incubated with 30 μM Cd or Hg for different periods, from 15 min to 24 h, and physiological parameters were assessed in parallel to the analysis of extracellular H_2O_2.

A very sensitive symptom of metal stress is the inhibition of growth (Ortega-Villasante *et al.*, 2005). This parameter increased with metal exposure time, being lower for Cd than for Hg after 24 h (12% and 29%, respectively; Fig. 3a), suggesting higher phytotoxicity of Hg. Regarding extracellular H_2O_2, as the incubation with toxic metals was prolonged, Amplex Red fluorescence increased (Fig. 3b). Treatment with 30 μM Cd

caused a modest increase up to 45 min, whereas in the presence of 30 μM Hg there was a remarkable surge up to 1.5 h. From then on, Cd-induced fluorescence decreased slightly, reaching control values. In contrast, plants exposed to 30 μM Hg suffered a rapid loss of extracellular H_2O_2 (minimum after 6.0 h), which dropped below control values (Fig. 3b). Therefore, a similar trend to that found for *in vivo* staining with H_2DCFDA was observed in Hg-treated seedlings (Fig. 2). Independent batches of seedlings at these incubation times were collected, pooled and used for the analysis of redox metabolites, activity of ROS-scavenging enzymes, gene expression and accumulation of Cd and Hg.

Table 2 Nonprotein thiol and ascorbate (AA) contents (nmol g^{-1} fresh weight) and percentage of oxidized homoglutathione (hGSSGh) vs total homoglutathione (hGSH) in *Medicago sativa* seedlings exposed to 30 μM (a) cadmium (Cd) or (b) mercury (Hg) for different times

(a)

	0 min	15 min	45 min	1.5 h	3.0 h	6.0 h	24.0 h
AA	1215 ± 99[a]	1375 ± 87[a]	1213 ± 89[a]	1535 ± 64[ab]	1859 ± 243[ab]	1367 ± 101[a]	1562 ± 106[a]
GSH	47.1 ± 11.4[a]	39.6 ± 17.0[a]	51.0 ± 29.6[a]	43.1 ± 10.2[a]	38.7 ± 26.2[a]	37.6 ± 11.1[a]	32.3 ± 16.2[a]
hGSH	281.2 ± 39.1[a]	310.7 ± 21.7[a]	306.4 ± 23.5[a]	294.8 ± 33.6[a]	352.1 ± 38.3[ab]	293.1 ± 31.7[a]	333.5 ± 21.3[ab]
GSSG	nd	nd	nd	nd	nd	nd	nd
hGSSGh	6.9 ± 1.9[ab]	5.9 ± 0.8[a]	5.9 ± 1.9[a]	5.7 ± 2.6[a]	3.6 ± 6.2[a]	6.6 ± 2.1[a]	7.5 ± 3.9[ab]
%hGSSGh	2.4	1.9	1.9	2.0	1.1	2.3	2.3

(b)

	0 min	15 min	45 min	1.5 h	3.0 h	6.0 h	24.0 h
AA	1101 ± 102[a]	1013 ± 129[a]	951 ± 88[a]	987 ± 69[a]	1190 ± 136[a]	1003 ± 78[a]	897 ± 88[a]
GSH	27.1 ± 3.4[a]	31.7 ± 5.8[a]	21.8 ± 6.4[a]	17.8 ± 3.4[a]	13.8 ± 1.3[a]	15.9 ± 4.1[a]	23.7 ± 4.7[a]
hGSH	300.1 ± 35.7[a]	295.6 ± 59.7[a]	209.2 ± 19.9[b]	196.1 ± 13.3[b]	215.5 ± 17.9[b]	191.1 ± 20.3[b]	183.8 ± 13.6[b]
GSSG	nd	nd	4.7 ± 0.5[ab]	5.4 ± 0.5[b]	2.9 ± 1.2[a]	3.7 ± 2.5[a]	3.9 ± 2.9[a]
hGSSGh	7.3 ± 1.1[a]	10.9 ± 11.4[ab]	31.2 ± 7.4[c]	38.3 ± 5.2[cd]	27.7 ± 3.4[c]	28.0 ± 4.7[c]	35.4 ± 3.6[cd]
%hGSSGh	2.3	3.6	12.9	16.2	11.5	12.8	16.0

