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Cd-induced changes in leaf proteome of the hyperaccumulator plant *Phytolacca americana*

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ABSTRACT

Cadmium (Cd) is highly toxic to all organisms. Soil contamination by Cd has become an increasing problem worldwide due to the intensive use of Cd-containing phosphate fertilizers and industrial zinc mining. *Phytolacca americana* L. is a Cd hyperaccumulator plant that can grow in Cd-polluted areas. However, the molecular basis for its remarkable Cd resistance is not known. In this study, the effects of Cd exposure on protein expression patterns in *P. americana* was investigated by 2-dimensional gel electrophoresis (2-DE). 2-DE profiles of leaf proteins from both control and Cd-treated (400 μM, 48 h) seedlings were compared quantitatively using ImageMaster software. In total, 32 differentially expressed protein spots were identified using MALDI-TOF/TOF mass spectrometry coupled to protein database search, corresponding to 25 unique gene products. Of those 14 were enhanced/induced while 11 reduced under Cd treatment. The alteration pattern of protein expression was verified for several key proteins involved in distinct metabolic pathways by immuno-blot analysis. Major changes were found for the proteins involved in photosynthetic pathways as well as in the sulfur- and GSH-related metabolisms. One-third of the up-regulated proteins were attributed to transcription, translation and molecular chaperones including a protein belonging to the calreticulin family. Other proteins include antioxidative enzymes such as 2-cys-peroxidase and oxidoreductases. The results of this proteomic analysis provide the first and primary information regarding the molecular basis of Cd hypertolerance in *P. americana*.

1. Introduction

Cd is highly toxic to all living organisms. The intensive application of phosphate fertilizers containing traces of Cd and industrial zinc mining is expanding the soil–Cd-contamination rapidly especially in the developing countries (Pinot et al., 2000; Peng et al., 2006). This situation is of particular concern in human health, because Cd is probably carcinogenic at low concentrations and could be easily taken in through foods produced in the Cd-contaminated soils (Wagner, 1993; Vido et al., 2001). Therefore, it is vital to eliminate Cd from the polluted areas. Phytoremediation is a promising approach making use of hyperaccumulator plants to remove Cd from the Cd-containing soils. This is because hyperaccumulator plants are, unlike many plants such as crops, capable of accumulating substantial amounts of heavy metals such as Cd in their aerial organs (Baker and Brooks, 1989; Lasat et al., 1998). The approach is principally advanced over other remediation means, *i.e.* soil removal/burial and chemical clean-up, since it is a non-second contaminating process and is ecologically-effective (Salt et al., 1998). Although field trials of phytoremediation were reported (Baker et al., 1991, 1994), large-scale application of the approach is, however, virtually hindered by the lack of ideal hyperaccumulator plants with sufficient biomass and high growth rates (Eapen and D'Souza, 2005). In terms of Cd hyperaccumulator plants, only a few species were reported (Van Rossum et al., 2004; Zhou and Qiu, 2005; Mari et al., 2006). To overcome the limitations, development of genetically modified plants with the desired characteristics mentioned above is largely in demand. To fulfill this, a better understanding of physiological and molecular regulation mechanisms of hyperaccumulating metals including Cd become essential. Considerable research has been conducted during the past decades to examine the physiological (Padmaja et al., 1990; Horváth et al., 1996; Sárvári et al., 1999; Perfus-Barbeoch et al., 2002) and biochemical changes (McCarthy et al., 2001; Sarry et al., 2006; Maksymiec, 2007; Kieffer et al., 2008, 2009) of plants in response to Cd stress in general as well as to study the molecular characteristics of Cd resistance in Cd hyperaccumulators (for reviews, see DalCorso et al., 2008; Krämer, 2010). In general, uptake of heavy metals such as Cd by plant cells causes physiological changes such

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as reduction in growth and photosynthesis, diminishing water and nutrient uptake, disruption of cellular transport, disturbance of redox control activity and eventually cell death (Ahsan et al., 2009). It is suggested to be similar regarding the primary process of metal uptake, accumulation, sequestration and detoxification among the hyperaccumulator and non-hyperaccumulator plants (Clemens, 2001). Regarding to the genes involved in homeostasis of specific metal ions, clear differences were revealed between *Arabidopsis thaliana* (non-hyperaccumulator) and its close relatives *Arabidopsis halleri* or *Thlaspi caerulescens* (Cd/Zn hyperaccumulators) (Becher et al., 2004; Weber et al., 2004; De Mortel et al., 2008). Several candidate genes such as *HMA4* (encoding the metal pump HMA4), *MTP1* (encoding the Metal Transport Protein 1), *ZNT1* (encoding a putative Cd/Zn transporter), and *SAT* (encoding serine acetyltransferase), have been identified and characterized (Pence et al., 2000; Dräger et al., 2004; Papoyan and Kochian, 2004; Freeman et al., 2005; Hanikenne et al., 2008). These findings have advanced current understanding of Cd hyperaccumulation at the genetic level. At protein level, however, experimental data are still limited (Tuomainen et al., 2006; Farinati et al., 2009). Conclusions toward the translational regulation of Cd hypertolerence are far from clear, probably due to the limited investigations in this area as well as to the complexity of proteins that contribute to hyperaccumulating properties (Plaza et al., 2007; Hanikenne et al., 2008).

