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Transcript accumulation of stress-related genes in *Vicia faba* roots under a short exposure to cadmium

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Abstract
Messenger RNA accumulation of various stress-related protein genes was monitored in response to Cd in *Vicia faba* plants. Metal treatment induced reactive oxygen species (ROS) production. H_2O_2 accumulated 12 h after the Cd exposure in *Vicia* roots proportionally to intracellular Cd content of treated plants and O_2^- by 24 h. The time course of oxidative stress evidenced by malondialdehyde (MDA) content indicated that lipoperoxidation occurred 24 h after Cd treatment. Prior to oxidative burst, the metal induced a similar dose-dependent kinetic of transcript accumulation for *Hsp70.1*, *MT2*, *GR1* and *Cu-ZnSOD* genes with a rapid increase or response at 12 h of Cd treatment, whereas the prolonged exposure to Cd resulted in reduction in the mRNA levels for all four genes. The pattern of *Cat* transcript accumulation was different from the other four genes and correlated with the intracellular oxidative burst. *Cat* transcripts were accumulated at 24 h in the *Vicia* roots, and this antioxidative enzyme gene was at high level of expression till the end of treatment concomitantly to ROS overproduction and MDA accumulation. The results indicate two different gene expression pathways activated by Cd-induced abiotic stress conditions and might be related to the cell oxidative status.

Keywords: *Cadmium, catalase, Hsp70, root, ROS, Vicia faba*

Abbreviations: *Cat*, catalase; Cd, cadmium; GR, glutathione reductase; Hsp70, heat shock proteins 70; LPO, lipid peroxidation; MDA, malondialdehyde; MT, metallothionein; NO, nitric oxide; PC, phytochelatin; ROS, reactive oxygen species; SE, standard error; SOD, superoxide dismutase.

Introduction

Cadmium (Cd) is one of the most toxic and ubiquitous pollutants. Over the past two centuries, anthropogenic and industrial activities have led to high emissions of Cd into the environment at concentrations significantly exceeding those coming from natural sources (Sanità di Toppi & Gabbrielli 1999). Metal overloads cause toxicity symptoms in most organisms. Its deleterious effects have been widely studied especially on higher plants, and knowledges gained from these studies were used for biotechnological application in phytoextraction of pollutants from soils (Dal Corso et al. 2008).

Due to its high mobility and water solubility, Cd enters readily the roots where it is mainly retained (Lux et al. 2011). The cellular toxicity can result from various direct as well as indirect effects of Cd. This heavy metal targets and damages a plethora of cellular activities and processes such as photosynthesis, carbohydrate and nitrate metabolism, water balance, DNA and lipid matrix, resulting in growth inhibition or in plant senescence or even in plant death (Dal Corso et al. 2008). An important factor of the Cd cellular toxicity is its chemical similarity with essential elements, in particular Zn, but also Ca and Fe. This could either deregulate the homeostasis of latter elements or cause their displacement from proteins. Moreover, the divalent cation, extremely thiol reactive, leads to protein inhibition activity or disruption of cellular structure (Hall 2002). Due to its redox potential (-820 mV), Cd is unable to catalyse redox reactions in biological systems. Nevertheless, it leads to cellular oxidative burst and formation of reactive oxygen species (ROS) such as superoxide radical (O_2^-) and hydrogen peroxide

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(H_2O_2). The metal might inhibit antioxidant enzymes, impair the respiratory chain or displace copper and irons in metalloproteins, which eventually trigger a Fenton reaction (Valko et al. 2005; Dal Corso et al. 2008; Rodríguez-Serrano et al. 2009). Cd^+-induced ROS such as hydrogen peroxide (H_2O_2) might also be produced by plasma membrane NADPH oxidase or originate in mitochondria (Romero-Puertas et al. 2004; Heyno et al. 2008).

Plants have evolved a complex network of homeostatic mechanisms to minimize the potential heavy metal damages. Cd within the cell is detoxified preferentially by binding to S-containing ligands such as metallothioneins (MTs), glutathione (GSH) and phytochelatins (PCs). Further the ligand–Cd complexes are most likely removed by sequestration from potentially sensitive organelles and structures (Cobbett & Goldsbrough 2002; Clemens 2006). Metabolites such as ascorbate (AA) and GSH, and antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase, GSH reductase (GR) and catalase (CAT) remove retroactively overproduced ROS from the Cd-intoxicated cells (Foyer & Noctor 2005; Cuypers et al. 2010).

For long, H_2O_2 was considered as toxic byproduct of aerobic metabolism, but this notion is rapidly changing in the light of the new regulatory functions of this molecule in cell signalling cascades (Neill et al. 2002b; Quan et al. 2008). It is now well-established that ROS are also key regulators in cellular defence mechanisms and resistance towards stress phytotoxicity. At low concentrations, ROS induce defence genes and adaptive responses, whereas at high concentrations, cell death is initiated (Vranová et al. 2002; Mittler et al. 2004; Cuypers et al. 2010).

