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ORIGINAL ARTICLE

Differential responses of microsomal proteins and metabolites in two contrasting cadmium (Cd)-accumulating soybean cultivars under Cd stress

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Abstract While there are significant genotypic differences in cadmium (Cd) uptake and distribution in soybean cultivars, little attention has been paid to the underlying molecular mechanisms. We adopted a comparative proteomic approach coupled with metabolite analysis to examine Cd uptake and translocation in two contrasting Cd-accumulating soybean cultivars, Enrei and Harosoy, which accumulate higher amount of Cd in the roots and aerial parts, respectively. Proteins extracted from the root microsomal fraction were evaluated by immunoblot analysis using different subcellular marker proteins. Analysis of control and Cd-exposed samples by two-dimensional gel electrophoresis coupled with mass spectrometry revealed a total of 13 and 11 differentially expressed proteins in the Enrei and Harosoy cultivars, respectively. Metabolome profiling identified a total of 32 metabolites, the expression of 18 of which was significantly altered in at least in one cultivar in response to Cd stress. Analysis of the combined proteomic and metabolomic results revealed that proteins and amino acids associate with Cd-chelating pathways are highly active in the Enrei cultivar. In addition, proteins associated with lignin biosynthesis are significantly upregulated in the Enrei cultivar under Cd stress. Our results indicate that in the Enrei cultivar, Cd-chelating agents may bind excess free Cd ion and that translocation of Cd from the roots to the aerial parts might be prevented by increased xylem lignification.

Keywords Cadmium · Lignifications · Metablomics · Proteomics · Soybean

Introduction

Among the heavy metals, cadmium (Cd) is one of the most toxic nonessential trace elements. Cadmium has a long biological half-life and can be readily taken up by roots and rapidly translocated to the aerial parts of plants (Wagner 1993). Cadmium ion is highly mobile, has a high water solubility and affinity for sulfhydryl groups of proteins and enzymes, and is chemically similar to other functionally active ions. Uptake of Cd ions leads to a number of negative effects, including inhibition of enzyme activity or disruption of protein structure, or displacement of essential elements resulting in deficiency effects, and inhibition of several biochemical pathways (Van Assche and Clijsters 1990; Hall 2002; Schützendübel and Polle 2002).

The toxic effect of Cd in plants, particularly in crop plants, has been extensively studied. It has been demonstrated that crop species differ widely in their sensitivity to and ability to accumulate Cd (Murakami et al. 2007). Moreover, the uptake of Cd by crops varies not only among species but also among cultivars (Grant et al. 2008). Arao et al. (2003) found considerable genetic variation in soybean cultivars that differed in the uptake, accumulation, and translocation of Cd from roots to aerial parts of the plant. A number of cultivars, such as Enrei, accumulate higher amounts of Cd in the roots; however, the

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translocation rate in the aerial portions of the plant is quite low compared with other cultivars that contain higher amounts of Cd in the seeds, such as Harosoy (Arao et al. 2003).

To cope with Cd stress, plants employ a range of cellular detoxification mechanisms. Cadmium tolerance or accumulation and detoxification in plants are apparently organ-specific, with pathways involving organs such as the cell wall, plasma membrane, cytosol, and vacuolar compartments (Hall 2002). Thus, it is believed that several genes or proteins are involved in Cd accumulation, translocation, and detoxification pathways in plants. Identifying the functional genes or proteins that are involved in organ-specific Cd-response pathways is a fundamental step in understanding the molecular mechanisms of detoxification or accumulation of toxic Cd in plant cells.

Roots are the first organ to contact heavy metals in soil solutions. The membrane is the first living cellular barrier to free inward diffusion of heavy metals, and is also considered to be the first target of Cd toxicity in plant root cells. Membrane proteins play important roles in various cellular processes and also modulate diverse signaling pathways during Cd stress. Plant cells posses a number of transporters that are directly involved in Cd uptake and translocation mechanisms (Cobbett et al. 2003; Hall and Williams 2003; Hanikenne et al. 2005). However, knowledge regarding the mechanisms that control the entrance of Cd and its translocation from root to shoot is still rudimentary. Identification of the genes encoding membrane proteins involved in these processes is a key issue for obtaining a better understanding of Cd accumulation, translocation, and detoxification processes.

The major advantage of proteomics is that it focuses on the functional translated portion of the genome. It has been suggested that a comparative proteomic approach involving tolerant and sensitive cultivars undergoing heavy metal stress could clarify the mechanism leading to heavy metal tolerance (Ahsan et al. 2009). Consistent with this suggestion, Hradilová et al.'s (2010) proteomic study suggested that tolerance in Cd-tolerant flax cultivars may involve Cd binding by ferritin and low-molecular-weight thiol peptides to maintain low Cd levels at sensitive sites.

Although the use of proteomic technologies has expanded rapidly in research involving Cd stress biology in plants, very few studies have investigated the subcellular and tissue-specific proteome responses under Cd stress (Ahsan et al. 2009). A recent comparative proteomic study of *Poplus* leaf and cambial tissue revealed an important tissue-specific response of plants under Cd stress (Durand et al. 2010). These studies improved our understanding of the total soluble proteome changes in response to Cd stress. However, limited information is available regarding how subcellular organs respond to Cd stress. Analyses of the subcellular proteome could provide a better understanding of the molecular mechanisms of Cd translocation and accumulation. In order to obtain a better insight into the molecular mechanism of Cd accumulation and translocation in soybeans, we employed a comparative proteomic approach coupled with metabolite analysis of root microsomal fraction proteins from the high and low Cd-accumulating cultivars, Enrei and Harosoy, respectively.