Phytolacca americana L. (hereafter *P. americana*) is a perennial plant frequently growing in the mining contaminated regions (Tie et al., 2005; Peng et al., 2006; Kim et al., 2008). A previous report showed that *P. americana* was capable of accumulating more than 0.24% Cd of leaf dry weight (Peng et al., 2008), which was significantly higher than that defined for Cd hyperaccumulators (Clemens, 2001). Recent experiments estimated that *P. americana* accumulated large quantity of Cd in the shoots at the concentrations well exceeding 100 μM (Liu et al., 2010). The remarkable capability of Cd accumulation, fast growth and high biomass, makes *P. americana* not only a potent plant species for Cd phytoremediation but also a valuable organism for studying molecular mechanisms of Cd hyperaccumulation in plant cells. In this study, we have investigated the proteomic changes of *P. americana* under Cd treatment. We have identified 32 protein spots that changed significantly in abundance based on the differential and quantitative analysis of 2-DE profiles using ImageMaster software. For several proteins their alterations were verified by immunoblot analysis.

2. Materials and methods

2.1. Chemicals and reagents

CHAPS, DTT, IPG buffer (pH 3–10), IPG DryTrip, Iodoacetamide and Thiourea were from GE Healthcare (GE Healthcare, Piscataway, NJ). Urea and sequencing-grade modified trypsin were obtained from Promega (Madison, WI). CBB G-250 was from Sigma (St. Louis, MO). All other chemicals and reagents used in the study were of analytical grade unless indicated otherwise. Deionized water produced by a Milli-Q system (Millipore, Billerica, MA) was used for all buffers.

2.2. Plant material, culture condition, and Cd treatment

Seeds of *P. americana* L. were surface sterilized in 70% ethanol then rinsed with sterile water. The seeds were placed on 1/2 (Murashige and Skoog, 1962) and grown in a controlled environment at 25 °C with continuous light (150 μmol photons·m^{-2} s^{-1}). After 2 weeks, the seedlings were transplanted to a vessel containing 1/2 strength Hoagland solution (Hoagland and Arnon, 1950) and grown for an additional week. The solution was aerated daily by air bubbling for 30 min and changed every 2 d. For Cd-treatment experiments, three replicates each consisting of seven seedlings were included for both control and Cd treatment. The 3-week-old plants were exposed to the nutrient solutions supplied with 0, 100, 200, 400, or 800 μM $CdCl_2$ and grown in the same controlled environment as described above. After 48 of Cd-treatment (or up to 96 h of Cd-treatment at 400 μM), the seedlings were harvested. The fourth expanded leaves as well as the roots were collected and then dried (for metal and chlorophyll analysis) or frozen in liquid nitrogen (for protein analysis) until use.

2.3. Cd and chlorophyll contents in plant tissues

Cd content in the leaf and root tissues was determined according to Gong et al. (2003). The tissues were collected and washed with distilled water then dried immediately at 105 °C for 48 h. The dried materials were ground into powder. Approximate 40 mg were digested in 5 mL of HNO_3 (48%, w/v) at 60 °C for 48 h. After dilution with Mili-Q water (1:20), Cd in the solution was measured using Inductive Coupled Plasma Emission Spectrometry instrument (Thermo 6300, USA). Cd concentrations in the tissues were calculated as mg per kg dry weight. Chlorophyll content was determined according to Arnon, (1949).

2.4. Protein extraction and separation by 2-DE

Proteins were extracted from leaves of *P. americana* L. accordingly to the protocols (Giavalisco et al., 2003; Cui et al., 2005) with modifications. Frozen leaf tissues were ground to a fine powder in liquid nitrogen and homogenized in ice-cold 50 mM Tris–HCl buffer (extraction buffer I, pH 7.8) containing glycerol (10%), β-mercaptoethanol (2%), EDTA (1 mM) and phenylmethylmethylsulfonyl fluoride (PMSF, 1 mM). Cells were then disrupted by 10 cycles of ultrasonication at 2 s/2 s (JY92-II Ultrasonic Crasher, Ningbo Scientz Biotechnology Co., Ltd, China) on ice in the presence of PMSF. After centrifugation at 40 000*g* at 4 °C for 30 min, the supernatant was collected constituting fraction I. The pellet was resuspended and protein extraction was repeated once in the same buffer. After transferring the supernatant to fraction I, the remaining pellet was resuspended in 100 mM phosphate buffer (extraction buffer II, pH 7.1) containing KCl (0.2 M), glycerol (10%), $MgSO_4$ (2 mM), CHAPS (4%, w/v), β-mercaptoethanol (2%), EDTA (1 mM) and 1 mM PMSF. After stirring at 4 °C for 40 min, 7 M urea, 2 M thiourea and 30 mM DTT were added and extracted for additional 40 min at room temperature. The mixture was then subjected to centrifugation at 40 000*g* for 30 min at 18 °C. The supernatant was collected constituting fraction II. Fraction I and II were combined and kept at −80 °C for proteomic analysis. Protein concentration was estimated according to Bradford (1976) with modifications described in Cui et al. (2009) using BSA as a standard.