This study describes the molecular response of *Vicia faba* to Cd connected with ROS production and oxidative status of the cell. Fabaceae species are highly sensitive to xenobiotics and largely used as plant models to study Cd-induced deleterious effect on DNA and membranes (Fusconi et al. 2006; Ünyayar et al. 2006). Recently, kinetics of early *in vivo* physiological and cytogenetic responses to Cd showed that the metal induced genotoxicity events prior to lipoperoxidation in *Vicia* roots (Souguir et al. 2011). Herein, we provide additional data on sequential molecular events affecting *Vicia* roots after Cd treatment. Cd accumulation in roots is related to that of H_2O_2, suggesting that this ROS molecule might act as an intermediate in the Cd signalling. Although heat shock proteins (*Hsp70.1*), MT (*MT2*), GSH reductase (*GR1*) and SOD (*Cu-ZnSOD*) protein genes showed a common transient pattern of mRNA accumulation, catalase (*Cat*) transcript accumulation appeared was delayed. This differential pattern of transcript accumulation shows distinct (unrelated) Cd/ROS regulations. The stress-related genes *Hsp70.1*, *MT2*, *GR1* and *Cu-ZnSOD* depend mainly on the initial stages of Cd incorporation and prior to oxidative burst within the cell, whereas *Cat* genes might be regulated by high H_2O_2 accumulation.

Materials and methods

Plant material

Seeds of *Vicia faba* L. var. Aguadulce were germinated on moistened paper at 25°C for 4–5 days and transferred into a hydroponic support (8-l containers/24 seedlings per container) in the following nutrient solution (pH 7): 3.9 mM $Ca(NO_3)_2$, 6.5 mM KNO_3, 2 mM $MgSO_4$, 0.9 mM KH_2PO_4 plus micronutrients: 90 μM Fe–EDTA, 2.7 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 4.5 μM H_3BO_3, 4 μM $CuSO_4$ and 2.0 μM $Mo_7O_{24}(NH4)_6$. Once roots had grown 2–3 cm in length (5 days), $CdCl_2$ was added (at concentrations of 50, 100 or 200 μM) in the hydroponic solutions for 48 h with a light/dark photoperiod of 16:8 h at 25°C. Six plants were used for each treatment and time point. All experiments were repeated three times using controls (without Cd^{2+}).

Total Cd content

Roots were washed carefully with distilled water before drying. Dried leaf and root tissues were ground and digested in 65% nitric acid (1 ml per 0.1 g of dry matter). The digested material was resuspended in distilled water and Cd contents were determined using a PerkinElmer atomic absorption spectrophotometer (PerkinElmer, Wellesley, MA, USA). Each measurement was carried out in triplicate.

H_2O_2 and O_2^- determination

$O_2^{-\cdot}$ production was measured by XTT reduction (sodium 3-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulphonic acid hydrate) according to Able et al. (1998). Root tissues (1 g) were homogenized in 1 ml of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 14, 000 rpm for 20 min. The reaction mixture contains the surpernatant in 50 mM Tris–HCl buffer pH 7.5 and 0.5 mM XTT. The XTT reduction was determined at 470 nm using 21.6 $mM^{-1}\,cm^{-1}$ as the extinction coefficient.

The intracellular H_2O_2 contents were determined according to Sergiev et al. (1997). Roots tissues (500 mg) were homogenized at 4°C with 5 ml 0.1% (w/v) trichloracetic acid and centrifuged at 12, 000 rpm for 15 min. The supernatant (0.5 ml) was added to 10 mM potassium phosphate buffer pH 7.0

(0.5 ml) and 1 M potassium iodide (1 ml). The absorbance was measured at 390 nm, and the content of H_2O_2 was calculated based on a standard curve using gradual H_2O_2 concentrations.

Lipid peroxidation

Lipid peroxidation (LPO) was estimated using malondialdehyde (MDA) content that is the major product of LPO. Root tissues (1 g) were homogenized in 10% (w/v) trichloracetic acid (10 ml) and centrifuged at 10,000 rpm for 10 min. An equal volume of 10% trichloracetic acid solution containing 0.5% 2-thiobarbituric acid was added to the supernatant. The sample was incubated at 95°C for 30 min, cooled quickly in an ice-bath and centrifuged at 10,000 rpm for 15 min. The absorbance was measured at 532 nm and corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated using 155 mM^{-1} cm^{-1} as an extinction coefficient.

RNA isolation and first strand cDNA synthesis

RNA extraction was performed using Tri-reagent (Euromedex, Souffelweyersheim, France), and the Euroscript Reverse Transcriptase (Eurogentec, Seraing, Belgium) was used for cDNA synthesis, both according to manufacturer's instructions. RNA integrity was verified on 1% agarose gel by detecting the three bands corresponding to ribosomal RNA 25S, 17S and 5S.

Primer design

Primers used for the amplification of target cDNA were designated according to their availability in the data bank (http://www.ncbi.nlm.nih.gov/). Both forward and reverse primers must frame a relatively short sequence (~150 bp) suitable for qPCR, designed with a GC percentage around 60% and a T_m between 58°C and 60°C. Primers for the internal control Actin and MT2 were designated on sequences of *Vicia faba* genes:

VfActin Fw: 5′-CAGCAGAGCGGGAAATTGTGAGGG,
VfActin Rev: 5′-AGGGCATCTGAATCTTTCAGCACCG,
VfMT2 Fw: 5′-GGAAGTAGCTGCAAGTGCGGCTC,
VfMT2 Rev: 5′-CCATCTCAGCACTCTCATATTGAGC.