Materials and methods

Plant growth, treatments and sample collection

Two contrasting Cd accumulator soybean (*Glycine max* L.) cultivars Enrei and Harosoy (Arao et al. 2003) were used as primary source of samples. Soybean seedlings were grown in a controlled growth chamber under white fluorescent light (300–350 μmol m^{-2} s^{-1}, 16 h light/8 h dark) at 25–20°C ± 2°C (day/night) temperatures and 75% relative humidity. Two-week-old seedlings were subjected to a solution culture containing 100 μM of Cd ($CdCl_2$, Wako, Osaka, Japan) for 3 days in controlled growth chambers. It has been reported in several biochemical and proteomic studies that a concentration of 50–100 μM Cd is considered as toxic to crop plants and able to regulate the protein and metabolite level significantly (Aina et al. 2007; Fagioni and Zolla 2009; Hradilová et al. 2010; Lee et al. 2010). Therefore, in the present study, 100 μM Cd was used to identify Cd stress responsive microsomal proteins in soybean roots. For copper (Cu) treatments, seedlings were exposed to 100 μM copper ($CuSO_4$, Wako, Osaka, Japan) for 3 days under the conditions mentioned. After each treatment, root samples (excised at 2 cm distance from the root–shoot transition zone) were harvested, followed by five washing steps with distilled water, immediately frozen in liquid nitrogen, and kept at −80°C. These samples were used for protein or other metabolite analyses.

Extraction of microsomal proteins

Microsome was fractionated from the roots according the protocol of Lee et al. (2004) with modifications. Briefly, ground root powder (5 g) was homogenized in 15 mL of ice-cold extraction buffer containing 0.5 M Tris–HCl (pH 8.0), 2 mM EDTA, 2 mM DTT, 0.25 M sucrose, and 1 mM phenylmethanesulfonyl fluoride followed by centrifugation at 8,000×*g* for 15 min at 4°C. After centrifugation, the membrane vesicles were pelleted from the resulting supernatant by centrifugation at 100,000×*g* for 1 h at 4°C. The pellet (contained microsomal fraction) was collected and rewashed with the same extraction buffer followed by centrifugation at 100,000×*g* for 1 h at 4°C.

The corresponding pellet consisting the microsomal fraction was resuspended with 1 mL lysis buffer containing 8.5 M urea, 2.5 M thiourea, 5% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1% dithiothreitol (DTT), and 1% Triton X-100. An equal volume of Tris–HCl saturated phenol (pH 8.0) was then added and mixed well by vigorous vortexing for 2 min followed by centrifugation at 3,500×g for 15 min. After centrifugation, the top phenol phase was collected, and proteins were precipitated by mixing with four volumes of cold methanol containing 0.1 M ammonium acetate at −30°C for 2 h.

Immunoblot analysis

Protein samples (25 µg) were separated on 15% SDS-PAGE and then transferred onto a PVDF membrane. The blotted membrane was blocked overnight at 4°C in TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 5% non-fat milk (Skim milk; Difco, MD, USA). The membrane was subsequently incubated with the polyclonal antibodies anti- calnexin (Nouri and Komatsu 2010), anti- HSP70 (H5147, Sigma-Aldrich, St. Louis, MO, USA), anti-ascorbate peroxidase (APX) (kindly provided by Dr. K. Yukawa, NICS, Tsukuba, Japan), anti-ATP-β (AS05 085, Agrisera, Vannas, Sweden) at 1:5,000 dilutions for 3 h at room temperature. Secondary antibodies were anti-mouse or anti-rabbit IgG with conjugated horseradish peroxidase (HRP; Bio-Rad). After incubation for 1 h with the appropriate HRP-conjugated secondary antibodies, the immunoblot signals were detected using an ECL plus western blotting detection kit (GE Healthcare, NJ, USA) following the manufacturer's protocols and visualized on X-ray films (Hyperfilm^{TM}, GE Healthcare).

Two-dimensional gel electrophoresis analysis and image acquisition

The purified protein pellet was air dried and re-suspended with solubilization buffer containing 8.5 M urea, 2.5 M thiourea, 5% CHAPS, 1% DTT, 1% Triton X-100, and 0.5% Biolyte (pH 5–8, Bio-Rad, Hercules, CA, USA). The solubilized protein was quantified using an RC-DC Protein Assay kit (Bio-Rad) and bovine serum albumin as the standard. For each sample, a total of 100 and 350 µg of solubilized proteins were applied to the immobilized pH gradient dry strips pH 3–10 (nonlinear, 11 cm, ReadyStrip, Bio-Rad) with 12 h rehydration following the manufacturer's instruction. Isoelectric focusing was performed using PROTEAN IEF Cell (Bio-Rad) at 20°C with a total of 35,000 V-h. After IEF, the IPG strips were equilibrated for 15 min in an equilibration buffer containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 2% DTT, followed by 15 min in an equilibration buffer containing 2.5% iodoacetamide. SDS-PAGE in the second dimension was carried out using 15% separation gel with 5% stacking gel at 30 mA for about 3 h, or until the dye line reached to the end of the gel. 2-DE gels were silver-stained using Sil-Best Stain Kit (Nacalai, Kyoto, Japan) or Coomassie Brilliant Blue (CBB) stained.

The silver-stained gels obtained from three biological replicates were scanned using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer; Bio-Rad). Spots were detected and quantified with the PDQuest software (ver. 8.0; Bio-Rad), on the basis of their relative volume. The amount of a protein spot was expressed as the volume of that spot, which was defined as the sum of the intensities of all pixels that make up the spot. To compensate for subtle differences in sample loading, gel staining, and destaining, the volume of each spot was normalized as a percentage of the total volume of all the spots present in the gel (Ahsan and Komatsu 2009). After automated detection and matching, manual editing was carried out.