2-DE of the leaf proteins was performed as described previously (Cui et al., 2005). For analytic and preparative gels, 100 μg and 400 μg proteins were used, respectively. Proteins were precipitated with methanol/chloroform according to the method described by Wessel and Flugge (1984). The protein precipitate was solubilized in 350 μL of an electrofocusing solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and 0.5% v/v IPG buffer (pH 3–10). The mixture was incubated at room temperature for 60 min. After centrifugation at 10 000*g* for 10 min, the supernatant was applied onto a linear IPG strip (pH 4–7, 18 cm, GE Healthcare). Rehydration loading and IEF were performed as described by Huang et al. (2002). Isoelectric-focusing was carried out at 20 °C and the running program was 300 V for 40 min, 500 V for 40 min, 1000 V for 1 h, and 10 000 V until a total of 80 000 V h was reached. Following the two-step equilibration, the second-dimen-

sion separation was carried out on 12.5% SDS–PAGE gel prepared according to Laemmli (1970) running at 20 mA/gel with Protean II xi cell (Bio-Rad, CA). Proteins were detected by silver nitrate using the kit (GE Healthcare) or Coomassie Brilliant Blue G-250 staining. Protein samples from three independent experiments were prepared and used for 2-DE. For the first dimension, the six IPG strips containing control or Cd-treated samples were run in parallel under the identical conditions described above. After isoelectric-focusing, the strips were immediately freezed at −80 °C. For SDS/PAGE, twin gels with control or Cd-treated samples from the same experiment were run at each time.

2.5. Image analysis of 2D gels

2-D gels were scanned using a UMAX PowerLook 2100XL scanner (Willich, Germany) with a resolution of 300dpi. 2-D gel image analysis software Image-Master 2D Platinum (version 5.0, GE Healthcare) was used for gel to gel matching, identification of differences in spot intensities between the control and Cd-treated samples, and spot quantification. After spot detecting and editing, a scatter plot was performed according to the manufacturer's instruction (GE Healthcare) to verify reproducibility of 2D-gels from different experiments. As described previously (Yang et al., 2007; Chen et al., 2010), relative quantification of each matched spot represented as volume% = {(volume of each spot)/(volume of total spots) × 100%} in one gel (*X*-axis) was plotted against with that in the matched CBB-stained gel (*Y*-axis). Protein spots showing 2-fold or above changes in abundance were defined as being differentially expressed.

2.6. Protein identification by mass spectrometry

MALDI-TOF/TOF analysis was performed on a mass spectrometry instrument Ultraflex MALDI-TOF/TOF II from Bruker Daltonics (Bremen, Germany). Excision of protein spots and sample preparation for MALDI-TOF analysis were done as described by Huang et al. (2002). For acquisition of mass spectra, 1 μL of samples was spotted onto a MALDI plate, followed by 0.1 μL of matrix solution (4 mg mL^{-1} R-cyano-4-hydroxycinnamic acid in 70% acetonitrile (ACN) and 0.1% TFA). Mass data acquisitions were piloted by FlexControl Software v3.0 using batched-processing and automatic switching between MS and MS/MS modes. All the MS survey scans were acquired over the mass range 700–3500 *m/z* in the reflectron positive-ion mode and accumulated from 1000 laser shots with acceleration of 22 kV. The MS spectra were externally calibrated using PeptideCalibStandard II (Bruker Daltonics) (757.40, 1046.542, 1296.685, 1347.735, 1619.822, 2093.086, 2465.198, and 3147.471) and resulted in mass errors of less than 10 ppm. The MS peaks were detected on minimum S/N ratio ⩾20 and cluster area S/N threshold ⩾6 without smoothing and raw spectrum filtering. Peptide precursor ions corresponding to contaminants including keratin and the trypsin autolytic products were excluded in a mass tolerance of ±0.2 Da. The filtered precursor ions with a user-defined threshold (S/N ratio ⩾100) were selected for the MS/MS scan. Fragmentation of precursor ions was performed using LIFT positive mode. MS/MS spectra were accumulated from 2000 laser shots. The MS/MS peaks were detected on minimum S/N ratio ⩾3 and cluster area S/N threshold ⩾5 with smoothing. Mass spectra were evaluated using flexAnalysis Software. Database searching for protein identification was performed via Mascot (http://www.matrixscience.com) using the NCBI database. Search parameters allowed for mass accuracy of ±100 ppm, one miscleavage of trypsin, oxidation of methionine, and carbamidomethyl of cysteine. Proteins were identified as the highest ranking results deduced by searching in the databases of NCBInr 20101113 against green plant.

ESI-MS/MS was performed on a quadrupole-time-of-flight mass spectrometer (Q-TOF-2; Micromass, Altrincham, U.K.) equipped with a nanoflow Z-spray source. The instrument was externally calibrated using the fragmentation spectrum of the doubly charged 1571.68 Da (785.84 *m/z*) ion of fibrinopeptide B from human. The peptides were loaded by nanoelectrospray with gold-coated borosilicate glass capillaries (Micromass). The capillary voltage was set to an average of 1000 V, with a sample cone working on 50 V. The collision energy was varied from 14 to 40 V, dependent on the mass and charge state of the peptides. Peptide precursor ions were acquired over the *m/z* range 400–1900 Da in TOF-MS mode. Multiply charged (2+ and 3+) ions rising above predefined threshold intensity were automatically selected for MS/MS analysis, and product ion spectra collected from *m/z* 50–2000. MS/MS data were processed using MassLynx (version 4.0; Micromass) with default parameters to generate the peak lists and searched in NCBInr 20101008 protein databases against green plant with Mascot MS/MS Ions Search program. Search parameters allowed for peptide mass tolerance ±1.2 Da, fragment mass tolerance ±0.6 Da, one miscleavage of trypsin, oxidation of methionine, and carbamidomethyl of cysteine.