Primers for *Hsp70.1*, *GR1*, *Cu-ZnSOD* and *Cat* were designated based on the sequence of the related species *Pisum sativum* and using sequence alignment with ClustalW software (http://align.genome.jp/) in order to locate highly conserved regions:

PsHsp70.1 Fw: 5′-TGTGCTTTGACATTGATGCCAACGG,
PsHsp70.1 Rev: 5′-ATCCTCAGACTTGTACTTCTCAGCC,
PsGR Fw: 5′-CGGATGTTGTGCTATTTGCCACTGG,
PsGR Rev: 5′-TCACCCACAGCCCATATGCTAGGG,
PsCu-ZnSODcy Fw: 5′-CTGGACCACATTTCAATCCTAATGG,
PsCu-ZnSODcy Rev: 5′-CTTTCCCAAGATCATCAGGATCGG,
PsCat Fw: 5′-TGAACAGCTTGCATTTTGTCCTGCC,
PsCat Rev: 5′-ATTGTTGTGGTGAGACCACTTGGG.

The identity of each reverse-transcription PCR product was confirmed by direct sequencing. PCR products were resolved on a 1.2% (w/v) agarose gel for size verification, purified with a DNA gel band purification kit (GFX™ PCR, Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to manufacturer's protocol and then sequenced using Genome Express services. Sequence homology searches were carried out using the BLAST search facility available through NCBI (http://www.ncbi.nlm.nih.gov).

Amplifications were performed with the following thermal profile: 30 s at 95°C, 30 s at a temperature chosen according to the T_m of the primers, 30 s at 72°C using *Taq* DNA polymerase (Sigma, St Louis, MO, USA) for a number of cycles to generate detectable signal for every sequence.

Quantitative real-time PCR

The quantitative assessment of mRNA levels was performed using the iCycler iQv3 (BIO-RAD, Hercules, CA, USA). Real-time quantitative PCR based on the fluorescence emitted by the amplification products in the presence of SYBR Green allows quantification of the accumulation of the target transcript relative to the *Actin* transcript used as reference. The reactions were prepared using the qPCR kit Mastermix for SYBR Green (Eurogentec) according to the manufacturer's protocol. The cDNA concentration used produced a threshold cycle (C_T) between 15 and 30 cycles. The abundance of targeted gene transcripts was normalized to *Actin* mRNA and set relative to control plants (no heavy metal exposure) according to the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001).

Statistical analysis

The results presented are the values ± standard error (SE) obtained from at least three independent experiments. Significant differences between treated and control plants are determined using ANOVA test ($p < 0.05$).

Results

Cd accumulation and toxicity symptoms in Vicia plants

Cd is a non-essential element for plants. However, when supplemented to the culture media, the metal was efficiently sequestered by roots under our experimental conditions. High Cd accumulation was first detected in treated roots after 12 h of metal exposure (Figure 1(A)). The Cd content increased by

24 h and almost doubled within 48 h when plants were constantly exposed to metal. A significant positive correlation was found between exogenously supplied and accumulated Cd at every time point ($R = 0.978$, $R = 0.986$, $R = 0.994$ at 12, 24 and 48 h, respectively at $p < 0.05$). Cd accumulation in shoot tissues was delayed and dramatically lower (25–50 times less) than the one measured in root tissues during the treatment (Figure 1(B)). It is interesting to note that the highest accumulation of Cd in aerial parts was detected when plants were treated with 100 μM Cd for 48 h, whereas the accumulation of the metal was similar when plants were treated either with 50 or with 200 μM Cd. Because 200 μM Cd led to necrotic symptoms (Figure 2), these results indicate that high Cd concentrations might reduce root system activity and inhibit the translocation of the metal towards the aerial part.

Cd induced phenotypic symptoms on both parts of the plant with higher visual damages on roots (Figure 2). At the end of the treatment (48 h), high Cd concentration (200 μM) affected *Vicia* growth. The aerial part was reduced in size, and roots showed strong ROS producing phenotypic symptoms such as brownishing, growth inhibition of the primary axis and quantitative reduction in size of secondary roots.

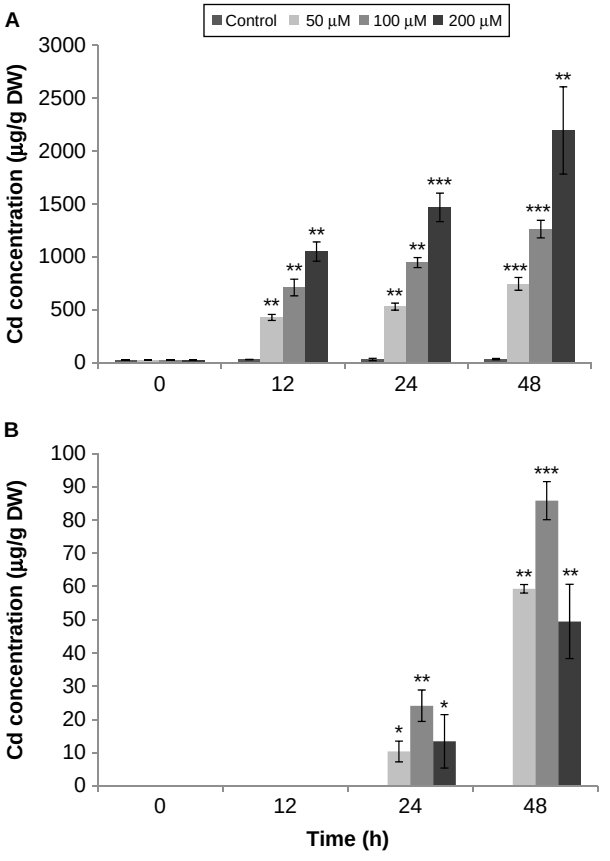

Figure 1. Metal accumulation in roots (A) and shoots (B) of hydroponically cultivated plants of *V. faba* var. Aguadulce treated with different concentrations of Cd. Data represent mean ± SE of three independent experiments (**$p < 0.01$; ***$p < 0.001$ compared with control).