In-gel digestion and nanoLC-MS/MS analysis

Proteins showed at least 1.5-fold differences in expression values compared with the control were considered as differentially expressed proteins and were subjected to analysis. Protein spots of interest were excised manually from CBB stained 2-DE gels and then alkylation and protein digestion with trypsin were performed using a robotic system (DigestPro96; Intavis AG, Koeln, Germany). The tryptic peptides were extracted from the gel grains with 0.1% trifluoroacetic acid in 50% acetonitrile three times. The generated peptides were purified using a NuTip C-18 (Glygen, Columbia, MD, USA). The desalted peptide solution was directly analyzed by nano liquid chromatography-tandem mass spectrometry (MS) as described previously (Komatsu et al. 2009). Using an Ultimate 3000 nanoLC (Dionex, Germering, Germany), peptides were loaded in 0.1% formic acid onto a 300 µm ID × 5 mm C_{18} PepMap trap column at a flow rate of 25 µL/min. Elution of the peptides from the trap column and their separation and spraying were done using 0.1% formic acid in acetonitrile at a flow rate of 200 nL/min on a Tip column (NTTC-360/75-3, Nikkyo Technos., Tokyo, Japan) with a spray voltage of 1.8 kV. A nanospray LTQ XL Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA) was operated in data-dependent acquisition mode with Xcalibur software (ver. 1.4, Thermo Fisher Scientific). Full scan mass spectra were acquired to cover a scan range of 100–2,000 m/z with a resolution of 15,000. The three most intense ions at a threshold above 1,000 were selected for collision-induced fragmentation in the linear ion trap at

normalized collision energy of 35% after accumulation to a target value of 1,000. Dynamic exclusion was employed within 30 s to prevent repetitive selection of the peptides.

MS data analysis

Tandem mass spectrum DTA files were converted to MGF files using BioWorks software (ver. 3.3.1, Thermo Fisher Scientific). The following parameters were set for creation of the peak lists: parent ions in the mass range with no limitation, one grouping of MS/MS scans, and threshold at 100. Precursor ion tolerance was 10.00 ppm. Data files were searched using the MASCOT search engine against the soybean genome sequence database and the NCBI (viridiplantae) database. To set the parameters for database search, carbamidomethylation of cysteines was considered as a fixed modification, and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Other parameters for search were peptide mass tolerance 10 ppm, fragment mass tolerance ±0.2 Da, and positive precursor peptide charge states of 1, 2, and 3. The instrument setting was specified as "ESI-Trap". Protein hits were validated if the identification was with at least five top-ranking peptides with the ions scores over the significance threshold level. Individual ions scores >32 against NCBI database indicate the probability of a true positive identification or extensive homology ($P < 0.05$). In the case of peptides matching multiple members of a protein family, the presented protein was selected based on the highest score and the highest-ranking member of matching peptides.

Metabolite analysis

Free amino acids were extracted by the methods described by Ohkama-Ohtsu et al. (2008) and Watanabe et al. (2008). A portion (100 mg) of soybean roots was homogenized in liquid nitrogen with a pestle and mortar. Ten volumes of methanol (10 μL mg^{-1} fresh weight) containing 8 μM of the internal standards, methionine sulfone (MES), which were used for compensation of the peak area after capillary electrophoresis-mass spectrometry (CE-MS) analysis, was added, and the mixture was vortexed again for 3 min at 4°C. The sample solution was then centrifuged at 20,400*g* for 3 min at 4°C. Chloroform (500 μL) and water (200 μL) were added to the supernatants, and the mixture was vortexed for 3 min, then centrifuged at 20,400*g* for 3 min at 4°C. The upper layer was evaporated for 30 min at 25°C in a centrifugal concentrator and then separated into two layers. The upper layer (100–200 μL) was centrifugally filtered through a Millipore 5-kD cutoff filter at 9,100*g* for 90 min. The filtrate was dried for 120 min in a centrifugal concentrator, and the residue was dissolved in 20 μL of water containing reference compounds (3-aminopyrrolidine).

Metabolites were extracted from three independent biological replicates for metabolome analysis. The final solution (20 μL) was used to quantify the contents of metabolites by cation analysis using capillary electrophoresis (CE)/MS. The CE/MS system and conditions were as described by Nakamura et al. (2009). The metabolites were separated and detected by a CE/MS system (Agilent CE system and 1100MSD MS, Agilent Technologies, Waldbronn, Germany). Cationic metabolites were separated in an uncoated fused-silica capillary using 1 M formic acid (pH 1.9) as the running buffer, and cationic metabolites were measured in the positive mode. The accuracy was determined by the measurement of known concentrations of selected metabolites.

Statistical analysis

The measured amino acids and spot intensities were statistically analyzed by one-way ANOVA and Least Significant Difference (LSD) test to determine significant differences among group means. Statistical analysis was carried out using the data obtained from three different sets of independent biological samples. $P \leq 0.05$ was considered to be statistically significant. The statistical package SAS (version 9.1, SAS/STAT Software for PC. SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Purification of soybean root microsomal proteins

To obtain significant biological results in a subcellular proteome analysis, it is vital to obtain a pure proteome sample. The purity of the proteins extracted from the root microsomal fraction was evaluated by immunoblot analysis using specific antibodies to known subcellular marker proteins, such as calnexin (as a microsomal marker), HSP70 (as a cytosolic marker), APX (as a cytosolic marker), and ATP-β (as a mitochondrial marker) (Fig. 1). We found that calnexin was predominantly present in the microsomal protein fraction; however, the antibody also cross-reacted with a constituent of the total soluble protein fraction, as a faint band was observed in immunoblot analysis of the cytosolic fraction. On the other hand, the cytosolic and marker proteins HSP70 and APX were present both in the total soluble and cytosolic fractions, whereas no band was observed for these proteins in the microsomal fraction. ATP-β was presented in all fractions; however, it was predominant in the microsomal fraction. These results indicated that the microsomal protein