2.7. SDS/PAGE and immunoblot analysis

Proteins were separated by SDS/PAGE (12.5% polyacrylamide, w/v) and transferred onto nitrocellulose membrane (GE Healthcare)

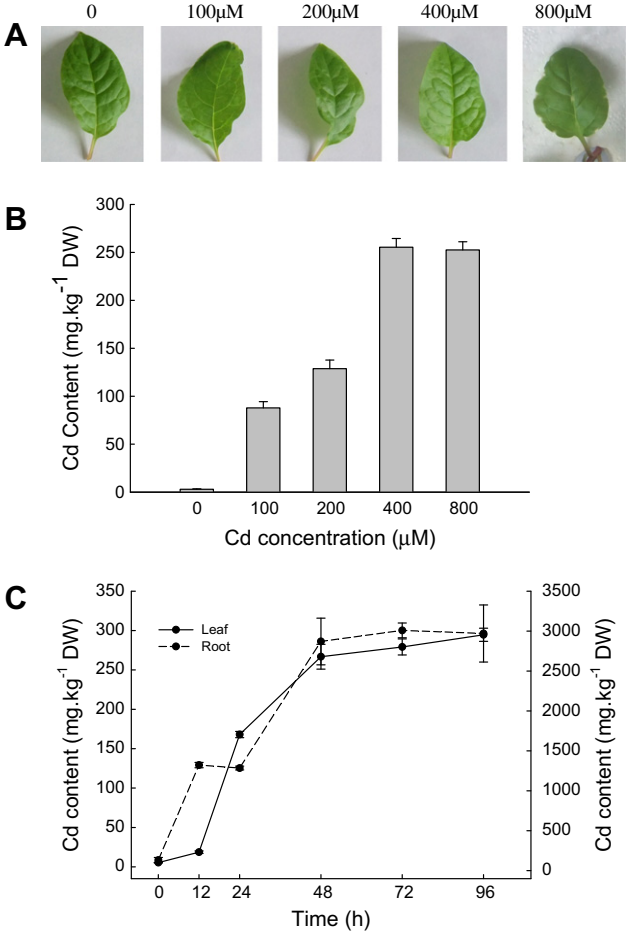

Fig. 1. Leaf morphology and Cd contents of *P. americana* grown under different Cd concentrations. (A) Morphology of the fourth expanded leaves collected from control (-Cd) and Cd-treated seedlings. (B) Leaf Cd content in the absence or presence of Cd for 48 h. (C) Cd content in leaf and root tissues under 400 μM Cd within 96 h. Measurements were performed in at least three independent experiments.