Concentration was calculated relative to GSH standards. Results are the mean of three independent assays. Different superscript letters denote significant differences between exposure times at $P < 0.05$.
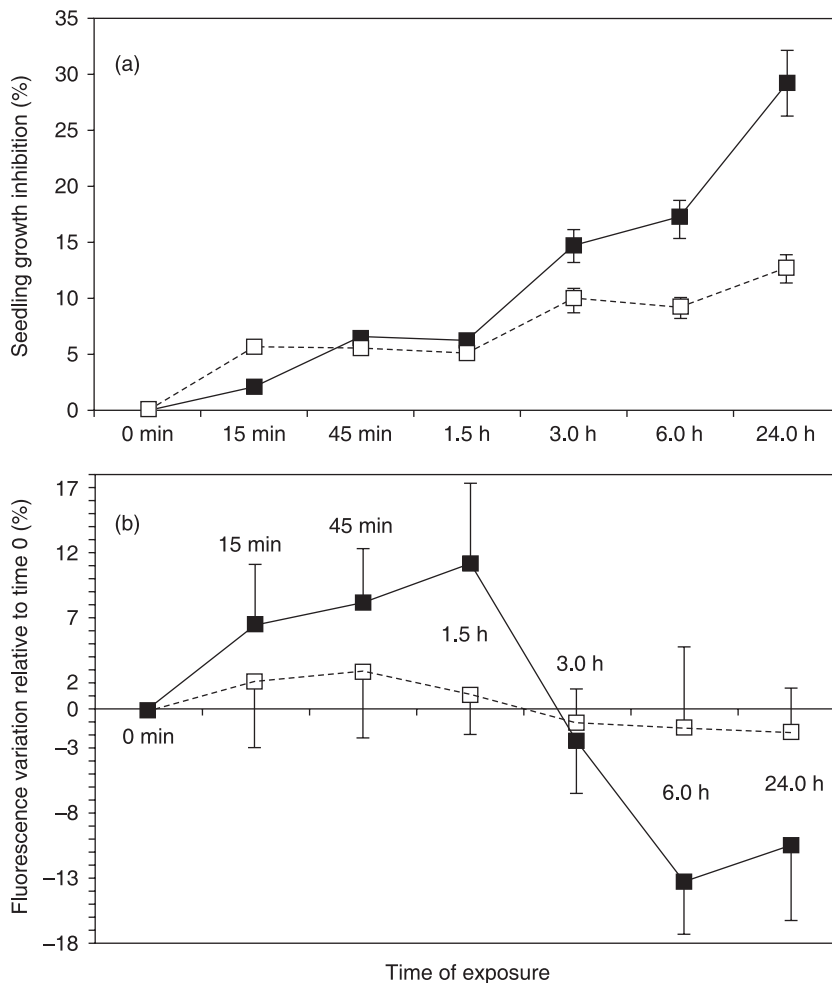GSH, glutathione; GSSG, oxidized GSH; nd, not detected.

Fig. 3 Effect of increasing incubation times of *Medicago sativa* seedlings exposed to 30 μM cadmium (Cd, open squares) and 30 μM mercury (Hg, closed squares) on (a) growth inhibition (%) and (b) relative extracellular hydrogen peroxide (H$_2$O$_2$) production (% of the control) measured using Amplex Red fluorescence. Data are the average of at least five independent experiments.

Analysis of redox metabolites The major nonprotein thiol obtained from alfalfa seedlings was hGSH; the concentration of GSH was about a tenth that of hGSH, in agreement with previous data (Table 2; Ortega-Villasante *et al.*, 2005). The other major redox metabolite was AA, which was found at similar concentrations to those found in several legumes, in the 1–2 mmol g^{-1} fresh weight (FW) range (Matamoros *et al.*, 2003).

Regarding the pools of hGSH and GSH, exposure to Cd had no significant effect but exposure to Hg led to a marked increase in hGSSGh with a subsequent depletion of hGSH (Table 2). The percentage of hGSSGh relative to total hGSH content increased from 2.3% to *c.* 16% after 1.5 h, thereafter reaching a plateau (Table 2). Similarly, Hg caused a slight increase in GSSG, but without a corresponding significant reduction in the GSH pool. Therefore, Cd had only a slight effect, which might indicate that this metal exerted a milder toxic effect. The concentration of AA was not affected when alfalfa seedlings were incubated with 30 μM Cd or Hg within this time frame (Table 2).