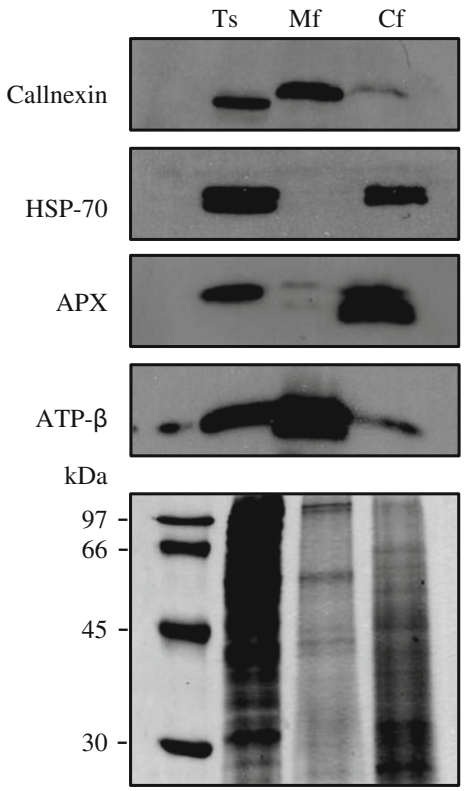

Fig. 1 Purity test of microsomal fractionated proteins of soybean roots. Immunoblot analysis was performed with specific sub-cellular marker antibodies raised against calnexin (microsomal marker), HSP70 and APX (cytosolic marker), and ATP-β (mitochondrial marker). The signal was detected by chemiluminescence after incubation with the appropriate HPR-conjugated secondary antibodies as described in the experimental procedures. CBB-stained proteins shown in the *lower panel* represent the SDS-PAGE analysis of total soluble (*Ts*), microsomal fraction (*Mf*) and cytosolic fraction (*Cf*) proteins. A total of 25 µg protein was loaded into each *lane*

fraction may have been contaminated with a few proteins from other subcellular organelles, but was enriched with microsome-associated proteins and was thus suitable for further proteomic analysis. Moreover, to confirm that the proteins we identified were indeed microsomal proteins, all identified protein sequences were searched using the PredictProtein (http://www.predictprotein.org) and/or TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) programs for predicted transmembrane helix. As summarized in Table 1, the identified proteins containing at least two transmembrane helices were predicted to be microsomal proteins.

Comparative two-dimensional gel electrophoresis analysis of microsomal proteins in two contrasting Cd-accumulating soybean cultivars

The primary objective of the present study was to identify the Cd-responsive microsome-associated proteins in the roots of two contrasting Cd-accumulating soybean seedlings. Changes in the root microsomal proteome resulting from Cd stress were evaluated using two-dimensional gel electrophoresis (2-DE) of proteins isolated from the purified microsomal fraction of control and Cd-exposed plants. The 2-DE gel pattern across the p*I* range 3–10 was detected in high resolution either by silver- or CBB staining. At least three biological replicates were used for image analysis. In the case of 2-DE analysis of root microsomal protein samples, approximately 400 protein spots were reproducibly detected in each silver-stained gel (Supporting Information, Fig. 1). The pattern of protein spots in analytical (silver-stained) and preparative (CBB-stained) gels was similar; however, many of the spots were either absent or not clearly detected in the CBB-stained gels (Supporting Information, Fig. 2). Protein spots that were reproducibly detected in each silver- or CBB-stained gel and that showed similar expression patterns were selected for further analysis.

Although the protein expression profiles of the Enrei and Harosoy cultivars were similar, the level of expression of some spots was clearly different between the control and Cd-treated samples in each cultivar (Fig. 2). Quantitative analysis using PDQuest software revealed a total of 13 and 11 proteins that differed by more than 1.5-fold in expression value in the high and low Cd-accumulating cultivars Enrei and Harosoy, respectively. Among the 13 differentially expressed proteins in cultivar Enrei, nine spots (spots 12–14, 17, 18, 23–25, and 29) increased and four spots (spots 15, 16, 27, and 28) decreased in abundance in response to Cd-stress. In the Harosoy cultivar, seven spots (spots 3–6, 10, 13, and 23) increased in abundance and four spots (spots 12, 16, 19, and 21) decreased in response to Cd stress (Supporting Information, Fig. 3).

Identification of Cd-responsive differentially expressed proteins from two contrasting Cd-accumulating soybean cultivars

Protein spots showing at least a 1.5-fold increase or decrease in abundance and a statistically significant with P value of ≤ 0.05 were excised from the preparative gels (CBB), digested with trypsin, and identified by nano LC-MS/MS analysis. A total of 22 proteins were identified with significant matches, while two spots showed no significant database matches (Table 1). Multiple spots corresponded to a single protein, such as alcohol dehydrogenase (spots 14 and 15) and lipoxygenase (spots 16, 17, and 18) in the Enrei cultivar, and glutamate dehydrogenase 1 (spots 4 and 5) in the Harosoy cultivar. Several proteins, including glutamine synthetase GS beta1 (GS1) and type IIIa membrane protein cp-wap13, increased in abundance in both cultivars, whereas peroxidase increased in abundance in the

Table 1 Cadmium-induced differentially expressed proteins in soybean root microsomal fraction identified by nanoLC-MS/MS analysis