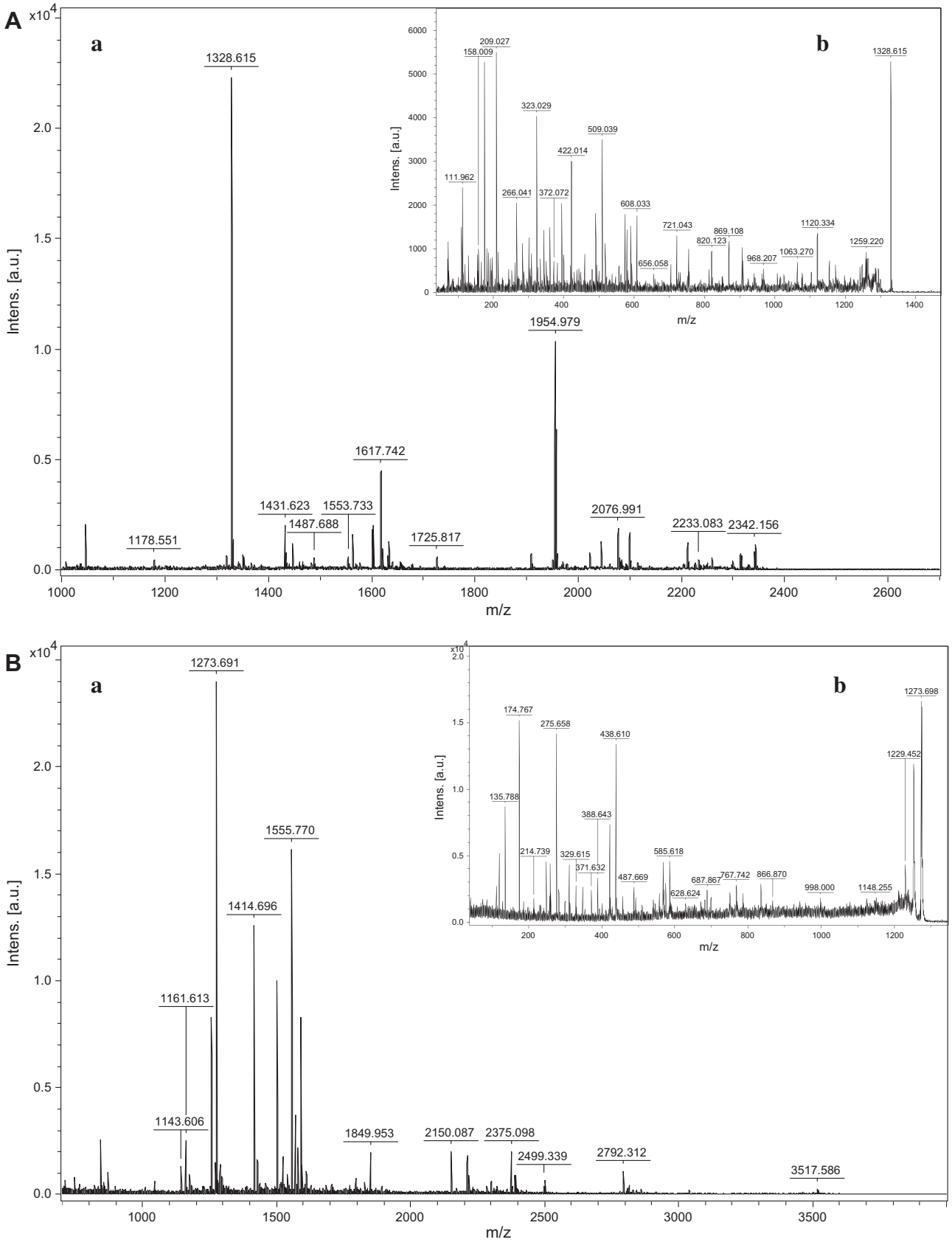

Fig. 2. MS spectra of spots 20 (A) and 66 (B) from the CBB-stained 2D gel of *P. americana* leaf proteins. (A) MS spectra of peptides generated by tryptic digestion of spot 20. (a) MALDI-TOF mass spectrum of peptides generated by tryptic digestion of protein spot 20; (b) MALDI-TOF/TOF spectrum of the precursor ion with *m/z* 1328.61. (B) MS spectra of peptides generated by tryptic digestion of protein spot 66. (a) MALDI-TOF mass spectrum of peptides generated by tryptic digestion of spot 66; (b) MALDI-TOF/TOF spectrum of the precursor ion with *m/z* 1273.69.

using electro-blotting for immunoblot analysis, as previously described (Zhang et al., 2009). Primary antibodies were purchased (Agrisera, Sweden) and used by following the instructions. The dilution for the specific antibodies used in this study is as follows: anti-AtpB (1:3000); anti-Hsp70 (1:3000); anti-Enolase (1:2000); anti-RbcS (1:5000); anti-RbcL (1:8000) and anti-Tubulin (1:1000). The binding of the cross-reacting antibody was detected using the ECL plus western blotting detection system (GE Healthcare). Protein content was estimated using the Bradford Microassay Kit (Bio-Rad).

3. Results and discussion

3.1. Leaf Cd accumulation under Cd treatment

It has been reported that Cd hyperaccumulation occurs in several plant species, including *P. americana* (Baker et al., 1994; Roger et al., 2001; Peng et al., 2006). To investigate how *P. americana* responds to Cd under hydroponic culture conditions, 3-week-old plants were transplanted and cultured in 1/2 strength Hoagland solution supplied with different Cd concentrations. As shown in Fig. 1, high Cd concentration (up to 400 μM) did not seem to affect the growth as demonstrated by leaf morphology. As the Cd concentration further increased, the symptom of Cd toxicity became apparent. Leaves from the seedlings grown at 800 μM showed a slight chlorosis starting at the leaf margins, a similar Cd symptom to that observed in other plant species such as *Spinacia oleracea* (Fagioni and Zolla, 2009) and *T. caerulescens* (Cosio et al., 2005). X-ray microanalysis indicated that in *T. caerulescens* expanding cells in leaf margins are more sensitive to Cd than other parts of a leaf. This may explain why chlorosis was at first observed in the leaf margins (Cosio et al., 2005). Chlorophyll content in the leaves of *P. americana* (Supplementary Fig. 1) was largely correlated with the morphological changes observed above. To confirm the capability of Cd hyperaccumulation of *P. americana*, the Cd content in the leaves from the seedlings grown at various Cd concentrations was examined. The Cd content in the leaves increased remarkably as the external Cd concentration was elevated from 0 to 400 μM (Fig. 1B) in 48 h. Although no Cd toxic symptom was observed after 48 h of treatment, the highest level of leaf Cd content (~260 mg kg^{-1} DW) was detected at 400 μM. This level of Cd accumulation is significantly higher than the levels generally described for Cd hyperaccumulators (Clemens, 2001), confirming *P. americana* as a Cd hyperaccumulator plant (Peng et al., 2006; Liu et al., 2010).

To determine the sampling time points for proteomic investigations, Cd content was monitored in seedlings grown in the presence of 400 μM Cd. As observed, Cd slowly accumulated in the leaves of *P. americana* during the first 12 h. Leaf Cd content increased almost linearly afterwards and reached near-maximum level at 48 h of Cd treatment (Fig. 1C). Prolonged Cd treatment (72 h, 96 h) retained high level of leaf Cd content, accompanied with different degree of chlorosis as mentioned above. Cd content in the roots showed similar alteration patterns in the duration of Cd treatments (Fig. 1C). These observations indicate that plant defense mechanisms are activated during the first 48 h. As an initial proteomic study aiming at revealing molecular networks underlying in the leaf tissue of *P. americana*, we therefore decided to harvest the leaves from the seedlings treated for 48 h for subsequent proteomic studies.

3.2. 2-DE and identification of proteins

To investigate proteomic changes of *P. americana* in response to Cd, we compared the 2-DE profiles of leaf proteins from the control and the Cd-treated plants. More than 500 intensive spots were reproducibly detected on the 2-D gels by Coomassie Brilliant Blue staining. To prove the reproducibility of the 2D-gels across different experiments, a scatter plots analysis was performed using ImageMaster 2D Platinum software (version 5.0, GE Healthcare) and the results for the control samples is shown in Supplemental Fig. 2. The correlation coefficients between the reference and the second and third 2D-maps were 0.967 and 0.962, respectively. These results indicate that the technical reproducibility of 2-DE from different experiments is sufficiently high to yield essentially identical results. Comparison of 2-DE profiles using ImageMaster 2D Platinum software revealed that about one hundred protein spots were enhanced or reduced at least 2-fold under Cd treatment. The differentially expressed protein spots were manually excised and subjected to in-gel trypsin digestion according to Huang et al. (2004). Since the genomic sequence information of *P. americana* is not available, MALDI-TOF/TOF and ESI-Q-TOF mass spectrometry were used for protein identification. The proteins were identified by combination of peptide mass fingerprint (PMF) and peptide sequence tags obtained by MALDI-TOF/TOF MS or ESI-Q-TOF MS coupled to protein database search, as described in previous reports for plant species lacking genomic sequence