APX and SOD activities The activities of two key enzymes involved in the detoxification of ROS, SOD and APX, were analysed, as it is known that they may be affected by heavy metals (Gomes-Junior *et al.*, 2006; Rellán-Álvarez *et al.*, 2006a). Interesting differences between Cd and Hg treatments in their effects on APX activity were observed (Fig. 4). The addition of 30 μM Cd led to minor alterations in APX activity over the course of the experiment (Fig. 4a), whereas APX activity was markedly increased in seedlings exposed to 30 μM Hg from 45 min to 6 h, decreasing from then on (Fig. 4d). It appears that there was a transient over-activation of APX after exposure to 30 μM Hg. Analysis of *M. sativa* protein extracts
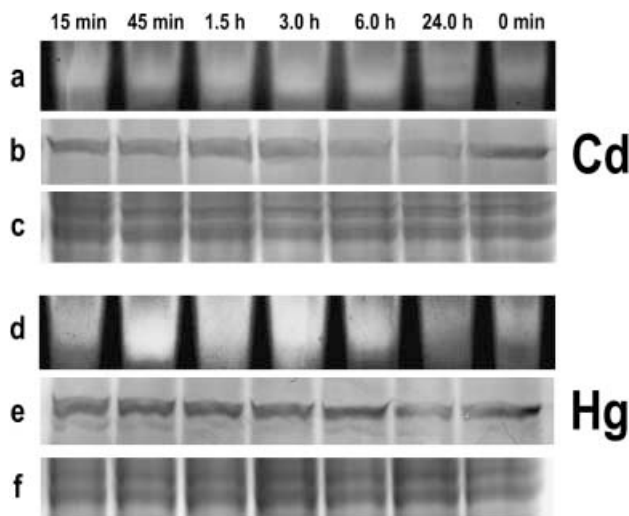

Fig. 4 Characterization of ascorbate peroxidase (APX) in *Medicago sativa* seedlings exposed for different times to 30 μM cadmium (Cd) or mercury (Hg). (a, d) APX activity in gel; (b, e) western blot immunodetection using anticytosolic APX antibody; (c, f) Coomassie blue staining of gels to assess protein loading.

by western blotting in nondenaturing gels revealed that the main band of activity corresponded to a cytosolic APX (cytAPX) isoform (data not show). Interestingly, a parallel immunodetection analysis by western blotting after denaturing polyacrylamide gel electrophoresis showed that the amount of cytAPX did not change significantly in any of the treatments (Fig. 4b,e). In contrast, five SOD isoforms were detected in alfalfa seedlings (Supplementary Material Fig. S2), with a very similar electrophoretic profile to that reported by Samis *et al.* (2002) for alfalfa leaves. The three fastest moving isoenzymes were inhibited by both H_2O_2 and KCN, indicating that they were copper/zinc SOD (Cu/ZnSOD). An intermediate isoform was inhibited only by H_2O_2, suggesting that it was an iron SOD (FeSOD). Finally, the slowest moving isoform was resistant to both inhibitors, confirming that this represented a manganese SOD (MnSOD) isoenzyme. None of the metal treatments at any incubation time had a significant effect on the activities of the different SOD isoforms found in alfalfa (Supplementary Material Fig. S2).

Gene expression analysis As there were changes in the pools of GSH/hGSH after Cd and Hg exposures, the expression of genes involved in their metabolism, i.e. *ECS*, *GS*, *hGS*, *GR1*, *GR2*, *GPX* and *PCS*, was analysed by semiquantitative RT-PCR. Because of its nonquantitative nature, only differences in relative amplicon abundance with a ratio of > 2.5 were considered relevant.