ROS accumulation and LPO in Vicia roots

We investigated the oxidative status of the roots during the Cd treatment. We measured O_2^- and H_2O_2 production in *Vicia* roots during the treatment period (Figure 3). The Cd-supplemented hydroponic culture induced O_2^- accumulation by 24 h with 200 μM Cd treatment. Longer treatment led to O_2^- overproduction (Figure 3(A)). ROS accumulation is dose-dependent for H_2O_2 (Figure 3(B)). Cd and H_2O_2 contents in roots were positively correlated ($R = 0.977$, $R = 0.987$, $R = 0.949$ at 12, 24 or 48 h, respectively, at $p < 0.05$). LPO evidenced by MDA production was kinetically evaluated also in roots (Figure 3(C)). MDA content was not significantly affected within 12 h, regardless of the exogenous Cd concentration. MDA accumulation increased significantly at 24 h and reached the highest level at the end of the treatment. At 48 h, MDA content was 128%, 180% and 209% higher than the control in root cells when treated with 50, 100, and 200 μM Cd, respectively.

Responses of stress-related genes in Vicia roots

The molecular response to the metal was assessed in roots, the organs that accumulated most Cd and

Figure 2. Images of hydroponically cultivated plants of *V. faba* var. Aguadulce treated with different concentrations of Cd. The phenotypic symptoms were dose dependent with high stunting and root brownishing.

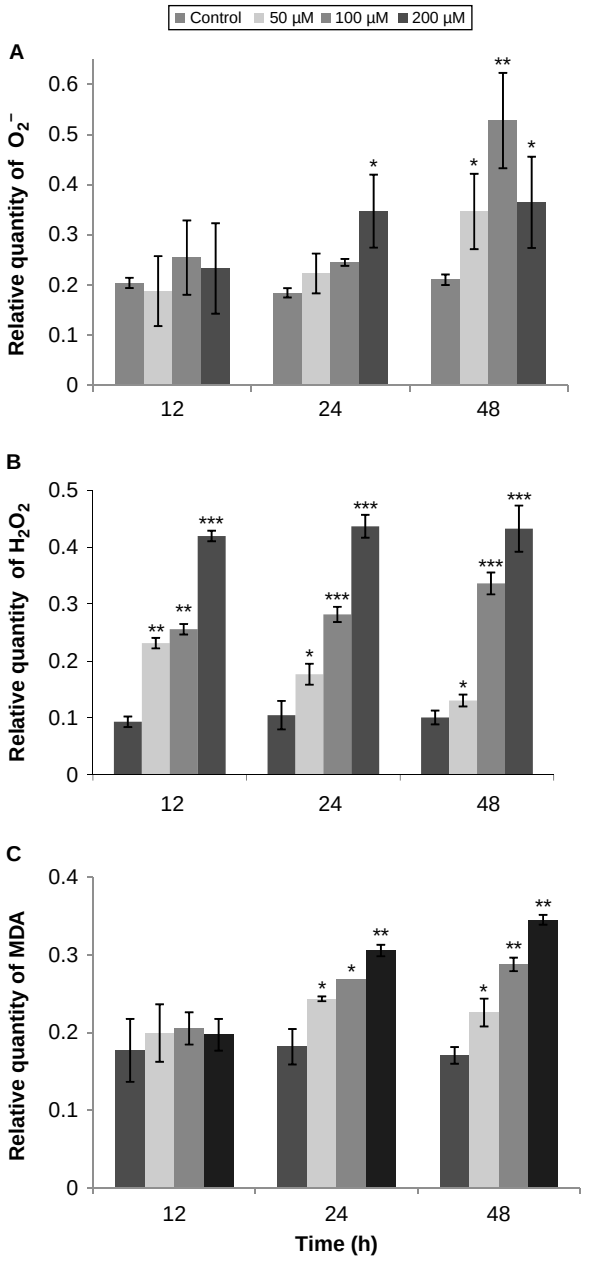

Figure 3. Evaluation of the oxidative status of root cells. Relative quantities of superoxide anion O_2^- (A), hydrogen peroxide H_2O_2 (B) and MDA (C) in roots of hydroponically cultivated plants of *V. faba* var. Aguadulce treated with different concentrations of Cd. Data represent mean ± SE of three independent experiments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with control).

displayed phenotypically the most rapid and profound effect of Cd treatment. Transcript levels were assessed by real-time quantitative PCR using RNAs isolated from roots of hydroponically cultivated *Vicia* plants (Figure 4). The primer pairs designated for *Hsp70-1*, *MT2*, *GR1*-, *Cu-ZnSOD*- and *Cat*-like protein genes allow the amplification of a single isoform (data shown only for *Hsp70-1* in Figure 4 (A)). Table I depicts the sizes and the homologies of PCR-amplified fragments to sequences in databases. Figure 4(A) shows transcript accumulation of *Hsp70-1* gene in *Vicia* roots treated with different concentrations of Cd over the period of treatment. High concentrations of Cd (100 µM and mainly 200 µM) induced the accumulation of *Hsp70-1* gene transcripts. However, the highest response of the *Hsp70-1* gene was obtained when plants were exposed for 12 h at 200 µM Cd, showing an acute response of the gene at the highest concentration. Heavy metal exposure for 24 h decreased *Hsp70-1* transcript accumulation except when the plants were exposed to 50 µM Cd. Prolonged exposure (48 h) reduced transcript accumulation to control levels in all Cd treatments.

