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RESEARCH PAPER

Proteome changes in *Arabidopsis thaliana* roots upon exposure to Cd^{2+}

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Abstract

Cadmium is a major environmental pollutant that enters human food via accumulation in crop plants. Responses of plants to cadmium exposure—which directly influence accumulation rates—are not well understood. In general, little is known about stress-elicited changes in plants at the proteome level. Alterations in the root proteome of hydroponically grown *Arabidopsis thaliana* plants treated with 10 μM Cd^{2+} for 24 h are reported here. These conditions trigger the synthesis of phytochelatins (PCs), glutathione-derived metal-binding peptides, shown here as PC2 accumulation. Two-dimensional gel electrophoresis using different pH gradients in the first dimension detected on average ~1100 spots per gel type. Forty-one spots indicated significant changes in protein abundance upon Cd^{2+} treatment. Seventeen proteins found in 25 spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Selected results were independently confirmed by western analysis and selective enrichment of a protein family (glutathione *S*-transferases) through affinity chromatography. Most of the identified proteins belong to four different classes: metabolic enzymes such as ATP sulphurylase, glycine hydroxymethyltransferase, and trehalose-6-phosphate phosphatase; glutathione *S*-transferases; latex allergen-like proteins; and unknown proteins. These results represent a basis for reverse genetics studies to better understand plant responses to toxic metal exposure and to the generation of internal sinks for reduced sulphur.

Key words: Cadmium, heavy metal accumulation, heavy metal tolerance, MALDI-TOF-MS, phytochelatins, proteomics.

Introduction

Cadmium (Cd) and other heavy metals such as lead (Pb) and mercury (Hg) are important health-threatening pollutants (Järup, 2003). Human activities, especially over the past 200 years, have resulted in the massive release of heavy metals into the environment (Nriagu and Pacyna, 1988). Major sources of Cd pollution are Zn smelting, coal burning, and the use of phosphate fertilizers (Pinot *et al*., 2000). Cd^{2+} ions are comparatively mobile in the geosphere, and concerns have been raised about accelerating accumulation of Cd in the human food chain (Nriagu and Pacyna, 1988). Uptake by crop plants is the main entry pathway into human and animal food. Plant responses to Cd^{2+} exposure are known to influence Cd accumulation rates. Enhanced intracellular binding, for instance, drives the accumulation of Cd (Clemens *et al*., 1999). Thus, it is important to identify the changes elicited by Cd^{2+} exposure in plants and eventually to understand their regulation as well as the molecular basis for the pronounced differences across plant species in Cd tolerance and accumulation rates (Wagner, 1993).

Cd is generally considered a non-essential transition metal since to date there is only one documented example of a protein requiring Cd as a cofactor, a Cd-specific carboanhydrase in the marine diatom *Thalassiosira weissflogii* (Lane *et al*., 2005). Still, Cd^{2+} ions can interfere with homeostatic pathways for essential metal ions. They enter plant cells most probably via uptake pathways for

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Abbreviations: DTT, dithiothreitol; GSH, glutathione; GST, glutathione *S*-transferase; LC–ESI-QTOF-MS, liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MT, metallothionein; PC, phytochelatin.

Ca^{2+}, Fe^{2+}, and Zn^{2+} (Clemens *et al*., 1998; Connolly *et al*., 2002). Also, even though Cd^{2+} ions are apparently non-essential and increased Cd exposure is a very recent phenomenon—at least on the evolutionary time scale—Cd detoxification pathways have been identified in organisms from bacteria to man. The main principles of Cd detoxification are the synthesis of intracellular high-affinity binding sites and removal from potentially sensitive sites by sequestration or efflux. Cd as a class B metal preferentially binds to S-containing ligands. Accordingly, the best known and presumably most effective chelators for Cd^{2+} ions are small cysteine-rich proteins (metallothioneins, MTs) and the cysteine-containing peptides glutathione (GSH) and phytochelatins (PCs). While in mammals MTs are proven to confer Cd^{2+} tolerance, the dominant Cd^{2+} detoxification pathway in plants, algae, many fungi, and apparently also in invertebrates, is PC-dependent. PC deficiency in these organisms results in severe Cd^{2+} hypersensitivity (Cobbett and Goldsbrough, 2002). PCs are peptides of the general structure $(\gamma\text{-Glu-Cys})_n$–Gly ($n$=2–11). In iso-phytochelatins, the terminal amino acid is a serine, glutamate, or β-alanine. They are non-ribosomally synthesized from GSH in a transpeptidase reaction (Vatamaniuk *et al*., 2004). Accumulation of PCs is triggered by exposure to various physiological and non-physiological metal ions. Cd^{2+} ions are the most potent activators of PC synthesis (Cobbett and Goldsbrough, 2002). As a consequence of PC formation and/or binding of Cd^{2+} ions directly to GSH, Cd^{2+} uptake into cells causes an increased demand for reduced sulphur. *Saccharomyces cerevisiae* cells, which detoxify Cd^{2+} in a GSH-dependent manner, therefore respond to Cd^{2+} exposure by the up-regulation of sulphur amino acid biosynthesis and GSH biosynthesis. Almost all enzymes of the respective pathways are more abundant in cells under conditions of Cd^{2+} stress than in control cells (Vido *et al*., 2001). Interestingly, *S. cerevisiae* cells additionally show a sulphur-sparing response upon Cd^{2+} exposure: highly abundant proteins of carbohydrate metabolism are replaced by isozymes with a lower S content in order to allocate more reduced sulphur to the production of GSH (Fauchon *et al*., 2002).

Much less is known in plants and other multicellular organisms about responses under toxic metal stress at the protein level. In general, there are few studies on stimulus-dependent changes in the proteome of plants, even though proteomics is recognized as a key approach to understanding biological systems (Baginsky and Gruissem, 2006). For the model species *Arabidopsis thaliana*, whose complete genome sequence was published in 2000 (Arabidopsis Genome Initiative, 2000), mostly organellar proteomes and the protein composition of subfractions such as particular membranes have been described to date (Peck, 2005; Baginsky and Gruissem, 2006). Examples are chloroplasts (Friso *et al*., 2004), vacuoles (Carter *et al*., 2004), and the plasma membrane (Marmagne *et al*., 2004). The few comparative experiments with *A. thaliana* have, for instance, investigated responses to bacterial challenge (Jones *et al*., 2004) or cold stress (Amme *et al*., 2006). A study was initiated on root proteome changes under Cd^{2+} stress with the following objectives: (i) to identify proteins potentially involved in Cd^{2+} detoxification, Cd accumulation, or the regulation of Cd^{2+} responses in plant roots; (ii) to find possible primary targets of Cd^{2+} toxicity; (iii) to gain insight into metabolic changes elicited by the generation of a strong intracellular sink for reduced sulphur; and (iv) to contribute to establishing reference data sets on plant proteome changes to environmental stimuli.