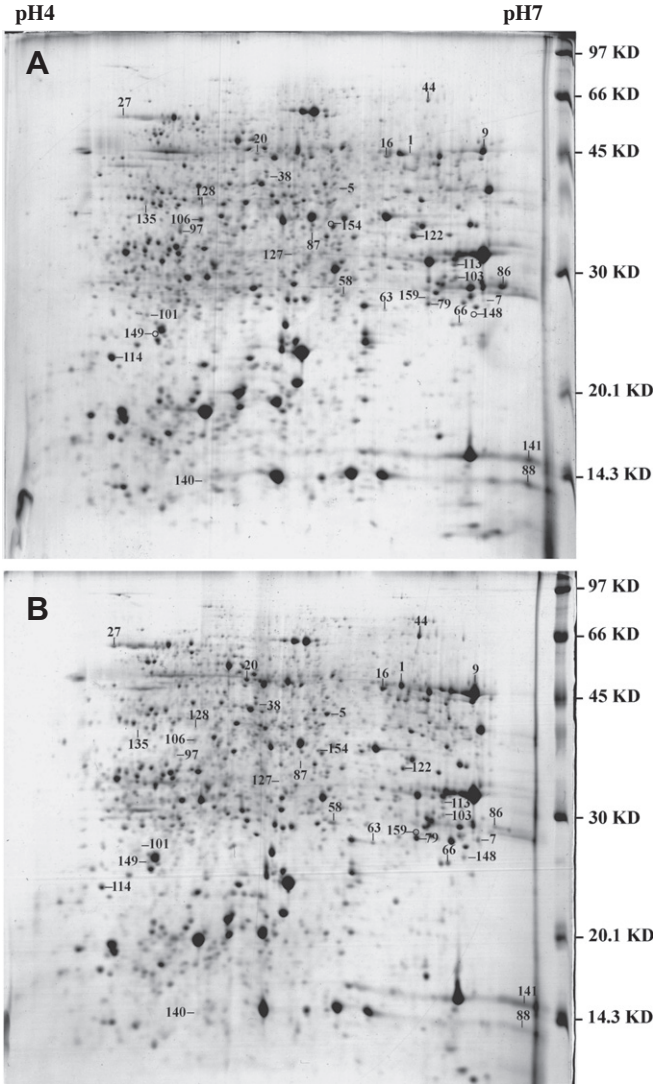

Fig. 3. CBB-stained 2-D gel maps of leaf proteins in *P. americana*. (A) Seedlings were grown under control (-Cd) condition; (B) Seedlings exposed to 400 μM Cd for 48 h. The proteins were resolved using a linear pH 4–7 IPG followed by 12.5% SDS–PAGE. Differentially expressed proteins identified are labeled in accordance with the numbers in Table 1.

Table 1
Differentially expressed proteins in *P. americana* L. leaves under control conditions and after 48 h of exposure to 400 μM Cd.

Spot no.	Protein name	NCBI accession No.	Species	MW (kDa)	pI	Cov. (%)	Mascot score	Fold change	Method
				Predic./ Exper.	Predic./ Exper.				
Photosynthesis and ATP production									
9, 114	Rubisco large subunit	gi|132013	*P. americana*	53.8/45.0, 27.2	6.6/6.6, 5.0	17	142	−2.24	M-TOF/ TOF
113	Rubisco large subunit	gi|113374116	*L. antipoda*	49.3/33.0	6.5/6.4	26	102	−1.94	M-TOF/ TOF
140	Rubisco large subunit	gi|20531018	*S. calymperes*	51.1/14.0	6.3/5.3	21	76	−2.03	M-TOF/ TOF
127	Rubisco large subunit	gi|298568058	*R. deamii*	49.2/35.0	6.3/5.5	37	107	−2.13	M-TOF/ TOF
103	Rubisco large subunit	gi|131993	*M. verticillata*	53.4/32.0	6.2/6.5	26	157	−2.15	M-TOF/ TOF
88	Rubisco small subunit	gi|223593	*N. tabacum*	14.6/14.2	5.0/6.8	44	75	−3.70	M-TOF/ TOF
87, 128	Glyceraldehyde-3-phosphate dehydrogenase, chloroplastic	gi|150261391	*S. oleracea*	39.7/38.0, 40.0	5.8/5.5, 5.3	29	91	−3.54	M-TOF/ TOF
106	Phosphoribulokinase	gi|125578	*M. crystallinum*	44.4/42.0	6.0/5.3	22	78	−2.96	M-TOF/ TOF
86	Carbonic anhydrase	gi|56562177	*S. lycopersicum*	34.8/30.0	6.7/6.7	25	82	−3.79	M-TOF/ TOF
159	Glyceraldehyde-3-phosphate dehydrogenase, cystosolic	gi|15222848	*A. thaliana*	37.0/36.0	6.7/6.6	25	178	Diminished	M-TOF/ TOF
16	Enolase	gi|33415263	*G. barbadense*	47.9/46.0	6.2/6.0	24	147	3.14	M-TOF/ TOF
20	ATP synthase CF1 beta subunit	gi|6017822	*P. americana*	53.0/51.0	5.3/5.2	34	335	2.96	M-TOF/ TOF
Sulfur and GSH metabolism									
1	S-adenosyl-L-homocystein hydrolase 2	gi|297831158	*A. lyrata subsp. lyrata*	53.9/50.0	5.6/5.8	15	166	5.23	M-TOF/ TOF
44	Cobalamin-independent methionine synthase, MS	gi|8134568	*M. crystallinum*	85.0/70.0	5.9/6.1	19	287	3.03	M-TOF/ TOF
38	S-adenosyl-methionine synthase, SMS	gi|48928010	*S. brevidens*	43.7/43.0	5.5/5.5	22	153	2.15	M-TOF/ TOF
7	Glutathione-S-transferase	gi|20197312	*A. thaliana*	24.1/28.0	6.1/6.5	19	93	4.61	M-TOF/ TOF
66	Glutathione-S-transferase	gi|158828314	*A. cebennensis*	26.7/25.6	5.2/5.8	44	71	2.09	M-TOF/ TOF
5	Sulfotransferase family protein	gi|15225893	*A. thaliana*	32.3/42.0	8.7/6.1	21	51	5.33	M-TOF/ TOF
97	Thi1 protein	gi|61679812	*A. thaliana*	30.1/33.0	5.9/5.2	33	80	−3.27	M-TOF/ TOF
Transcription, translation and chaperones									
149	Sig1	gi|2706544	*S. alba*	54.4/29.0	9.6/5.0	28	81	Induced	M-TOF/ TOF
135	NAC domain protein	gi|224144749	*P. trichocarpa*	41.3/40.0	8.1/5.2	22	71	−2.05	M-TOF/ TOF
122	Elongation factor 2	gi|32400836	*T. aestivum*	18.6/32.0	5.8/6.1	20	113	−2.01	M-TOF/ TOF
27	Heat shock cognate 70	gi|123620	*S. lycopersicum*	71.0/60.0	5.1/5.0	33	174	2.50	M-TOF
58	Calreticulin family protein	gi|116784673	*P. sitchensis*	46.0/30.1	4.5/6.0	22	189	2.03	ESI-Q-TOF