Figure 4(B)–(D) depicts the dramatic changes in transcript abundance of *MT2*, a heavy metal-chelating protein gene, and *GR1* and *Cu-ZnSOD*, two antioxidative enzyme genes. These three gene transcripts accumulated rapidly in *Vicia* roots 12 h after the exposure at 200 µM Cd. For MT2, the transcript accumulation was exclusively triggered for the highest Cd concentration in hydroponic medium (200 µM). The intracellular Cd concentration at 12 h, ~1000 µg/g DW (Figure 1(A)), might represent a threshold content to activate early the expression of *MT2* gene producing adequate quantity of the chelating protein in root cells. The decreases in *MT2* transcript level were observed with longer Cd treatments in plants (24 and 48 h). *GR1* and *Cu-ZnSOD* transcripts were dramatically enhanced at 12 h Cd treatment (eight and six times on average, respectively, compared with control plants) and decreased when plants were Cd-treated for longer periods (Figure 4(C),(D)). The pattern of mRNA abundance for all the four mentioned genes showed a curve (bell-shape)-pattern of accumulation with the highest point at 12 h exposure to 200 µM Cd. The gene transcript accumulation showed a recovery profile and mRNAs returned to control levels after a prolonged exposure. However, Cd and/or H_2O_2 root content continued to increase at 24 or 48 h of Cd exposure. Taking these results together, the transient mRNA accumulation strongly argues that Cd or H_2O_2 mode of action in gene induction or transcript accumulation is bivalent and when a threshold level is passed, gene upregulation ceases or mRNA stability declines, or both.

The *Cat* transcript accumulation was analysed as well (Figure 4(E)). The profile of gene induction or mRNA abundance was different. Higher Cd content induced higher transcript accumulation. This transcript accumulation was enhanced 24 h after Cd exposure with no significant decrease at the end (48 h) of the treatment. Since *Cat* is directly involved in H_2O_2 detoxification and H_2O_2 content was barely higher at 12 h of Cd exposure (Figure 3(B)), this can suggest that high H_2O_2 accumulation triggers the expression of the *Cat* gene (Figure 4(E)).

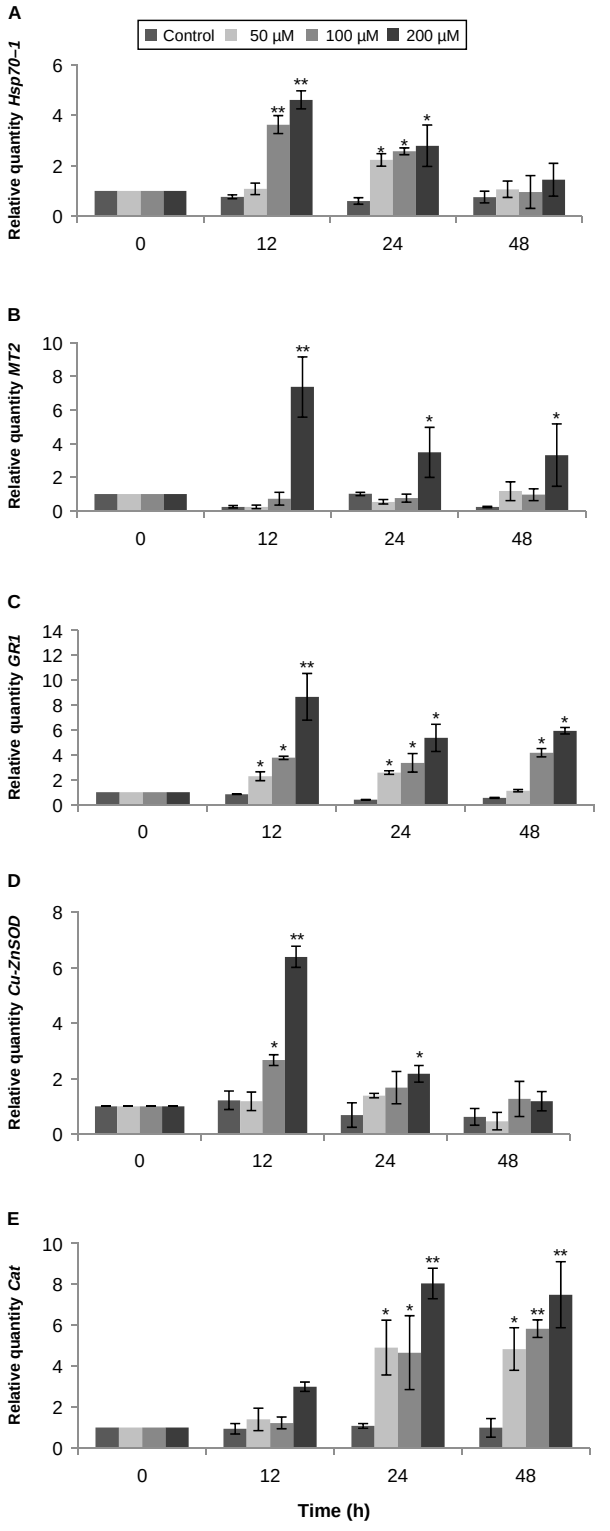

Figure 4. Kinetic of stress-related transcript accumulation during Cd treatments of hydroponically cultivated plants of *V. faba* var. Aguadulce. The amount of transcript encoding *Hsp70.1* (A), *MT2* (B), *GR1*- (C), *Cu-ZnSOD*- (D) and *Cat*- (E) like protein genes was quantified by real-time quantitative PCR and normalized to the amount of the housekeeping *Actin* transcripts. Values are expressed relative to the control (no Cd treatment) value. Bars represent mean values ± SE from three C_T values of three independent experiments (*$p < 0.05$; **$p < 0.01$ compared with control).