Materials and methods

All chemicals used were, if not further specified in the text, p.a. or electrophoresis grade. All electrophoresis units employed were from Amersham Biosciences.

Plant growth

Arabidopsis thaliana plants were cultivated as described by von Roepenack-Lahaye *et al*. (2004). Briefly, surface-sterilized seeds of ecotype Col-O were sown on agarose plugs (0.5%, w/v) and grown hydroponically in one-tenth Hoagland nutrient solution No. 2 (pH 5.3–5.5; Sigma-Aldrich, St Louis, MO, USA). The medium was supplemented with iron, chelated by *N*,*N*-di-(2-hydroxybenzoyl)-ethylenediamine-*N*,*N*-diacetic acid (HBED) to a final concentration of 5 μM Fe-HBED. Seedlings were grown for 6 weeks in hydroponic greenhouse boxes containing 10 plantlets each. The nutrient solution in the boxes was changed on a weekly basis and was aerated through 0.2 μm filters. Light conditions in the growth cabinet were fixed to 230–240 $\mu E\ m^{-2}\ s^{-1}$ and a photoperiod of 8 h of light/16 h of dark at 23 °C, day and night. Roots of plants were harvested ~1 h into the light period, pooled, and stored at –80 °C.

Phytochelatin analysis

A 100 mg aliquot of root tissue was homogenized in 200 μl of 0.1 M HCl spiked with 2.0 μl of 10 mM PC_2-NH_2 (=26.9 μg) using ultrasound (10 min). Following centrifugation at 19 000 *g*, 50 μl of the supernatant were incubated with 6 μl of 20 mM TRIS-(2-carboxyethyl)phosphine hydrochloride (TCEP) and 50 μl of MH_3BO_3 buffer, pH 9.2, for 30 min at ambient temperature in a brown Eppendorf tube. After addition of 10 μl of 50 mM monobromobimane (mBrB) (in CH_3CN), the mixture was incubated for 30 min at 45 °C. Through addition of 60 μl of 1 M methanesulphonic acid (MSA), the reaction was quenched. Before analysis by capillary liquid chromatography coupled to electrospray ionization quadrupole time-of-flight (LC–ESI-QTOF) mass spectrometry, the sample was filtered using a 0.45 μm PTFE syringe filter. A 2 μl aliquot of labelled extract was injected onto a Phenomenex Luna C8 column (particle size 3 μm, pore size 100 Å, length 150 mm, i.d. 0.3 mm). Separation was performed using the following gradient system: solvent A=H_2O/0.1% formic acid; solvent B=CH_3CN/0.1% formic acid; 0–5 min 95% A, 5% B; 5–40 min linear from 5% B to 22% B, 40–50 min 95% B, 50–60 min 5% B; flow rate: 5 $\mu l\ min^{-1}$. LC–ESI-QTOF mass spectra (positive ion mode) were recorded on an API QSTAR Pulsar Hybrid Quadrupole TOF instrument (Applied Biosystems, Foster City, CA, USA). The ion spray voltage was +5.5 kV; detected mass range, 910–930 Da; scan-rate, 0.5 s^{-1}; declustering potential 1, 50 V; declustering potential 2, 15 V. Each

sample was analysed twice, i.e. two extracts were run per sample. The signals for dialkylated PC2-NH_2 (*m/z* 919.0–919.5 $[M+H]^+$) and dialkylated PC2 (*m/z* 920.0–920.5 $[M+H]^+$) were integrated. PC2 was quantified based on calibration curves recorded for PC2 and PC2-NH_2 standards. PC2 and PC2-NH_2 standards were synthesized on an Abimed (Langenfeld, Germany) Economy Peptide Synthesizer EPS 211 using *N*-α-Fmoc-L-glutamic acid α-tert-butyl ester (Novabiochem, Läufelfingen, Switzerland).

Sample preparation for 2D PAGE

For 2D electrophoresis, *A. thaliana* root protein was isolated essentially according to a method described by Peck *et al.* (2001): 1.5 g of frozen root tissue was ground in liquid nitrogen using a pestle and mortar. The resulting powder was homogenized in 3 ml of cold extraction buffer [100 mM HEPES-KOH, pH 7.5, 5% (v/v) glycerol, 5 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 1% (v/v) protease inhibitor cocktail for plant cells (Sigma-Aldrich)] and incubated for 10 min on ice. To remove cell debris, the sample was filled into micro-centrifuge tubes and centrifuged at 13 000 rpm for 10 min at 4 °C. The supernatant was transferred to fresh tubes and again centrifuged at 13 000 rpm for 10 min to remove other remaining particles. The clarified supernatant was then mixed with an equal volume of TRIS-buffered phenol, pH 8 (Roth, Germany), and incubated for 5 min on ice. Subsequently, the mixture was centrifuged at 5000 rpm for 10 min at 4 °C to separate the aqueous from the phenol phase. The upper phase was removed and the remaining phenol phase was back-extracted twice with re-extraction buffer [100 mM TRIS, pH 8.4, 20 mM KCl, 10 mM EDTA, 0.4% (v/v) 2-mercaptoethanol] without disturbing the interphase. Protein was then precipitated from the separated phenol phase by incubation with 5 vols of 0.1 M ammonium acetate in methanol at –20 °C for at least 3 h. The precipitated protein was pelleted by centrifugation, washed once in 3 ml of chilled ammonium acetate/methanol and twice in 3 ml of 80% (v/v) acetone/20% (v/v) 50 mM TRIS–HCl, pH 7.5 to remove the phenol. After a final centrifugation step, the protein pellet was air-dried and resolubilized in lysis buffer [7 M urea, 2 M thiourea, 5 mM potassium carbonate, 0.4% (w/v) SDS, 50 mM dithiothreitol (DTT), 1 mM Pefabloc (Fluka), 2% (v/v) IPG buffer (Amersham Biosciences) pH 3–10 NL, 4% (w/v) CHAPS]. The protein content was quantified using the 2D-quant kit (Amersham Biosciences) with bovine serum albumin (BSA) in lysis buffer as standard.