SP	Homologous protein name	Acc. no	S	SC (%)	M	Id (%)	Theo Mw/p*I*	Exp Mw/p*I*	Fold	*P* value[a]	TM Pred[b]
↑E-12	Glutamine synthetase GSbeta1	AAG24873	265	39	20	100	39.1/5.48	40.0/5.3	>2.21	0.0095	Yes
↑E-13	Type IIIa membrane protein cp-wap13	AAB61672	311	38	41	100	40.0/6.24	38.0/5.5	>3.01	0.026	Yes
↑E-14	Alcohol-dehydrogenase	AAC97495	451	18	55	100	37.0/6.13	41.0/5.6	>3.92	0.0011	Yes
↓E-15	Alcohol-dehydrogenase	AAC97495	976	32	110	100	37.0/6.13	41.0/6.1	<0.23	0.0008	Yes
↓E-16	Lipoxygenase	AAA03726	252	25	22	100	95.0/5.65	38.0/5.7	<0.52	0.0056	Yes
↑E-17	Lipoxygenase-5	ABX60410	76	8	7	100	91.3/6.08	36.0/5.7	>2.30	0.0011	Yes
↑E-18	Lipoxygenase-4	P38417	382	24	63	100	96.6/5.71	32.0/5.2	>3.37	0.0022	Yes
↑E-23	Guanine nucleotide-binding protein subunit beta-like protein	O24076	101	27	7	100	36.0/7.07	33.0/7.5	>24.9	0.0008	Yes
↑E-24	F1-ATPase alpha subunit	AAQ74612	99	18	7	100	44.8/6.90	25.0/5.5	>3.91	0.0034	Yes
↑E-25	26S proteasome AAA-ATPase subunit RPT4a	ADD09611	108	19	9	100	50.1/8.84	23.0/5.8	>2.16	0.0265	Yes
↓E-27	Not Hit	–	–	–	–	–	–	24.0/6.5	<0.60	0.0131	–
↓E-28	Not Hit	–	–	–	–	–	–	39.0/6.8	<0.41	0.0061	–
↑E-29	Unknown (peroxidase)	ACU19453	571	44	55	100	38.5/6.01	27.0/5.3	>7.84	0.0002	Yes
↑H-3	Glutamine synthetase GSbeta1	AAG24873	446	52	69	100	39.1/5.46	40.0/5.3	>2.60	0.0003	Yes
↑H-4	Type IIIa membrane protein cp-wap13	AAB61672	204	35	32	100	40.0/6.24	38.0/5.5	>2.70	0.0022	Yes
↑H-5	Glutamate dehydrogenase 1	CAI53673	462	57	40	100	44.8/6.04	43.0/5.6	>15.7	0.0031	Yes
↑H-6	Glutamate dehydrogenase 1	CAI53673	258	54	24	100	44.8/6.04	44.0/5.9	>2.64	0.0285	Yes
↑H-10	26S proteasome regulatory particle non-ATPase subunit 8	ACF06631	128	26	8	100	34.8/5.85	33.0/5.3	>2.39	0.0026	Yes
↓H-12	Lipoxygenase	AAA03726	171	17	15	100	95.0/5.65	32.0/5.2	<0.48	0.020	Yes
↑H-13	Unknown	ACU21161	359	46	26	100	25.5/5.51	26.0/5.5	>2.30	0.0274	Yes
↓H-16	Unknown	ACU19758	177	40	9	100	37.1/6.97	24.0/5.9	<0.43	0.0041	Yes
↓H-19	Glyceraldehyde-3-dehydrogenase C subunit	ABA07956	112	23	5	100	36.8/6.72	25.0/6.4	<0.49	0.0284	Yes
↓H-21	Unknown	ACU19453	327	56	35	100	38.5/6.01	39.0/6.5	<0.45	0.0119	Yes
↑H-23	Proteasome subunit alpha type-7	Q9SXU1	221	29	26	100	27.1/6.86	32.0/6.7	>1.64	0.0170	Yes

SP spot number in 2-DE gel as shown in supplementary Fig. 1. E, Enrei; H, Harosoy., *Acc. No* accession number of NCBI database, *S* Mascot score, *SC* sequence coverage, *M* number of matched peptides, *ID* identity, *Theo* theoretical molecular weight and p*I*, *Exp* experimental molecular weight and p*I*, *Fold change* increased (>) or decreased (<) compared with the control plant

[a] The protein spots showed a significant change in abundance (fold change) by a factor >1.5-fold compared to the control analyzed by LSD test. A *P* value of ≤0.05 was considered statistically significant

[b] Transmembrane helix prediction (http://www.predictprotein.org and/or http://www.ch.embnet.org/software/TMPRED_form.html)

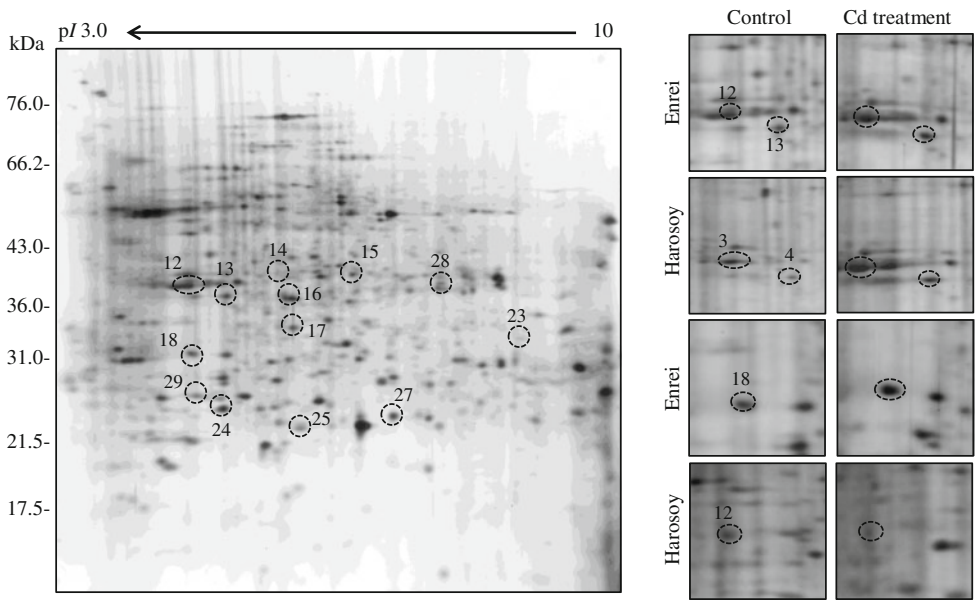

Fig. 2 Representative 2-DE gel images of microsomal proteins of soybean roots. A total of 100 μg proteins was extracted, separated by 2-DE, and visualized with silver staining. The *circled areas* indicate proteins that were differentially expressed in control and treated samples. *Right panel* represents the close-up views of some identified proteins that were differentially expressed in control and Cd-treated samples in both Enrei and Harosoy cultivars

Enrei cultivar, but decreased in the Harosoy cultivar (Fig. 2). Peroxidase was an example of a protein that showed cultivar-specific expression, as its expression increased in the Enrei cultivar but not in the Harosoy.