Discussion

A range of Cd concentrations over a short period of treatment (up to 48 h) was used in our experimental conditions to assess the *Vicia* response at a molecular level. We applied Cd concentrations (50–200 µM) that mimic environmental heavy metal concentrations in polluted soils (Nagajyoti et al. 2010). Previous results showed that this Fabaceae species is sensitive to heavy metals causing genotoxicity and oxidative stress in root tips (Ünyayar et al. 2006; Souguir et al. 2008, 2011). Roots were mainly affected by Cd treatment since this organ sequestered rapidly and highly the metal and showed pronounced phenotypic symptoms of toxicity. This difference in Cd accumulation between roots and shoots has also been detected in tobacco plants, in which overexpression of *Arabidopsis* PC synthase enhances Cd tolerance and accumulation without any further translocation to the shoot (Pomponi et al. 2006).

The short-term kinetic of Cd treatment on hydroponically cultivated *Vicia* plants allowed to analyse the early events underlining transcript accumulation profiles of different stress-related genes in roots: (i) *Hsp* gene, an environmental toxicology stress marker; (ii) *MT* gene coding for a metal-binding protein and (iii) *GR*, *SOD* and *Cat* genes coding for markers of enzymatic ROS scavenging mechanism.

Hsp transcripts are potential molecular biomarkers of environmental stress. Indeed, they are more sensitive than traditional indices such as growth inhibition (Ireland et al. 2004). Hsps are constitutively expressed stress-proteins and upregulated proportionally to the degree of the stress (Bierkens et al. 1998). Their role is to protect and repair protein folding under stress conditions (Timperio et al. 2008). In higher plants, the Hsp70 chaperone family could further provoke heavy-metal tolerance by preventing the membrane damage (Neumann et al. 1994; Romero-Puertas et al. 2004). In our experimental conditions, Cd induced *Hsp70.1* transcript accumulation during the treatment, suggesting that *Vicia* plants sense the potent metallic stress. In these adverse conditions, the dose-dependent *Hsp70.1* induction suggested that this stress-related chaperone family counteracts the metal-induced proteotoxicity by activating protecting mechanisms against protein damage and destabilization of cellular homeostasis (Feder & Hofmann 1999). Recently, the effects of heavy metals, both toxic (Pb, Cd) and essential (Cu, Zn) on the ultrastructure and the induction of *Hsp70*, have been studied in the aquatic moss *Leptodictyum riparium* (Esposito et al. 2012).

The physiological regulation of metal-binding proteins MTs, GSH recycling enzymes GRs and peroxide neutralizing enzymes SODs are well-

Table 1. Homologies of quantitative PCR-amplified fragments to sequences in the databases.

PCR fragments	Length[a] (pb)	Accession number	Homology[b]	BLAST score
Actin	147	EU884301	*Vicia faba* cultivar long pod actin mRNA (AY338230.1)	3e-70
Hsp70.1	136	EU884304	*Pisum sativum* (little marvel) HSC71.0 mRNA (Z32537.1)	3e-64
MT2	109	EU884305	*Vicia faba* clone 038 H06 MT mRNA (EU920050.1)	2e-49
GR1	105	EU884307	*Pisum sativum* mRNA for GR (X98274.1)	4e-47
Cu-ZnSOD	177	EU884303	*Pisum sativum* mRNA for Cu-ZnSOD (AB189165.1)	7e-8
Cat	132	EU884302	*Pisum sativum* mRNA for CAT (X60169.1)	5e-62

[a] Sequence provided by MWG/OPERON.
[b] GenBank accession numbers of sequences homologous to quantitative PCR fragments are in parentheses.

characterized relative to heavy metal stress (Seregin & Ivanov 2001; Rodríguez-Serrano et al. 2009). Previous studies have shown that *MTs* and *GR* genes were expressed at high levels under heavy metal treatment (Cobbett & Goldsbrough 2002; Zimeri et al. 2005; Goupil et al. 2009). Tamás et al. (2008) showed that MT and GR were upregulated under Cd treatment in barley root. Little is known about the transcriptional regulation of *SODs* genes by heavy metals. We analysed the kinetic of transcript accumulation of the *MT2*, *GR1*- and *Cu-ZnSOD*-like protein genes for 48 h. The transcript accumulation for the three genes showed a similar pattern to the one detected for *Hsp70.1* during the treatment, i.e. the highest response 12 h after Cd treatment followed by a decrease for prolonged Cd exposure in the highest concentrated culture (200 μM). Because *Hsp70.1*, *MT2*, *GR1* and *Cu-ZnSOD* transcripts all accumulated early under Cd treatment, a coordinated and combinatorial type of tolerance mechanism involving protein damage repair, metal chelation and antioxidative metabolism should take place in *Vicia* roots under the Cd stress. Cd as a highly generative ROS metal stimulated enzymatic antioxidant metabolism involving GSH. The profile of mRNA accumulation of the stress-related genes within 12 h showed that they should be coordinately stimulated and should be driven by a common transcriptional activation mechanism.

Cat gene expression was temporarily delayed. Like SODs, *Cat* is directly involved in enzymatic ROS scavenging mechanisms. SODs act as the first line of defence against ROS, dismutating superoxide to H_2O_2. Cat subsequently detoxifies H_2O_2 (Apel & Hirt 2004). Both enzymes were sequentially regulated in *Vicia* roots during the Cd stress while *SOD* transcripts accumulated earlier than *Cat* transcripts.