Different times of exposure to 30 μM Cd did not alter the expression of the genes analysed (Fig. 5). However, 30 μM Hg clearly affected accumulation of some of the transcripts. With the exception of the *PCS*, *GPX* and *GS* genes, which were expressed at similar levels at all times analysed, the genes were induced in a transient manner (Fig. 5). Hg produced a clear induction of the *GR1* (cytosolic) and *GR2* (plastidic) genes, with maximum expression being reached at 3 h after metal supply and decreasing thereafter (Fig. 5). Similarly, the *ECS* and *hGS* genes were expressed transiently, also reaching maximum transcript accumulation after 3 h (Fig. 5). Notably, this pattern can be linked to GSH|hGSH depletion (Table 2), and the maximum activity of cytAPX (Fig. 4), observed in Hg-treated seedlings.

Accumulation of heavy metals In the light of the different phytotoxic effects observed for Cd and Hg, the amount of toxic metals readily accumulated in the seedlings was determined. Both metals followed a saturation pattern, with plateaus after 3–6 h of exposure to 30 μM Cd or Hg (Fig. 6). Interestingly, Hg accumulated to a much higher extent, its concentration in alfalfa seedlings being approximately one order of magnitude higher than of Cd.

Discussion

The effect of Cd and Hg in early oxidative stress responses was studied in alfalfa seedlings using different methodologies,
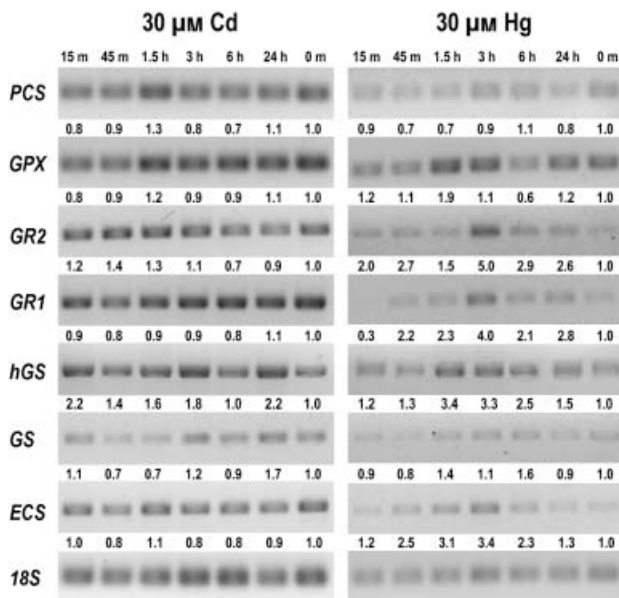
Fig. 5 Gene expression analysis (reverse transcriptase–polymerase chain reaction (RT-PCR)) of phytochelatin synthase (*PCS*), glutathione peroxidase (*GPX*), glutathione reductase 2 (*GR2*), glutathione reductase 1 (*GR1*), homoglutathione synthetase (*hGS*), glutathione synthetase (*GS*) and γ-glutamylcysteinyl synthetase (*ECS*) in *Medicago sativa* seedlings after time-elapsed exposure to 30 μM cadmium (Cd) or 30 μM mercury (Hg). Values are the relative variation of band intensity compared with exposure time 0, normalized against the product of the 18S rRNA amplicon. Representative results obtained for three independent assays are shown.

Fig. 6 Time–course of accumulation of cadmium (Cd) and mercury (Hg) in alfalfa (*Medicago sativa*) seedlings. The metal dose was 30 μM Cd or Hg. The data are the average of three replicates. FW, fresh weight.

including fluorimetry, which allowed us to monitor the accumulation of intracellular ROS and extracellular H_2O_2 with the fluorescent probes H₂DCFDA and Amplex Red, respectively. For the first time, it was possible to visualize the immediate cellular damage caused by these toxic elements in intact root cells, as was previously reported for tobacco BY-2 cells in culture (Olmos *et al.*, 2003; Garnier *et al.*, 2006). The maximum toxic metal dose used in this work was 30 μM, in contrast to the extremely high concentrations of Cd used for BY-2 cells (up to 3 mM). Interestingly, induction of oxidative stress showed a scattered spatial pattern (see films in Supplementary Material) similar to that observed in pea (*Pisum sativum*) roots treated with 50 μM Cd for 15 d (Rodriguez-Serrano *et al.*, 2006) and maize (*Zea mays*) seedlings subjected to a 50 μM aluminium (Al) treatment *in vivo* (Jones *et al.*, 2006). The pattern of metal-induced cell damage could be explained by differential uptake of the metals or the fluorescent probes, as a result, in turn, of differences in the physiological status of the cells.