(*continued on next page*)

Table 1 (*continued*)

Spot no.	Protein name	NCBI accession No.	Species	MW (kDa)	pI	Cov. (%)	Mascot score	Fold change	Method
				Predic./ Exper.	Predic./ Exper.				
Antioxidant and oxidoreductases									
79	2-Cys-peroxiredoxin precursor	gi|6002472	*Brassica rapa subsp. pekinensis*	30.1/29.5	6.0/6.0	8	82	2.04	ESI-Q-TOF
63	Benzoquinone reductase	gi|124488474	*G. hirsutum*	21.7/23.7	6.1/6.2	13	72	2.08	ESI-Q-TOF
101	Cytochrome P450 DDWF1	gi|74273619	*G. hirsutum*	56.8/28.0	8.8/5.1	22	76	−3.08	M-TOF/TOF
154	Highly similar to auxin-induced protein belong to aldo/keto reductase family	gi|2462763	*A. thaliana*	37.8/38.0	5.7/5.5	19	90	Induced	M-TOF/TOF
141	Actin	gi|116222105	*P. cristatum*	38.5/14.0	5.3/6.8	22	148	−2.00	M-TOF/TOF
148	Predicted protein	gi|255073625	*Micromonas sp. RCC299*	32.3/28.0	9.5/6.5	28	78	Induced	M-TOF/TOF

information (Hajheidari et al., 2007; Cui et al., 2009). Fig. 2 shows spectra of two identified proteins. In spectrum A (spot 20) three sequence tags acquired by MALDI-TOF/TOF MS were used in the Mascot search (http://www.matrixscience.com), and all of them matched to ATP synthase CF1 beta subunit of *P. americana.* Spectrum B represents the spectra used for identification of glutathione-S-transferase (spot 66) of *P. americana.* Since the identification is based on cross-species, the matching results of three distinct spots (spot 9, 20, 114) to the proteins of *P. americana* in the protein database exhibit a high confidence in the algorithm applied in the study.

3.3. Differentially expressed proteins in response to Cd treatment

In total, 32 identified protein spots exhibited statistically significant increases or decreases in response to Cd treatment at 48 h time point (Fig. 3), corresponding to 25 unique gene products. Of those, 14 proteins were increased at least 2-fold while 11 were decreased more than 2-fold after Cd treatment (Table 1). For further verification of the changes in proteomic profiling, key proteins representing the distinct pathways, *i.e.* Rubisco large and small subunits, ATP synthase beta subunit, Hsp70 and enolase, were analyzed by immuno-blotting. As shown in Fig. 4, the alteration pattern of those proteins is in agreement with that revealed by 2D-gels. The differentially expressed proteins identified in the present work are summarized and listed in Table 1. The physiological implications of the Cd-affected proteins are discussed below.