The early and transient overexpression of the isoform genes (*Hsp70.1, MT2, GR1* and *Cu-ZnSOD*) in the Cd kinetic treatment suggested that common signalling molecules could be involved in their concerted upregulation. The H_2O_2 production rose proportionally to the Cd taken up by the roots, arguing that H_2O_2 molecules might be one of the Cd-signalling intermediates. This ROS, unlike O_2^-, is a stable and diffusible molecule with a well-documented dual action. At low concentrations, this oxidizing agent acts as a molecular signal mediating the acquisition of stress tolerance. Higher H_2O_2 concentrations lead to oxidative damages in plant cells (D'Autréaux & Toledano 2007; Quan et al. 2008). It has been shown that it acts as a modulator for various H_2O_2-sensitive genes including those used in our study: antioxidative enzymes, defence and stress-related proteins (Neill et al. 2002a; Timperio et al. 2008).

ROS are known to attack the highly unsaturated fatty acids of membrane systems to induce LPO, which is an autocatalytic process and may cause peroxidative tissue damage. The level of LPO, measured as MDA content, has been considered an indicator of metal-induced oxidation in cell membranes (Ippolito et al. 2011; Rahman et al. 2012). In our experiments, the MDA content allowed us to distinguish among the signal and pro-oxidative effect of H_2O_2 in the time course of Cd treatment. In the early stage (12 h) following Cd exposure, the H_2O_2 concentration and substantial transcripts accumulation of *Hsp70.1*, *MT2*, *GR1* and *Cu-ZnSOD* protein genes occurred without any great MDA increase that could be assumed; at this time point, H_2O_2 might act as a signal. Since root intracellular H_2O_2 accumulation was almost similar at 12 h of 200 μM and at 24 h of 100 μM Cd treatment while the induction for all the four genes was different, it can be proposed that cell senses and responds rapidly to acute toxic Cd concentrations by the expression of these genes. Prolonged exposures either damage the cellular homeostasis or the specific transcriptional activation ceases. We could not exclude the possibility of specific mRNA degradation or differentiation in transcript stability. Furthermore, after 24 and 48 h Cd exposure, the H_2O_2 burst and *Cat* transcript accumulation occurred with MDA production. At these time points of Cd treatment, the transcriptional activation of *Cat* genes was maintained to detoxify accumulated H_2O_2 in *Vicia* root cells.

In conclusion, our work showed that unrelated pathways might be involved in the transcriptional activation of metallic stress-induced genes in *Vicia* roots. Since the low concentration of Cd/H_2O_2 could

induce *Hsp70.1*, *MT2*, *GR1* and *Cu-ZnSOD* protein–gene transcription activation in the first 12 h of the Cd treatment, the higher intracellular concentration of Cd/H_2O_2 produced 24 h after Cd treatment could induce the accumulation of *Cat* transcripts. *Cat* gene might not be involved in early molecular events leading to cellular homeostasis protection but as a vital modulator of secondary response involved in cellular detoxication mechanisms.

It can be suggested that ROS/H_2O_2 within the first 12 h of Cd treatment acts as signal molecule involved in acclimatory signalling triggering tolerance to the metal stress in *Vicia* roots. Other key signalling components, such as nitric oxide (NO), play a pivotal signalling role in defence gene expression (Neill et al. 2002a, 2002b; De Michele et al. 2009). As shown by De Michele et al. (2009), NO is temporarily produced prior to H_2O_2 accumulation and leads to cell death. In *Vicia* roots, cell death is potentially triggered by 24 h Cd treatment of plants (Souguir et al. 2011). Whether this gaseous reactive molecule is involved in the Cd-treated response process will be further investigated.

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References

Able AJ, Guest DI, Sutherland MW. 1998. Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with a virulent zoospores of *Phytophthora parasitica* var *nicotianae*. Plant Physiol 117: 491–499.

Apel H, Hirt H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–399.

Bierkens J, Maes J, Van der Plaetse F. 1998. Dose-dependent induction of heat shock protein 70 synthesis in *Raphidocelis subcapitata* following exposure to different classes of environmental pollutants. Environ Pollut 101: 91–97.

Clemens S. 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie 88: 1707–1719.

Cobbett CS, Goldsbrough P. 2002. Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. Annu Rev Plant Physiol Plant Mol Biol 53: 159–182.

Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, et al. 2010. Cadmium stress: An oxidative challenge. Biometals 23: 927–940.

Dal Corso G, Farinati S, Maistri S, Furini A. 2008. How plants cope with cadmium: Staking all on metabolism and gene expression. J Int Plant Biol 50: 1268–1280.

D'Autréaux B, Toledano MB. 2007. ROS as signalling molecules: Mechanisms that generate specificity in ROS homeostasis. Mol Cell Biol 8: 813–824.

De Michele R, Vurro E, Rigo C, Costa A, Elviri L, Di Valentine M, et al. 2009. Nitric oxide is involved in cadmium-induced programmed cell death in Arabidopsis suspension culture. Plant Physiol 150: 217–228.

Esposito S, Sorbo S, Conte B, Basile A. 2012. Effects of heavy metals on ultrastructure and HSP70S induction in the aquatic moss *Leptodictyum riparium* Hedw. Int J Phytoremediation 14(4): 443–455.

Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Ann Rev Physiol 61: 243–282.

Foyer C, Noctor G. 2005. Oxidant and antioxidant signalling in plants: A re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ 28: 1056–1071.