2D protein gel electrophoresis

Arabidopsis thaliana root proteins were separated according to a protocol described by Görg *et al.* (2000). Isoelectric focusing: for analytical gels, sample aliquots of 100 µg (pH gradient 4–7) or 150 µg (pH gradient 5–6) of protein were diluted with rehydration buffer [8 M urea, 2% (w/v) CHAPS, 0.3% (w/v) DTT, 0.5% (v/v) IPG buffer pH 4–7 or 5–6 depending on the pH gradient used, 0.002% (w/v) bromophenol blue] to yield 450 µl. These mixtures were then loaded onto 24 cm IPG strips (Amersham Biosciences) covering pH gradients of 4–7 or 5–6 using the rehydration method. For micro-preparative gels, the strips were rehydrated with samples containing 250 µg (pH 4–7) or 300–450 µg (pH 5–6) of protein. Isoelectric focusing including low-voltage rehydration was performed on an IPGphor electrophoresis unit (Amersham Biosciences). Basically, for both pH gradients, the following voltage programme was applied: 12 h at 50 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 1.5 h gradient to 8000 V, whereas the final focusing step was adapted to the pH gradient used: 8 h at 8000 V for pH 4–7 and 9 h at 8000 V for pH 5–6. Following IEF, the strips were equilibrated for application to the second dimension (SDS–PAGE) for 15 min in equilibration buffer [50 mM TRIS–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.01% bromophenol blue] plus 1% (w/v) DTT and another 15 min in equilibration buffer plus 2.5% (w/v) iodoacetamide. Subsequently, the IPG strips were placed on top of self-cast large format SDS–polyacrylamide gels (24 cm×20 cm, 12.5% Duracryl Proteomics Solution) and sealed with warm agarose solution (0.5% agarose, 0.002% bromophenol blue in TRIS/glycine running buffer). Electrophoresis was carried out with a TRIS/glycine buffer system in an EttanDalt *twelve* chamber (Amersham Bioscience) and run at 25 °C and constant power (10 W per gel) until the dye front reached the bottom of the gels (~6 h). Gels were fixed in 40% (v/v) ethanol/10% (v/v) acetic acid overnight. Analytical gels were stained with silver according to Blum *et al.* (1987) with slight modifications. Briefly, after fixation, the gels were washed twice with 30% (v/v) ethanol and once with deionized water (Millipore system) for 20 min each. Gels were then sensitized with 0.02% sodium thiosulphate (w/v), rinsed with water, and incubated in 0.2% (w/v) silver nitrate plus 0.075% (v/v) formaldehyde for 20 min. After additional rinsing steps, the protein spots were visualized by incubation in developer solution [3% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde] until background signals started to appear. To stop the staining process, the gels were transferred to a new tray filled with 5% (v/v) acetic acid. Finally, the gels were washed in water, transferred to storage solution [20% (v/v) ethanol, 3% glycerol] and scanned using a scanner generating 16 bit greyscale images (UMAX PowerLook III, Labscan 3.01). Preparative gels were stained with colloidal Coomassie (Neuhoff *et al.*, 1988) or alcoholic Coomassie (Coomassie R 250) according to Fairbanks *et al.* (1971), but with accelerated de-staining accomplished by microwaving the gels.

Image analysis

Sixteen bit digitalized images of the gels were loaded into the Proteomweaver 1.3–2.3 (Definiens) software and spot patterns were analysed with respect to spots appearing to be differentially regulated after Cd^{2+} treatment. Gel matching was applied using the multiple spot-to-spot mapping resulting in the comparison of each individual gel image with all other gel images. Quantitative estimates for individual spots were obtained after each gel was normalized using a pre-match normalization factor of 1. Quantification was performed with a total number of 13 gels (seven gels for the Cd-treated group and six gels for the control group) originating from three independent experiments. Spots appearing in <4 gels in at least one group were considered as artefacts and not analysed further. Proteins were initially considered as up- or down-regulated when the average spot volume (integral of spot area and height) changed at least 2-fold upon stimulation. The candidate spot lists generated according to these criteria by the ProteomWeaver software were re-analysed in a second step and statistically evaluated manually. The G value (α=0.05) for each candidate spot volume was calculated to eliminate any outliers using the test of Grubbs (1969). Only one outlier per group was allowed. The outliers found were mostly due to incorrect spot detection or spot position close to the edge of a gel. Significance was then tested by performing a *t* test.

Protein identification

Validated protein spots were manually picked from gels and prepared for tryptic digestion. Coomassie-stained spots were washed three times (water; 50% acetonitrile; and 100% acetonitrile) and dried at room temperature. Silver-stained spots were destained using the SilverQuest destaining kit (Invitrogen GmbH, Karlsruhe, Germany) followed by two additional washing steps with 50% and 100% acetonitrile and drying of the spots. In-gel digestion was carried out by adding 5 µl of digestion buffer [5 mM NH_4HCO_3, 5% acetonitrile, 0.2 µg ml^{-1} porcine trypsin (proteomic grade; Roche, Penzberg, Germany)]. The gel plugs were incubated for 4 h at 37 °C. After incubation, 1 µl of the sample solution was transferred to a

400 μm Anchor Chip MALDI Plate. The target plate had been prepared with 0.7 μl of 2.5-dihydroxybenzoic acid (DHB; 10 g l^{-1} in water) added to each anchor. Tryptic peptide maps were recorded on a Bruker Reflex III time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) using the company's software packages (flexControl, XTOF, flexAnalysis). After internal calibration with the trypsin autolysis peaks, the search lists representing the peptide mass fingerprints were compared with genomic databases (MSDB, Matrix Science, London, UK; MIPS *Arabidopsis thaliana* Genome Database, http://mips.gsf.de). This was done using the MASCOT search engine (Matrix Science) at a tolerance of 0.1 Da.

Western blot analysis of ATP sulphurylase

For 2D western blot analysis, 100 μg of protein were loaded on 18 cm IPG strips and separated essentially as described above. However, the IEF programme was shortened (final focusing step 4 h at 8000 V) according to the reduced strip length compared with analytical 2D gels, and a Hoefer SE 600 chamber (Amersham Biosciences) was used to run the second dimension on 12.5% acrylamide gels. Blotting was done overnight using a tank blot system (Bio-Rad) on nitrocellulose membranes in accordance with standard protocols.