Previous analysis of ROS production by H₂DCFDA in alfalfa seedlings exposed to 3, 10 and 30 μM Cd for 6 and 24 h revealed increases in cellular levels of oxidative stress and death (Ortega-Villasante *et al.*, 2005). In the case of Hg we were able to measure only low amounts of oxidative stress induction as
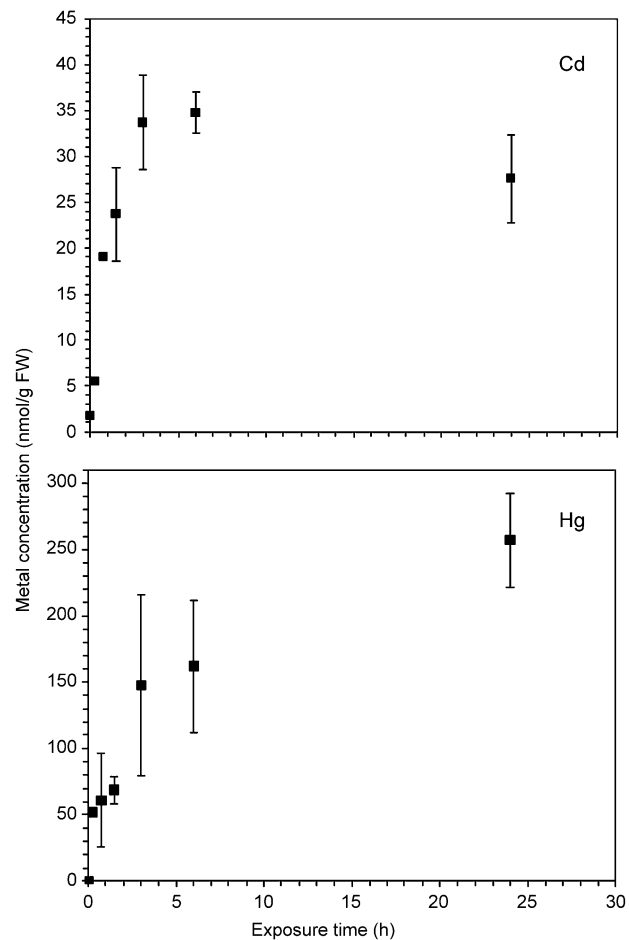
a result of the large amount of cell death. However, by shortening the observation window, we have found in this study that within a few minutes Hg causes a potent and transient induction of oxidative stress (Fig. 2 and Supplementary Material Film S3). Changes in intracellular ROS accumulation were consistent with those of extracellular H_2O_2, when the metal exposure ranged from 15 min to 24 h (Fig. 3). The oxidative burst was followed by dramatic cell death, the extent of which was much greater than that produced by Cd within the first 2 h of metal exposure (Fig. 2, Supplementary Material Fig. S1). The extremely high concentration of Cd used by Garnier *et al.* (2006) at exposure times of > 2 h rapidly increased the number of dead tobacco cells, which was associated with a transient accumulation of extracellular H_2O_2 within the first 30 min, similar to the pattern found for Hg-treated seedlings in this work. Therefore, under extreme toxic conditions it is feasible that cells are rapidly poisoned and become metabolically nonfunctional, being unable to oxidize H₂DCFDA or Amplex Red.

In comparing the changes in extracellular H_2O_2 detected using Amplex Red (Fig. 1) with those in intracellular ROS production measured using H_2DCFDA (Fig. 2), we noticed an interesting discrepancy. Within the same time period, extracellular H_2O_2 increased constantly in Hg-treated roots, whereas intracellular ROS accumulated in a transient manner. In contrast, the production of intracellular ROS rose constantly in Cd-exposed seedlings, with a smaller increase in extracellular H_2O_2. This suggests that the two metals triggered different mechanisms of toxicity, and that Hg probably caused the release of larger amounts of apoplastic ROS.