Fusconi A, Repetto O, Bona E, Massa N, Gallo C, Dumas-Gaudot E, et al. 2006. Effects of cadmium on meristem activity and nucleus ploidy in roots of *Pisum sativum* L. cv. Frisson seedlings. Environ Exp Bot 58: 253–260.

Goupil P, Souguir D, Ferjani E, Faure O, Hitmi A, Ledoigt G. 2009. Expression of stress-related genes in tomato plants exposed to arsenic and chromium in nutrient solution. J Plant Physiol 166: 1446–1452.

Hall JL. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53: 1–11.

Heyno E, Klose C, Krieger-Liszkay A. 2008. Origin of cadmium-induced reactive oxygen species production: Mitochondrial electron transfer versus plasma membrane NADPH oxidase. New Phytol 179: 687–699.

Ippolito MP, Fasciano C, d'Aquino L, Tommasi F. 2011. Responses of antioxidant systems to lanthanum nitrate treatments in tomato plants during drought stress. Plant Biosyst 145(1): 248–252.

Ireland HE, Harding SJ, Bonwick GA, Jones M, Smith CJ, Williams JHH. 2004. Evaluation of heat shock protein 70 as a biomarker of environmental stress in *Fucus serratus* and *Lemna minor*. Biomarkers 9: 139–155.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402–408.

Lux A, Martinka M, Vaculík M, White PJ. 2011. Root responses to cadmium in the rhizosphere: A review. J Exp Bot 62: 21–37.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. Trends Plant Sci 9: 490–498.

Nagajyoti PC, Lee KD, Sreekanth TVM. 2010. Heavy metals, occurrence and toxicity for plants: A review. Environ Chem Lett 8: 199–216.

Neill S, Desikan R, Clarke A, Hurst RD, Hancock JT. 2002a. Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot 53: 1237–1247.

Neill S, Desikan R, Hancock J. 2002b. Hydrogen peroxide signalling. Curr Opin Plant Biol 5: 388–395.

Neumann D, Lichtenberger O, Günther D, Tschiersch K, Nover L. 1994. Heat shock proteins induce heavy metal tolerance in higher plants. Planta 194: 360–367.

Pomponi M, Censi V, di Girolamo V, de Paolis A, di Toppi LS, Aromolo R, et al. 2006. Overexpression of *Arabidopsis* phytochelatin synthase in tobacco plants enhances Cd^{2+} tolerance and accumulation but not translocation to the shoot. Planta 223: 180–190.

Quan L-J, Zhang B, Shi W-W, Li H-Y. 2008. Hydrogen peroxide in plants: A versatile molecule of the reactive oxygen species network. J Int Plant Biol 50: 2–18.

Rahman MM, Chongling Y, Rahman MDM, Islam KS. 2012. Effects of copper on growth, accumulation, antioxidant activity and malondialdehyde content in young seedlings of the mangrove species *Kandelia candel* (L.). Plant Biosyst 146(1): 47–57.

Rodríguez-Serrano M, Romero-Puertas MC, Pazmiño DM, Testillano PS, Risueño MC, del Río LA, et al. 2009. Cellular response of pea plants to cadmium toxicity: Cross-talk between

reactive oxygen species, nitric oxide and calcium. Plant Physiol 150: 229–243.

Romero-Puertas MC, Rodriguez-Serrano M, Corpas FJ, Gomez M, del Rio LA, Sandalio LM. 2004. Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. Plant Cell Environ 27: 1122–1134.

Sanità di Toppi L, Gabbrielli R. 1999. Response to cadmium in higher plants. Environ Exp Bot 41: 105–130.

Sergiev I, Alexieva V, Karnov E. 1997. Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. CR Acad Bulg Sci 51: 121–124.

Seregin IV, Ivanov VB. 2001. Physiological aspects of cadmium and lead toxic effects on higher plants. Russ J Plant Physiol 48: 523–544.

Souguir D, Ferjani E, Ledoigt G, Goupil P. 2008. Exposure of *Vicia faba* and *Pisum sativum* to copper-induced genotoxicity. Protoplasma 233: 203–207.

Souguir D, Ferjani E, Ledoigt G, Goupil P. 2011. Sequential effects of cadmium on genotoxicity and lipoperoxidation in *Vicia faba* roots. Ecotoxicology 20: 329–336.

Tamás L, Dudíková J, Ďurčekova K, Halušková L, Hottová J, Mistík I, et al. 2008. Alterations of the gene expression, lipid peroxidation, proline and thiol content along the barley root exposed to cadmium. J Plant Physiol 165: 1193–1203.

Timperio AM, Egidi MG, Zolla L. 2008. Proteomics applied on plant abiotic stresses: Role of heat shock proteins (HSP). J Proteomics 71: 391–411.

Ünyayar S, Çelik A, Çekiç FO, Gözel A. 2006. Cadmium-induced genotoxicity, cytotoxicity and lipid peroxidation in *Allium sativum* and *Vicia faba*. Mutagenesis 21: 77–81.

Valko M, Morris H, Cronin MT. 2005. Metals, toxicity and oxidative stress. Curr Med Chem 12: 1161–1208.

Vranová E, Inzé D, Breusegem FV. 2002. Signal transduction during oxidative stress. J Exp Bot 53: 1227–1236.

Zimeri AM, Dhankher OP, McCaig B, Meagher RB. 2005. The plant MT1 metallothioneins are stabilized by binding of cadmium and are required for cadmium tolerance and accumulation. Plant Mol Biol 58: 839–855.