One-dimensional SDS–PAGE and western blot analysis were carried out according to standard protocols. The Precision Plus Protein standard (Bio-Rad) was loaded next to 20 μg of total root protein extracted as mentioned above. Acrylamide concentration was the same as described for 2D gels. For immunoblot analysis, 1:1000 rabbit polyclonal antisera against *A. thaliana* ATP sulphurylase (APS; courtesy of Drs Stan Kopriva and Rüdiger Hell) as well as a secondary anti-immunoglobulin G antibody raised in goat and conjugated to alkaline phosphatase (1:3000, Sigma-Aldrich) were used. Cross-reacting bands were detected with the BCIP-NBT system. Equal loading was confirmed by staining the blot with amido black.

GST pull-down assay

An aliquot of 500 μg of protein [1 μg μl^{-1} in extraction buffer: 100 mM HEPES-KOH, pH 7.5, 5% (v/v) glycerol, 5 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 1% (v/v) protease inhibitor cocktail for plant cells (Sigma-Aldrich)] was diluted in 1× phosphate-buffered saline (PBS), 0.1% Triton X-100 to a total volume of 750 μl. The sample was incubated with 250 μl of equilibrated GSH–Sepharose beads (Pharmacia) for 1.5 h at 4 °C to pull down glutathione *S*-transferases (GSTs). The beads were washed three times with 1× PBS and the proteins were eluted in rehydration buffer [8 M urea, 2% (w/v) CHAPS, 0.3% (w/v) DTT, 0.5 % (v/v) IPG buffer pH 3–10, 0.002 % (w/v) bromophenol blue, 10 mM GSH], to yield 350 μl. The complete GST fraction was subjected to 2D PAGE as described above and stained with an MS-compatible silver staining protocol according to Shevchenko *et al.* (1996).

Results

Roots are the site of impact under most natural conditions of toxic metal exposure. It was therefore decided to grow *A. thaliana* plants hydroponically and to treat them via the addition of Cd^{2+} ions to the growth medium. The analysis was also focused on root tissue because the response is likely to be synchronized here, while it is not clear when and how much Cd^{2+} reaches the shoot. Plants were treated with a Cd^{2+} concentration that did not cause visible symptoms up to the time point of analysis (24 h). Prolonged exposure to 10 μM Cd^{2+} (7 d), however, led to severe chlorosis in leaves (not shown). Cultivation in the presence of Cd^{2+} elicited a significant accumulation of PCs, a reliable marker for Cd^{2+} responsiveness (Cobbett and Goldsbrough, 2002). PC2 levels increased within 24 h by more than a factor of 6 (Fig. 1) as determined by capillary LC–ESI-QTOF-MS and based on the use of PC2-amide as an internal standard. After 7 d of exposure, PC2 accumulation reached a concentration >80-fold higher than in untreated controls (not shown).

In order to maximize the number of identifiable significant changes in the root proteome under these conditions, (i) a comparatively large number of samples were analysed and (ii) the 2D gel electrophoresis was performed using both a regular pH 4–7 and a 'zoom' gradient of pH 5–6 to increase resolution in the area with the highest spot density. Large-format high resolution gels of samples from three independent experiments (performed over a time span of 8 months) were run, silver-stained, and analysed using the ProteomWeaver software. On average, 1080 spots were detectable in pH 4–7 gels and 1038 in pH 5–6 gels. Of these, 752 could be matched in pH 4–7 gels and 672 in pH 5–6 gels. Figure 2 shows representative annotated images for control and Cd^{2+}-treated samples, separated in pH 4–7 and pH 5–6 gels. Protein spots showing a significant difference in abundance between control and Cd^{2+}-treated samples were selected for identification. Listed in Table 1 are all spots that after statistical re-analysis showed a >1.5-fold change (P <0.05) in at least one of the gradients. Additionally, changes are listed that were supported by more than one observation, e.g. proteins detected in two spots. Figure 3 highlights examples of protein spots showing significant differences in intensity. Using a pH 4–7 gradient in the first dimension, 17 spots were found to show higher intensity (i.e. greater spot volume) upon 24 h Cd^{2+} treatment and two to show lower intensity. Five of the 17 spots were not detectable in control samples. Within the pH 5–6 gradient analysis group, 20 spots showed higher intensity in extracts of Cd^{2+}-treated roots (one of these was not

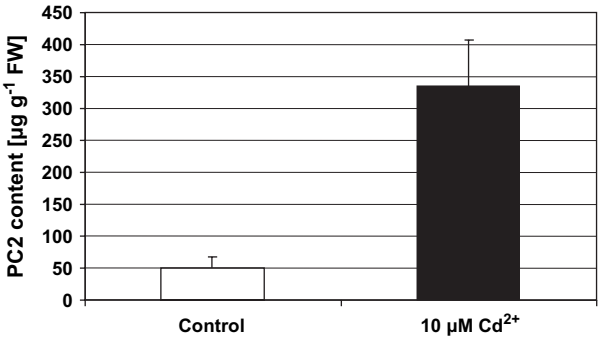

Fig. 1. Accumulation of PC2 upon exposure to Cd^{2+}. Monobromobimane-derivatized root extracts of control (white bar) and 24 h Cd^{2+}-treated plants (black bar) were analysed by capillary LC–ESI-QTOF-MS. PC2 was quantified using PC2 amide as an internal standard. Error bars represent the SD of three independent experiments.