Spot 29 from the Enrei cultivar gel, and spots 13, 16, and 21 from the Harosoy gel, matched proteins that were annotated as unknown. To obtain functional information about these proteins, BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) was used to investigate their homology to other proteins in the database. We found that these proteins are homologous to certain proteins with at least 50% homology (Supplementary Table 1). More than 50% similarity at the amino acid level indicates that homologous proteins might have a similar function (Yan et al. 2006). Based on homology, the putative physiological functions of the unknown proteins we identified included detoxification, protein metabolism, energy and metabolism, and development.

To examine whether the expression of some proteins of interest, such as GS1, type IIIa membrane protein cp-wap13, and lipoxygenase, is particular to Cd stress or if they are expressed in response to other redox heavy metal stresses, soybean seedlings were exposed to a solution containing 100 μM Cu and roots were collected for 2-DE as described earlier. Proteins previously identified as being responsive to Cd stress were analyzed for potential differential expression in response to Cu stress using silver-stained gels. Comparative analysis revealed that expression of type IIIa membrane protein cp-wap13 and lipoxygenase was not altered as a result of Cu stress, whereas GS1 was differentially expressed under Cu stress compared with controls (Fig. 3). These results suggest that the expression of GS1 could be regulated by redox metal stress, whereas the expression of type IIIa membrane protein cp-wap13 and lipoxygenase may be involved in the response to Cd toxicity.

Metabolite response of two contrasting Cd-accumulating soybean cultivars under Cd stress

To gain a better insight into the profile of Cd-responsive metabolites, particularly free amino acids, metabolites from control and Cd-exposed roots of Enrei and Harosoy cultivars were analyzed by CE-MS. A total of 32 metabolites were analyzed using this procedure (Supplementary Table 2), and the abundance of 18 of these metabolites significantly changed in response to Cd stress in at least one cultivar (Fig. 4). Amino acids associated with heavy metal detoxification processes, such as glycine, glutamine, serine, methionine, lysine, arginine, and proline, showed the most prominent changes in response to Cd stress. Metabolites associated with ATP biosynthesis, such as adenine, adenosine, and guanine, were markedly upregulated (as much as fivefold) in both cultivars (Fig. 4), whereas methionine, which is associated with various secondary metabolites potentially involved in Cd detoxification processes, decreased in abundance by at least twofold in both cultivars. Some metabolites were differentially expressed in the two cultivars in response to Cd stress (Fig. 4). For example, metabolites associated with glutathione biosynthesis, such as glutamine and glycine, increased in the Enrei cultivar but decreased in the Harosoy cultivar, whereas proline (a key metabolite involved in Cd stress tolerance mechanisms) decreased in the Enrei cultivar and increased in the Harosoy cultivar.

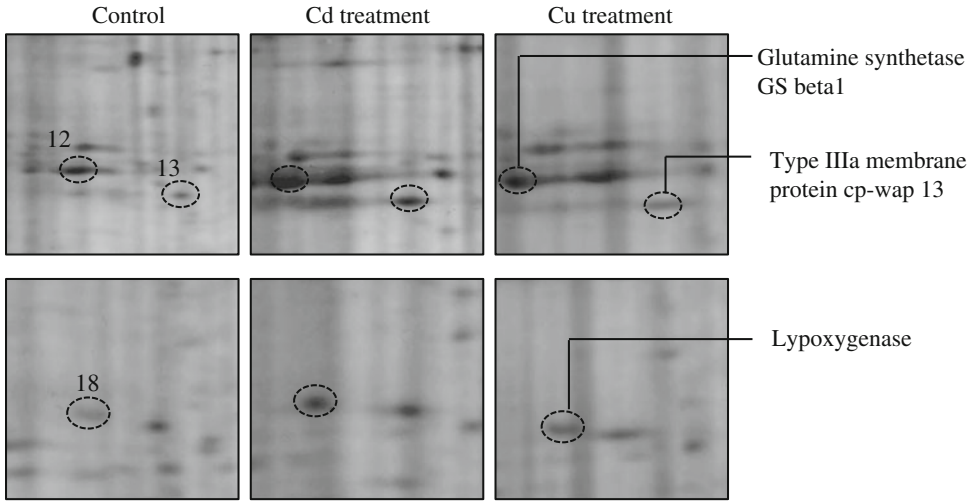

Fig. 3 Differential expression of some target proteins in response to Cd and Cu stress. *Spots 12*, *13,* and *18* correspond to glutamine synthetase GS beta, type IIIa membrane protein cp-wap 13 and lypoxygenase, respectively. Proteins were extracted from the control, 100 μM of Cd, and/or 100 μM of Cu-treated roots and separated by 2-DE and detected by silver staining as described in Sect. 2