The increase in H_2O_2 produced by Hg and Cd could be primary attributable to an effect of these metals on NADPH oxidase, which is known to be involved in H_2O_2 generation, which in turn is thought to initiate a signalling cascade for stress responses (Maksymiec & Krupa, 2006). Thus, extracellular H_2O_2 accumulation was impaired in Cd-treated alfalfa seedlings preincubated with the NADPH oxidase inhibitor DPI (Fig. 1). A similar impairment was found with tobacco BY-2 cells treated with 1–3 mM Cd (Olmos *et al.*, 2003; Garnier *et al.*, 2006), and roots of pea plants exposed for 15 d to 50 μM Cd (Rodriguez-Serrano *et al.*, 2006). Curiously enough, in Hg-treated plants, DPI inhibited H_2O_2 generation only slightly (Fig. 1), suggesting that the underlying mechanism of H_2O_2 production caused by Hg differs from that caused by Cd.

APX activity was affected by Hg similarly to H_2O_2 formation, showing a transient activation after 3 h (Fig. 4d). Conversely, APX activity was only moderately affected by Cd (Fig. 4a) and H_2O_2 accumulated only slightly (Fig. 3b). There did not appear to be any alteration in the amount of cytAPX (Fig. 4e), suggesting that there might have been post-translational activation of the enzyme. There is little information on the effect of short-term treatment with Hg or Cd on APX activity. However, some evidence indicates that, at moderate Cd doses (5–10 μM) and longer exposure times, the activities of peroxidases, APX included, are increased (Schützendübel & Polle, 2002; Schützendübel *et al.*, 2002). Nevertheless, under acute toxicity (over 40 μM) or prolonged treatments, a general failure of metabolism might lead to the attenuation of overall enzyme activities (Schützendübel *et al.*, 2001, 2002). Indeed, this was observed in maize plantlets treated with 6 or 30 μM Hg for 7 d, in which both activity and protein concentration of cytAPX were reduced (Rellán-Álvarez *et al.*, 2006a). As this reduction was not observed in alfalfa seedlings treated for < 24 h with 30 μM Hg, it is conceivable that experiments of longer duration may be needed to detect protein degradation. This could also explain in part the lack of response in SOD activity observed (Supplementary Material Fig. S2). Our results are not in accordance with those obtained in pea roots grown in 50 μM Cd for 14 d (Rodriguez-Serrano *et al.*, 2006), where a clear diminution in the activity of Cu/ZnSOD isoforms was observed.

A relationship between GSSG and hGSSGh accumulation and the amount of oxidative stress has been demonstrated, as

the maximum oxidation of hGSH and GSH (*c.* 16%) was coincident with the peak of extracellular H_2O_2 and APX activity in 30 μM Hg-treated seedlings (Table 2). In contrast, 30 μM Cd had little effect on these redox metabolite pools. Data available on Cd-treated plants in short-term experiments indicate that GSH depletion appeared only when plant material suffered extensive stress damage. This was found in *Arabidopsis* seedlings grown in liquid culture in the presence of 100 μM Cd for 24 h (Xiang & Oliver, 1998), or an *Arabidopsis* cell culture maintained for 24 h with 50 and 200 μM Cd (Sarry *et al.*, 2006). In such conditions, a high concentration of GSSG was found, about 20% of the total pool (Xiang & Oliver, 1998), similar to values for the Hg treatment in this work (up to 16%). We also observed a stronger effect of Hg than Cd on oxidation of GSH and hGSH in maize roots, in an analysis of GSSG and hGSSGh concentrations in plants exposed for 7 d to 30 μM Cd compared with 6 and 30 μM Hg (Rellán-Álvarez *et al.*, 2006a). Therefore, it appears that GSSG and hGSSGh accumulate only under acute oxidative damage.

The remarkable effect of Hg on GSH|hGSH was also observed at the cellular level in the depletion of MCB *in vivo* staining (Supplementary Material Fig. S1). This result is in agreement with previous observations carried out after 6 and 24 h of exposure (Ortega-Villasante *et al.*, 2005), where Hg had a slightly stronger effect on MCB fluorescence quenching. Nevertheless, the fluorescence depletion could be attributed not only to oxidation of GSH|hGSH, but also to competition between metals and MCB for free thiol groups of GSH|hGSH.