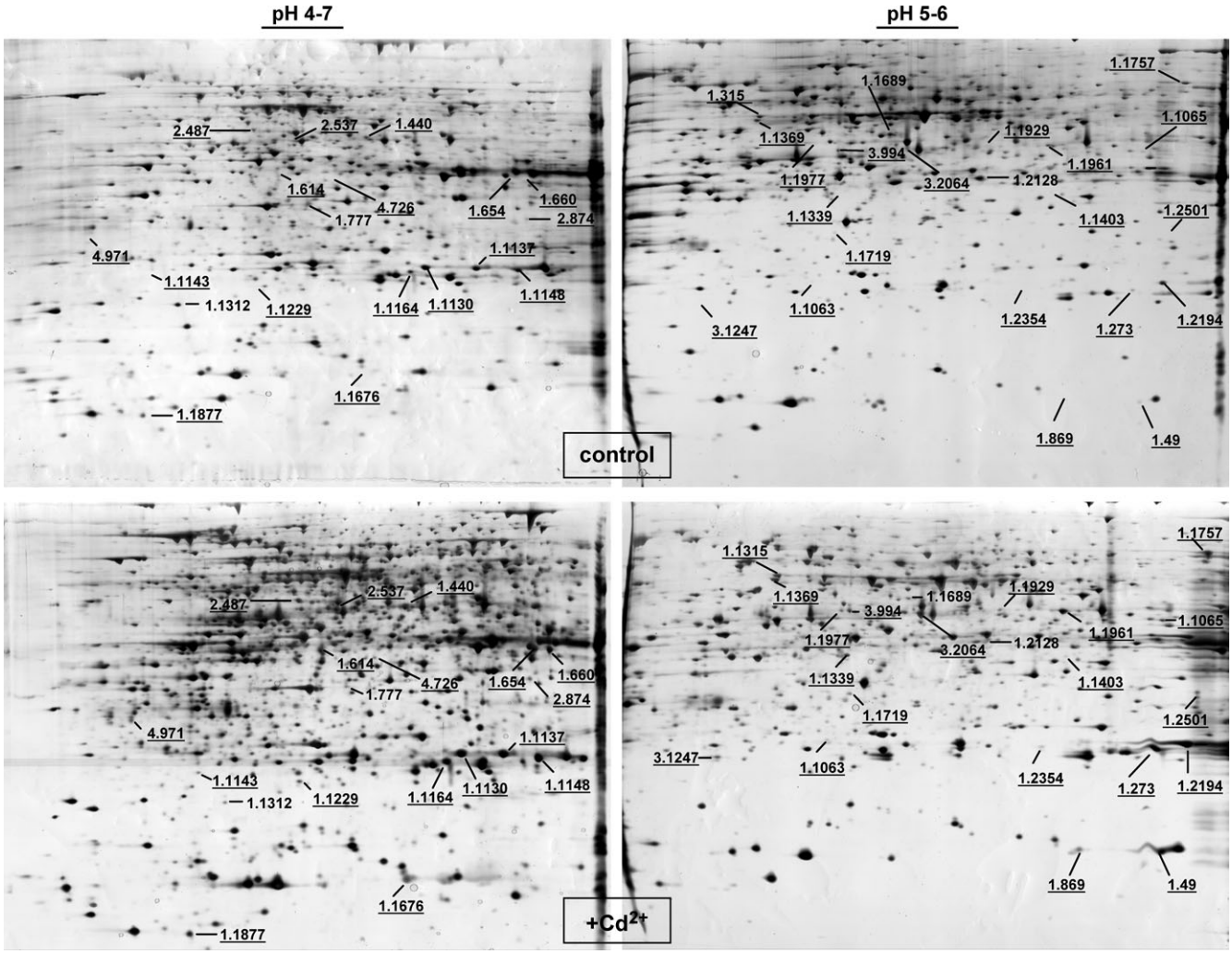

Fig. 2. Annotated images of silver-stained two-dimensional gels. All samples were analysed with pH gradients 4–7 (left) and pH 5–6 (right) in the first dimension. Shown are the separations of soluble proteins from control (top) and Cd^{2+}-treated (bottom) roots. Underlined spot identities indicate higher abundance under the latter conditions.

detectable in control extracts) and two spots indicated lower abundance. For identification, proteins were excised either from the original silver-stained gels or from preparative Coomassie-stained gels, subjected to trypsin digestion, and analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The resulting spectra were used to query databases for protein identification. For 25 of the 41 protein spots (=61.0%), an identification with a significant score (P <0.05) and at least five matching peptides was achieved. These 25 spots represent 17 different proteins. Table 1 shows a summary of the results including statistics of intensity data, Mowse scores, number of matched peptides, and annotation. Five proteins were identified in both types of 2D gels.

Most of the identified proteins fell into one of four classes: metabolic enzymes, GSTs, proteins annotated as similar to latex allergens, and hypothetical/unknown proteins. The metabolic enzymes include APS1, glyceraldehyde 3-phosphate dehydrogenase, alanine aminotransferase, dioxygenase, NADP-dependent malic enzyme, and glycine hydroxymethyltransferase. All three GSTs belong to the plant-specific phi class (Dixon *et al*., 2002). The two proteins annotated as latex allergen-like are a pathogenesis-related protein of the PR-10 family, also classified as Bet v I (Pfam domain 00407) (At4g23670), and a patatin-like phospholipase (At2g26560). The only proteins outside these categories were subtilase, i.e. a serine peptidase, and RNA helicase.

The validity of the proteome data was tested independently in two ways. First, for one of the proteins, APS1, which was identified in both gradients, the apparent increase in abundance under Cd^{2+} stress was checked by SDS–PAGE/western analysis. In Fig. 4, 2D gel and western results are shown. The antibody staining clearly confirmed the data of the proteome analysis both in one-dimensional and

Table 1. *Root proteins detected in pH 4–7 and/or pH 5–6 gels as showing significant changes in abundance upon exposure to 10 μM Cd^{2+} for 24 h*

The list contains protein spots that indicated a quantitative change (fold change and *P*-value are given) as well as protein spots that were not detectable in extracts from control samples (iwc=induced without counterspot). Included in the list are all proteins that showed a significant change in abundance by a factor >1.5 ($P < 0.05$) for at least one spot and in at least one pH gradient.