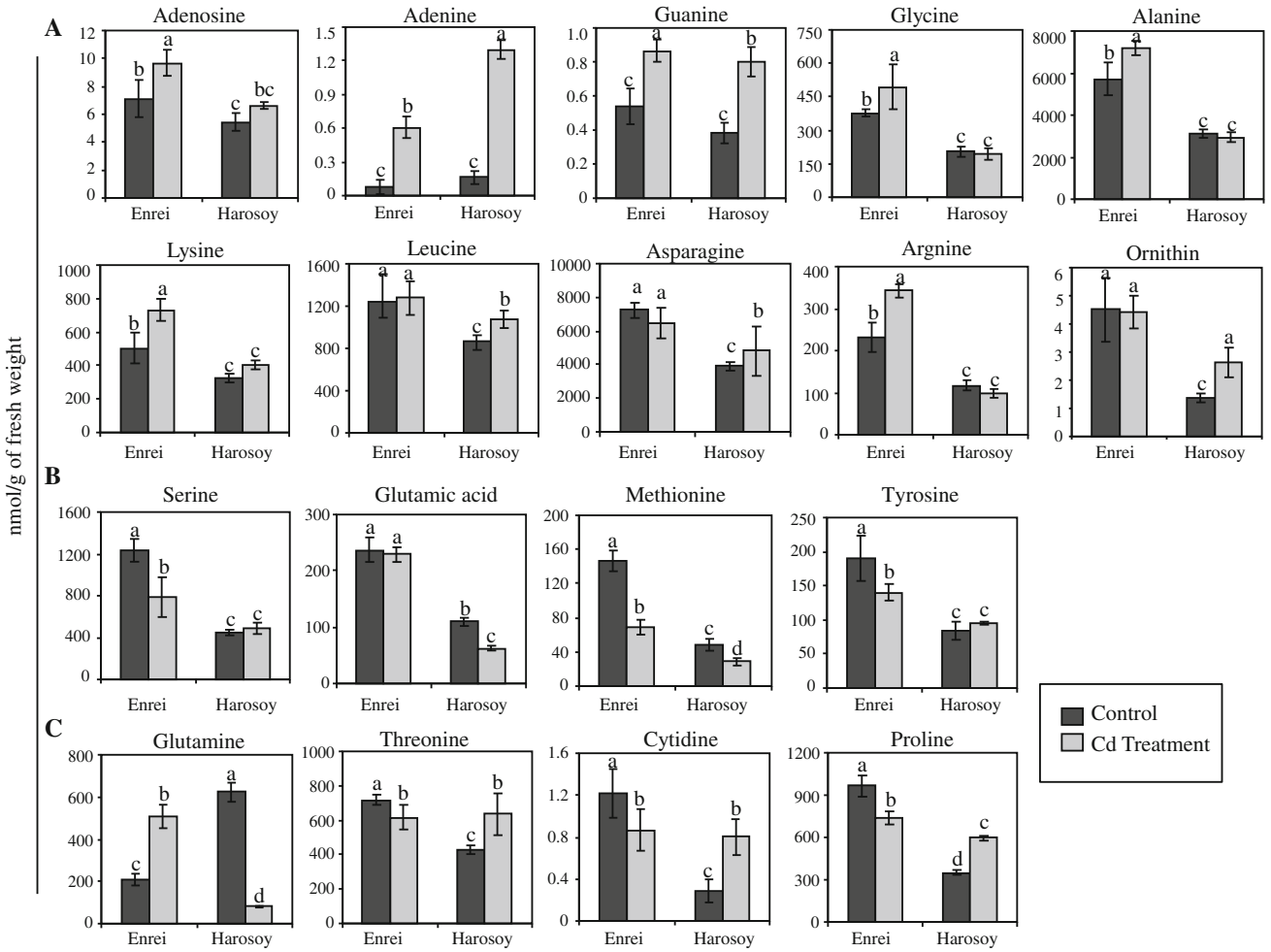

Fig. 4 Analysis of metabolites activities of soybean roots under Cd stress. **a** Metabolites that were significantly increased in both or at least in one cultivar under Cd stress, **b** metabolites that were significantly decreased in both or at least in one cultivar under Cd stress, and **c** indicates metabolites that were changed in opposite manner in both cultivars in response to Cd stress. The height of each *bar* represents the average of three individual identical experiments (±SE). *Different letters* above the *bars* indicate a statistically significant difference between the control and treated samples according to LSD test ($P < 0.05$)

Discussion

Arao et al. (2003) investigated the genotypic differences associated with Cd uptake and translocation in different soybean cultivars. They found that the Enrei cultivar accumulated Cd in higher amounts in roots than did the Harosoy cultivar. However, the Harosoy cultivar accumulated higher amounts of Cd in the aerial parts of the plant. Their results clearly indicated that the rate of Cd accumulation and translocation differed in these two cultivars. The primary aim of the present study was to characterize the differentially expressed Cd-responsive microsomal proteins in these two contrasting Cd-accumulating soybean cultivars, with the goal of enhancing understanding of the molecular mechanisms involved in Cd translocation and accumulation.

Proteomic analysis of microsomal proteins in the two cultivars revealed that certain proteins, including GS1, type IIIa membrane protein cp-wap13, lipoxygenase, and proteasome subunits were common to both cultivars but their expression varied in response to Cd stress. In this study, we found that GS was upregulated in both cultivars; however, GS expression in the control and treated samples of the Enrei cultivar was significantly higher than it was in the Harosoy cultivar. GS is involved in the synthesis of glutathione (GSH) via the glutamate biosynthesis pathway (Sarry et al. 2006; Semane et al. 2010). Upregulation of GS has also been reported to occur in other plants in response to Cd stress (Sarry et al. 2006; Kieffer et al. 2008; Hradilová et al. 2010; Semane et al. 2010). Consistent with the upregulation of GS, our metabolite analysis revealed that glutamine expression was markedly upregulated (at least 2.5-fold) in the Enrei cultivar, but was downregulated in the Harosoy cultivar. Increased glutamine production has also been described in Cd-exposed *Arabidopsis* (Sun et al. 2010). We found that several metabolites associated with GSH and phytochelatin (PC) biosynthesis, such as glycine, serine, glutamate, and lysine, accumulated differentially between the two cultivars in response to Cd stress. Glycine is a key precursor in the GSH biosynthesis pathway. In the Enrei cultivar, glycine expression increased significantly in response to Cd stress, whereas no significant change was observed in the Harosoy cultivar.