Transcription of genes related to GSH|hGSH synthesis (*ECS* and *hGS*) and recycling (*GR1* and *GR2*), measured upon exposure to 30 μM Cd or Hg (Fig. 5), followed the pattern of other physiological alterations. This is in agreement with the findings of Sävenstrand & Strid (2004), who observed in *P. sativum* that Hg induced GSH-related genes to a higher extent than Cd. Under extreme stress conditions produced by 100 μM Cd, *Arabidopsis* seedlings grown in liquid culture showed a similar time–course of expression of *ECS*, *GS* and *GR* (Xiang & Oliver, 1998). Moreover, the transient activation of genes related to GSH|hGSH metabolism in Hg-treated alfalfa seedlings agrees with previous reports on *Brassica juncea* (Schäfer *et al.*, 1998) and barley (*Hordeum vulgare*) (Finkemeier *et al.*, 2003), where plants were subjected to extensive stress damage with higher doses of Cd or longer exposure times. The apparent decrease in gene expression found after > 3 h of Hg treatment might reflect cellular poisoning, similarly to APX activity and generation of extracellular H_2O_2. The negligible influence of Cd on the expression of GSH-related genes, such as *ECS*, was also reported in *Arabidopsis* cultured cells after short-term treatments (up to 8 h) even for very high metal doses, from 50 μM to 1 mM (Sarry *et al.*, 2006).

The extent of physiological changes produced by Cd and Hg during the first hours of metal exposure seemed to depend on the metal concentration in the tissue, as it correlated with

the accumulation of both metals before reaching a plateau (Fig. 6). The greater toxicity of Hg could be attributed, among other causes, to a higher Hg accumulation in plant tissues and/or stronger interaction with cell components. Indeed, the maximum concentration of Hg attained was one order of magnitude higher than that of Cd (Fig. 6). Similar differences were found in maize seedlings grown for 7 d with 6 and 30 µM Cd or Hg (Rellán-Álvarez *et al.*, 2006a).

The moderate changes in plants produced by Cd might suggest a homeostatic response (Mithöfer *et al.*, 2004), in which ROS signalling networks would be involved (Pastori & Foyer, 2002; Mittler *et al.*, 2004). It would therefore be interesting to investigate in depth the transport and fate of metals using more sensitive techniques than those employed in this work.

Acknowledgements

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Confocal imaging of *Medicago sativa* root cells stained with monochlorobimane (MCB), which labels glutathione/homoglutathione (GSH|hGSH) to give a blue-green fluorescent adduct (blue pseudo-colour) and propidium iodide (PI) counterstaining (red), after 120 min of immediate exposure to control (a), 30 μM cadmium (Cd) (b), and 30 μM mercury (Hg) (c).

Fig. S2 Superoxide dismutase (SOD) activity *in gel* of *Medicago sativa* seedlings exposed to either 30 μM cadmium (Cd) or 30 μM mercury (Hg) at different times. On the right is shown the identification of the isoforms on the basis of inhibition with 5 mM H_2O_2 (copper/zinc SOD (Cu/ZnSOD) and iron SOD (FeSOD)) and 5 mM KCN (Cu/ZnSOD).

Film S1 Film showing complete series of images of Fig. 2(a). Confocal imaging is shown of control *Medicago sativa* root cells stained with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA). Images were collected every 5 min. A final image was obtained after 120 min with propidium iodide (PI) counterstaining to show dead cells. The scale bar at the end is 200 μm.

Film S2 Film showing complete series of images of Fig. 2(b). Confocal imaging is shown of 30 μM cadmium (Cd)-treated *Medicago sativa* root cells stained with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA). Images were collected every 5 min. A final image was obtained after 120 min with propidium iodide (PI) counterstaining to show dead cells. The scale bar at the end is 200 μm.

Film S3 Films showing (a) complete series of images of Fig. 2(c) and (b) another equivalent experiment. Confocal imaging is shown of 30 μM mercury (Hg)-treated *Medicago sativa* root cells stained with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA). Images were collected every 5 min. A final image was obtained after 120 min with propidium iodide (PI) counterstaining to show dead cells. The scale bar at the end is 200 μm.

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