Spot ID		AGI code	Annotation	Fold induction		*P*-value		Mol. wt (Da)	pI	Score		No. of peptides matched	
pH 4–7	pH 5–6			pH 4–7	pH 5–6	pH 4–7	pH 5–6			pH 4–7	pH 5–6	pH 4–7	pH 5–6
1.440	1.1961	At3g22890	ATP sulphurylase, APS1	2.2	1.9	<0.001	0.01	51 484	6.34	93	92	9	6
1.1130	1.2194	At4g02520	Atpm24.1 glutathione *S*-transferase, AtGSF2	2.0	2.2	0.05	0.14	24 114	5.92	156	213	10	13
1.1137		At4g02520	Atpm24.1 glutathione *S*-transferase, AtGSTF2	1.8		0.02		24 114	5.92	131		10	
1.1148		At1g02920	Glutathione *S*-transferase, AtGSTF7	2.5		0.002		23 554	6.3	134		9	
1.1164	1.273	At1g02930	Glutathione *S*-transferase, AtGSTF6	4.3	5.4	0.002	0.007	23 486	5.8	114	82	7	5
1.1676	1.49	At4g23670	Putative major latex protein, Bet v I, PR-10 family	5.7	2.7	0.02	0.03	17 621	5.91	64	140	4	9
2.537	3.2064	At2g26560	Similar to latex allergen from *Hevea brasiliensis*, patatin-like phospholipase	iwc	5.8	<0.001	100	44 326	5.57	87	80	8	7
1.614		At5g65140	Trehalose-6-phosphate phosphatase	1.7		0.02		43 371	9.42	65		6	
1.654		At1g13440	Glyceraldehyde 3-phosphate dehydrogenase	iwc		<0.001		37 004	6.67	83		21	
1.660		At1g13440	Glyceraldehyde 3-phosphate dehydrogenase	iwc		<0.001		37 004	6.67	108		15	
1.777		At5g57410	Unknown protein	0.3		0.01		42 804	5.94	60		6	
1.1143		At5g59190	Peptidase/subtilase	iwc		<0.001		74 373	5.72	52		5	
1.1229				2.1		0.02							
1.1312				0.2		0.007							
1.1877		At1g36440	Hypothetical protein	1.9		0.008		46 183	6.09	65		6	
2.487				8.6		<0.001							
2.874				9.9		<0.001							
4.726				iwc		0.03							
4.971				4.9		<0.001							
	1.315	At1g17290	Alanine transaminase (ALAAT1)		1.3		0.13	53 955	5.39		97		7
	1.869				3.1		0.05						
	1.1063				2.6		0.006						
	1.1065				2.1		0.02						
	1.1339	At5g64250	2-Nitropropane dioxygenase-like protein		1.8		0.05	36 067	5.38		88		5
	1.1369	At1g17290	Alanine transaminase (ALAAT1)		2.1		0.01	59 534	5.91		87		7
	1.1403				1.9		0.02						
	1.1689	At5g11170	DEAD box RNA helicase RH15-like		0.4		<0.001	48 324	5.42		71		5
	1.1719	At3g48670	Putative protein; MIPS: myosin heavy chain		5.1		<0.001	75 177	5.64		74		5
	1.1757	At5g11670	NADP-dependent malic enzyme		1.7		0.02	64 413	6.01		204		16
	1.1929				2.6		0.01						
	1.1977				2.9		0.04						
	1.2128				0.6		0.02						
	1.2354				2.2		0.03						
	1.2501				4.3		<0.001						
	3.994	At1g36370	Glycine hydroxymethyltransferase		3.0		0.04	66 294	8.07		57		5
	3.1247				iwc		0.004						

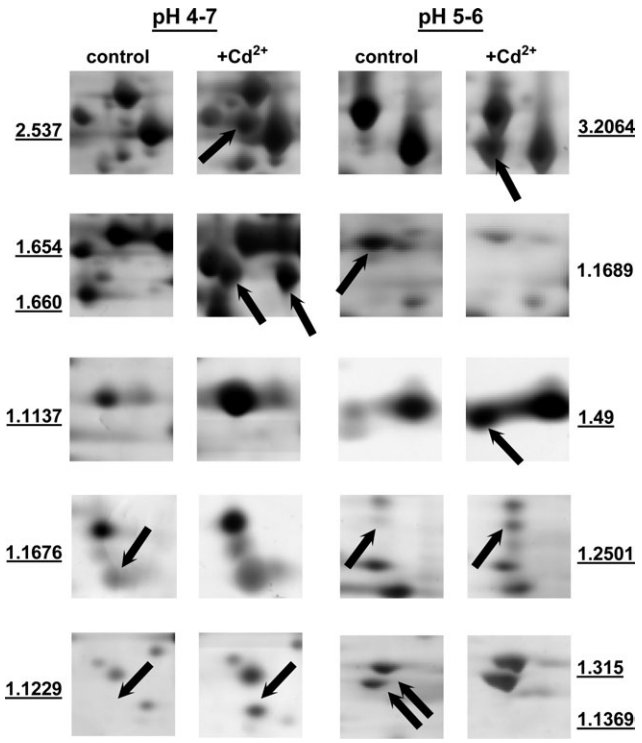

Fig. 3. Examples of protein spots from pH 4–7 and pH 5–6 gels that showed changes in abundance. For protein identities, refer to Table 1. Underlined spot identities indicate higher abundance in extracts of Cd^{2+}-treated roots. Spots 2.537 and 3.2064 (first row) represent the same protein and serve as an example to illustrate the difference in resolution between pH 4–7 and pH 5–6 gels.

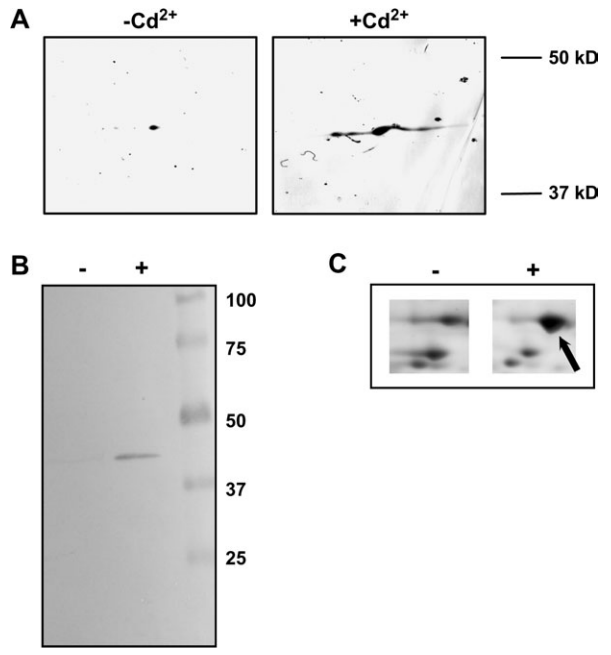

Fig. 4. Confirmation of higher ATP sulphurylase abundance in Cd^{2+}-treated roots. Soluble root protein from control and Cd^{2+}-treated plants was separated by two-dimensional (A) and one-dimensional (B) gel electrophoresis. Western blots were stained with an ATP sulphurylase antiserum. For comparison, the respective 2D gel image is shown (C).

in two-dimensional gels. Secondly, because three of the identified proteins belonged to the large class of GSTs, GSH-binding proteins were selectively enriched by affinity chromatography and the 2D maps of extracts derived from control and Cd^{2+}-treated samples (Fig. 5) were compared. The up-regulation of GSTs was clearly detectable. Four different GSTs and a putative protein (At5g48160) were identified in nine protein spots (Table 2). Three of the four GSTs were identical to the ones already identified as being more abundant in Cd^{2+}-treated roots.