While the amount of serine and lysine decreased and increased, respectively, as a result of Cd stress in both cultivars, the amount of glutamate decreased significantly in the Harosoy cultivar. Serine is a precursor in the glycine and cysteine biosynthesis pathways (Sarry et al. 2006). Thus, increased consumption of serine for glycine and cysteine biosynthesis during Cd stress (for possible use in GSH and PC production) could be the reason for the observed decrease in serine content. It has been suggested that glutamate can be produced as a byproduct of lysine metabolism (Galili et al. 2001; Stepansky et al. 2006); thus, the increase in lysine production we observed suggests that increasing lysine stores might be a mechanism the Enrei cultivar employs to maintain the internal glutamate level in root cells. Taken together, these results indicate that Cd-chelating peptides or molecules are highly active in the Enrei cultivar, and that they may form a complex upon binding Cd ions that are then translocated and deposited in the vacuoles. Therefore, it might be possible that the translocation rate of free Cd ions from the root to shoot in the Enrei cultivar was considerably lower than that in the Harosoy cultivar.

This hypothesis could be supported by the observed upregulation of lipoxygenase and peroxidase in the Enrei cultivar. In plants, lipoxygenase has different isoforms with different functions; however, it also plays an important role in the octadecanoid pathway leading to the biosynthesis of jasmonic acid (JA). Upregulation of JA biosynthesis-related genes or proteins in response to heavy metal stress has been demonstrated in several studies (Ahsan et al. 2008; Xue et al. 2008). Moreover, JA levels have been shown to increase under heavy metal exposure, suggesting that JA may participate in the response to heavy metal toxicity by regulating cellular responses (Maksymiec et al. 2005; Rodríguez-Serrano et al. 2006). In accordance with these observations, Xiang and Oliver (1998) demonstrated that genes of the GSH biosynthesis pathway were significantly upregulated in response to JA. In addition, JA and its conjugates, the small signaling molecules methyl jasmonate (MeJA) and jasmonoyl-isoleucine (JA-Ile), which are collectively referred to as jasmonates (JAs), play an active role in the regulation of monolignol biosynthesis in plants (Pauwels et al. 2008). Lignin is a natural branched plant biopolymer generated by monolignols (Boerjan et al. 2003). Formation of a monolignol radical is catalyzed by an oxidative enzyme. Many studies have suggested that peroxidases are involved in several physiological and biochemical processes, and the most intensively studied functions of peroxidases are their putative role in lignification (Marjamaa et al. 2009). It has been demonstrated that increased expression of lignin biosynthesis genes (e.g., genes for peroxidase and laccase) is accompanied by a rise in the lignin content in the roots of Cd-treated soybeans (Yang et al. 2007). Moreover, a comparative transcriptome analysis of *Arabidopsis* and the metal hyperaccumulator *Thlaspi caerulescens* revealed that the high expression of lignin biosynthesis genes corresponds to the deposition of lignin in the endodermis, of which there are two layers in *T. caerulescens* roots and only one in *A. thaliana* (van de Mortel et al. 2006). Taken together, these studies clearly indicate that lignification in roots is positively correlated with heavy metal stress, and that lignification might play a protective role in preventing Cd translocation from roots to the aerial parts of the plant.

In this study, the Golgi-associated protein type IIIa membrane protein cp-wap13 was significantly upregulated under Cd stress in both cultivars; however, no expression of this protein was observed in response to Cu stress. The highest concentration of type IIIa membrane protein cp-wap13 was found in the plasmodesmata (Epel et al. 1996). Cell wall polysaccharides are synthesized in the Golgi complex from UDP-sugars and are then exported to the cell matrix in Golgi-derived vesicles (Shoresh and Harman 2008). Several studies have reported that the cell wall structure is modulated in response to Cd stress (Douchiche et al. 2007, 2010). Our observation that type IIIa membrane protein cp-wap13 is upregulated in response to Cd stress in both cultivars suggests that this protein is a general Cd stress defense protein that inhibits Cd uptake.

We also found that proteasome subunits were differentially regulated (mostly upregulated) in both cultivars in response to Cd stress. Using biochemical and proteomics approaches, Polge et al. (2009) recently presented the first evidence that under Cd stress, the proteasome proteolytic pathway is upregulated at both the RNA and protease activity levels in *Arabidopsis* leaves and that this increase in proteolysis may play a role in degrading oxidized proteins generated by metal stress.

Although a couple of proteins and metabolites were also identified as being differentially expressed in both soybean cultivars we examined, the role these proteins play in response to Cd stress could not be determined because their function was unclear or the available information was insufficient to provide a sustainable hypothesis for their role in Cd uptake and translocation. However, the results of the present study did reveal that a combined proteomic/metabolomic approach is a useful strategy for comprehensive analysis of the molecular mechanisms adopted by soybean plants to overcome Cd stress.

In summary, the higher accumulation of Cd in Enrei cultivar roots may be due to higher amounts of Cd-chelating agents in these plants, such as GSH, PCs, and organic acids that bind free Cd ions and deposit them in vacuoles. Upregulation of proteins associated with lignin biosynthesis in Enrei cultivar roots also suggests that lignification in xylem vessels inhibits the translocation of Cd from roots to the aerial parts of the plant in this cultivar. Although we did not analyze the content of GSH, PCs, or lignin in the two cultivars studied, results of our proteomic and metabolomic analyses suggest that future studies should focus on a number of proteins and metabolites to obtain a better understanding of the molecular mechanisms involved in Cd translocation in soybean cultivars. We believe that analysis of the subcellular proteomes of organs such as the cell wall, cytosol, and vacuoles, coupled with analysis of Cd-chelating metabolites and complexes in the roots and shoots of the Enrei and Harosoy cultivars, would clarify the molecular mechanisms controlling Cd translocation and accumulation in metal hyperaccumulating and non-hyperaccumulating soybeans.

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