Discussion

Given the non-essential nature of Cd^{2+}, surprisingly specific and well-regulated responses to Cd^{2+} exposure have been identified in the unicellular organisms *S. cerevisiae* and *Schizosaccharomyces pombe*. In *S. cerevisiae*, the sulphur amino acid biosynthesis pathway is activated by Cd^{2+}, but not by other abiotic stress treatments (Vido *et al.*, 2001). *Schizosaccharomyces pombe* shows up-regulation of a set of Cd^{2+}-specific genes (Chen *et al.*, 2003). Much less is known about changes in multicellular organisms exposed to toxic metal ions, because studies at the proteome level have been lacking until recently (Sarry *et al.*, 2006). In order to establish a starting point for the elucidation of plant Cd^{2+} responses and to identify proteins of potential biotechnological interest that might contribute to cadmium detoxification and accumulation, changes in the root proteome of *A. thaliana* were determined by high-resolution 2D-PAGE and MALDI-TOF-MS. Plants were exposed to a sublethal toxic dose of Cd^{2+}, which elicited the expected metabolic changes as indicated by the accumulation of PCs. The use of two different pH gradients in the first dimension clearly increased the number of detected changes. More than half of the differentially expressed proteins could be identified from either Coomassie- or silver-stained gels.

Of the identified changes in metabolic enzymes, the up-regulation of an APS was to be expected. PC synthesis and chelation of Cd^{2+} by GSH generate a strong sink for reduced sulphur. APSs catalyse the first step in S assimilation, the activation of sulphate. Up-regulation of APS transcript levels in the presence of Cd^{2+} has been found before (Heiss *et al.*, 1999; Weber *et al.*, 2006). The present 2D gel and western data show that this key step in the pathway is also regulated at the protein level, an observation supported by de Knecht *et al.* (1995), who demonstrated strongly enhanced APS enzyme activity in *Silene vulgaris* under Cd^{2+} exposure. No other component of S assimilation, cysteine biosynthesis, or GSH biosynthesis was found in the present study. Since (i) only proteins are identified that showed a significant change in abundance, and (ii) the coverage of the proteome is limited [sulphate transporters, for instance, which have also been

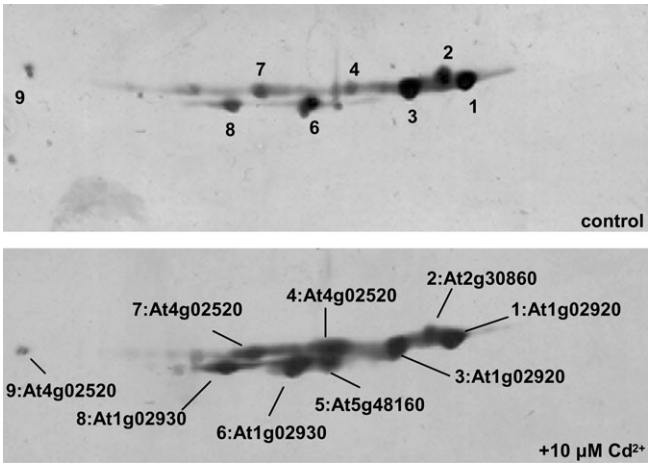

Fig. 5. Separation of GSH-binding proteins by two-dimensional gel electrophoresis. Protein extracts from control (top) and Cd^{2+}-treated (bottom) samples were subjected to affinity chromatography on GSH–Sepharose. The GSH eluate was separated on a pH 3–10 two-dimensional gel and silver stained. Spots were picked and analysed by MALDI-TOF-MS. Details of the identification are listed in Table 2.

Table 2. *Proteins identified by MALDI-TOF-MS following affinity chromatography on GSH–Sepharose and two-dimensional gel electrophoresis (see Fig. 5)*

Spot ID	AGI code	Protein name	Molecular weight (Da)	pI	Mowse score	No. of peptides matched
1	At1g02920	GSTF7	23 586	6.14	182	11
2	At2g30860	GSTF9	24 131	6.17	115	10
3	At1g02920	GSTF7	23 583	6.14	190	12
4	At4g02520	GSTF2	24 114	5.92	171	12
5	At5g48160	Putative protein	66 126	6.43	89	8
6	At1g02930	GSTF6	23 471	5.80	141	9
7	At4g02520	GSTF2	24 114	5.92	156	10
8	At1g02930	GSTF6	23 471	5.80	168	10
9	At4g02520	GSTF2	24 114	5.92	95	10

shown to be up-regulated under conditions of heavy metal stress (Saito, 2004), are unlikely to be present in gels separating soluble proteins], conclusions cannot be drawn about other S assimilation enzymes. However, a related cross-species study on rapid transcriptome changes in *A. thaliana* and *A. halleri* under metal excess indicated that the up-regulation of sulphate activation is indeed the most pronounced and robust response to Cd^{2+}-induced consumption of reduced sulphur. One of only four genes belonging to the 'core response' shared by the two species encodes an APS (Weber *et al.*, 2006). Since there is little evidence for *APS* up-regulation under sulphur-depleted conditions, this result was interpreted as further evidence for the suggestion by Heiss *et al.* (1999) that distinct signalling cascades are activated, depending on whether there is sulphur starvation or a strong intracellular sink for sulphur.

An increase in glycine hydroxymethyltransferase abundance might represent a second direct consequence of activated PC synthesis as glycine is a component of PCs. In Cd^{2+}-exposed *A. thaliana* cell cultures, a different isoform of this enzyme (At4g37930) is up-regulated (Sarry *et al.*, 2006). In fact, the recurring themes in all global analyses of *A. thaliana* Cd^{2+} responses at the transcriptome or proteome level published to date are the up-regulation of S assimilation and the modulation of amino acid biosynthesis, albeit that different components of the respective pathways have been identified (Kovalchuk *et al.*, 2005; Weber *et al.*, 2006). It is generally assumed that amino acids and other molecules such as polyamines play an important role in excess metal detoxification (Sharma and Dietz, 2006). Other consistent metabolic changes are not apparent from the published studies, most probably due to the fact that different systems (cell cultures, roots, and leaves) and different conditions have been investigated.

Higher levels of a trehalose-6-phosphate phosphatase might indicate an accumulation of trehalose in *A. thaliana* roots under Cd^{2+} stress. Trehalose is a disaccharide that has long been known as a stress protectant from bacteria and yeast but has only recently been found in plants. Overproduction of trehalose in transgenic rice plants enhances salt tolerance and influences the mineral status (Garg *et al.*, 2002). Thus, trehalose could also potentially have a protective function under the protein denaturing conditions of Cd^{2+} exposure.

The significance of the other changes in metabolic enzymes is not obvious. No indication of a sulphur-sparing response as described for *S. cerevisiae* (Fauchon *et al.*, 2002) was found. The up-regulated isoforms of GAPDH and malic enzyme are—according to microarray and expressed sequence tag (EST) data—the major ones expressed in *A. thaliana* roots. Therefore, they are unlikely to represent S-depleted isoforms specifically induced under S-limiting conditions like the pyruvate decarboxylase Pcd6 and other isozymes in *S. cerevisiae*.

Five of the identified proteins can be designated typical stress proteins. So-called pathogenesis-related proteins of the PR-10 or Bet v I family have been found in extracts of stress-treated plant samples before, for instance, after herbicide treatment of grapevines (Castro *et al.*, 2005) and in Cu-stressed birch leaves and roots (Utriainen *et al.*, 1998). The function of PR-10 proteins is unclear. One recent hypothesis states that they represent steroid carriers (Markovic-Housley *et al.*, 2003). The other protein annotated as latex allergen-like is homologous to phospholipases A of the patatin-type. One might speculate about a possible role in the mediation of Cd^{2+} responses.

Finally, three GSTs of the plant-specific phi class were more abundant under Cd^{2+} stress. They were each found in several positions on the 2D maps for crude extracts and for enriched fractions. This was also described by Sappl *et al.* (2004). GSTs are assumed to play a protective role against

xenobiotics and oxidative stress. Transcript levels are typically up-regulated under various stress conditions (Dixon *et al*., 2002). Recent studies showed that the respective proteins are also more abundant (Sappl *et al*., 2004; Smith *et al*., 2004). Interestingly, under conditions of Cu^{2+} excess, the exact same GSTs as identified in this study (AtGSTF2, AtGSTF6, and AtGSTF7) were more abundant in *A. thaliana* seedlings (Smith *et al*., 2004). The latter two were also identified in Cd^{2+}-treated cell cultures (Sarry *et al*., 2006). Within the large GST family in *A. thaliana*, the identified proteins are encoded by genes which belong to the most highly expressed ones (Smith *et al*., 2004). As to their functions, they are poorly understood for plant GSTs in general. The plant-specific phi class might have evolved to counteract the consequences of generation of reactive oxygen species during photosynthesis (Edwards *et al*., 2000). Besides the well-established conjugation of xenobiotics, GST activities include GSH peroxidation and the binding of metabolites such as flavonoids. The latter was also demonstrated recently for AtGSTF2 (Smith *et al*., 2003). Still, it remains to be determined what the functional significance of up-regulation in cells under metal stress is, to what extent GSTs can exert a protective effect on metal-treated plants, and whether the metal induction is a direct result of GSH depletion (Smith *et al*., 2004) or rather triggered by the occurrence of oxidative stress.

An important question of stress physiology is, which of the changes detected under certain conditions are specific responses to the stimulus under investigation and which ones are secondary responses to damage. Addressing this question requires large data sets generated for a high number of stress conditions. At the proteome level, these are not available yet. Therefore, transcript data were used here which might be informative as they at least allow differentiation between common stress responses and candidates for specific responses. The Genevestigator database, containing the results of >2000 Affymetrix ATH1 gene chip experiments performed with *A. thaliana* (Zimmermann *et al*., 2004), was queried. An analysis of the genes encoding the proteins identified in the proteome analysis revealed that they cluster into two groups: genes that are barely responsive to stress treatments and genes that are responsive to a large number of stress treatments. Genes belonging to the former category encode the APS, the NADP-dependent malic enzyme, the GAPDH, the unknown protein At5g57410, the putative protein At3g48670, the subtilase, and the trehalose-6-phosphate phosphatase. The alanine aminotransferase, the glycine hydroxymethyltransferase, the 2-nitropropane dioxygenase-like protein, the patatin-like phospholipase, the PR-10 protein, and the three GSTs are encoded by genes that show pronounced changes in transcript abundance under numerous conditions. Reverse genetics studies will be needed to determine whether especially any of the potentially more Cd^{2+}-specific responses have a measurable effect on Cd accumulation and/or tolerance.

The underlying sensing and signal transduction mechanisms that are involved in transcriptome and proteome changes under Cd^{2+} exposure are largely unknown. There is very little information about the type of signalling molecules and signalling cascades required for responses to Cd^{2+} in plants. Available evidence indirectly implicates the phytohormone jasmonate and phosphorylation cascades. Jasmonate was proposed to be involved in the transcriptional control of GSH biosynthesis genes under metal excess, because jasmonate treatment, but not application of other stress signals such as salicylic acid or H_2O_2, led to an up-regulation of *gsh1* and *gsh2* (Xiang and Oliver, 1998). A possible phosphorylation dependence of transcriptional Cd^{2+} responses in *A. thaliana* was found for some of the genes identified in a fluorescence differential display screening. Pretreatment with the kinase inhibitor staurosporine prior to Cd^{2+} application suppressed the increase in transcript abundance (Suzuki *et al*., 2001). Furthermore, mitogen-activated protein kinases (MAPKs), which are well known as mediators of stress responses, were found to be activated under Cu^{2+} and Cd^{2+} excess in *Medicago sativa*. Differences in kinetics and in dependence on various tested MAPK kinases led the authors to suggest that distinct metal-specific signalling pathways exist in plants (Jonak *et al*., 2004). A direct functional link between signal pathways and responses at the transcript and/or protein level has not been established yet. There are no reports on Cd^{2+} responses in jasmonate-insensitive mutants, for instance, and targets of the metal stress-activated MAPKs are unknown.

Recent data from both *S. cerevisiae* and *S. pombe* demonstrated that specific Cd^{2+}-triggered signalling does exist in nature (Barbey *et al*., 2005; Harrison *et al*., 2005). Available microarray data on Cd^{2+}-treated *A. thaliana* roots at least indicate that Cd^{2+}-specific responses might also be trackable in plants (Weber *et al*., 2006). It will be important to see which of the known signalling pathways in plants are involved in mediating the now identified Cd^{2+}-induced changes in the proteome and what upstream factors are essential for the expression of these Cd^{2+} responses. This might also contribute to shedding light on the largely unknown signalling pathways involved in regulation under sulphur nutritional stress (Saito, 2004).

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