3.4. Calvin–Benson cycle and ATP production

The largest group of proteins differentially expressed under Cd treatment was attributed to the proteins involved in photosynthesis (Table 1). These include Rubisco large subunit (spot 9, 103, 113, 114, 127) and small subunit (spot 88), the chloroplastic glyceraldehyde-3-phosphate dehydrogenase (spot 87, 128), phosphoribulokinase (spot 106) and carbonic anhydrase (spot 86) (Fig. 3, Table 1). Quantitative analysis of 2D-gels revealed that the level of these proteins was reduced 2.0–3.8-fold under Cd treatment (Fig. 3). Compared to that in the absence of Cd, the level of the large and small subunits of Rubisco decreased 2.0- and 3.7-fold, respectively (Fig. 3), which was further confirmed by immunoblot-experiments (Fig. 4). As for phosphoribulokinase, nearly 3-fold reduction was observed. Down-regulation of Rubisco, a key enzyme of Calvin-Benson cycle for CO_2 assimilation, has also been observed in numerous non-hyperaccumulator organisms (Hajduch et al., 2001; Kieffer et al., 2008) as well as in hyperaccumulator plants such as *T. caerulescens* (Tuomainen et al., 2006) under Cd treatment. Beside the reduction in protein amount, a significant inhibition of Rubisco activity was also reported (Nováková et al., 2004). Since Rubisco and phosphoribulokinase represent the enzymes catalyzing irreversible reactions in Calvin–Benson cycle, their drastic reduction in abundance and enzyme activity strongly suggests that efficiency of CO_2-fixation was decreased under Cd treatment. It has been proposed previously that Cd severely compromises Rubisco in higher plants (Nováková et al., 2004). In contrast to the enzymes involved in photosynthesis mentioned above, the level of enolase (spot 16) involved in the glycolytic pathway was enhanced remarkably under Cd treatment as demonstrated by both 2D-gel (Fig. 3) and immuno-blotting (Fig. 4). An increase in abundance of enolase was frequently observed in different organisms under a variety of stress conditions including Cd exposure (Vido et al., 2001; Lee et al., 2002; Sarry et al., 2006). Recently, the association of enolase with the tonoplast was clearly demonstrated in *Mesembryanthemum crystallinum* in response to salt stress treatment, highlighting the importance of this enzyme in plant tolerance to high salinity (Barkla et al., 2009). It would be of interest

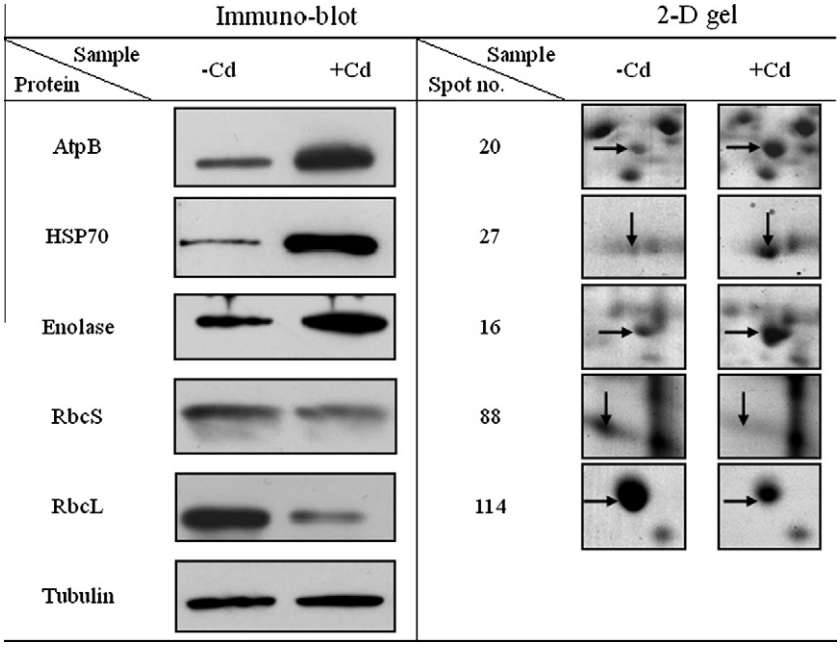

Fig. 4. Immunoblot detection of selected proteins among the proteins identified by 2-DE. All immunoblot experiments were done three times, and the similar results were obtained.

to determine the specific role of enolase in coping with Cd cytoxicity in *P. americana*. In addition, we found that the β subunit of CF1 ATP synthase (spot 20), a peripheral subunit of the ATP synthase in thylakoids, increased nearly 3-fold under Cd treatment (Figs. 3 and 4). This may be an indication of alteration in energetic status in the chloroplasts of *P. americana* under such toxic conditions.

3.5. Sulfur and GSH metabolism

The second largest group of differentially expressed proteins consisted of proteins involved in sulfur and GSH metabolism. These include S-adenosyl-methionine synthase (SAM synthetase, spot 38), cobalamin-independent methionine synthase (MS, spot 44), S-adenosyl-L-homocystein hydrolase 2 (SAH2, spot 1), putative sulfotransferase (spot 5) and glutathione-S-transferase (GST, spot 7, 66). In contrast to the proteins involved in Calvin–Benson cycle for CO_2 fixation in photosynthesis described above, all proteins related to sulfur metabolism identified in the present work were found enhanced under Cd treatment (Table 1). The levels of SAM synthetase, methionine synthase and glutathione-S-transferase were previously shown increased in various plant species exposed to Cd stress (Sarry et al., 2006; Kieffer et al., 2008). In this work, we identified an additional protein which has not been reported heretofore: S-adenosyl-L-homocystein hydrolase 2, an enzyme catalyzing hydrolysis of the substrate to form homocystein and adenosine. Our data showed that the protein level of SAM synthetase, methionine synthase and S-adenosyl-L-homocystein hydrolase 2 increased up to 5.2-fold in the leaf proteome of *P. americana* exposed to Cd treatment (Fig. 3, Table 1). Since all these enzymes are involved in catalyzing the intermediate reactions in regeneration of methionine and the methyl cycle (Vido et al., 2001; Sarry et al., 2006), such a remarkable accumulation of these proteins may lead to an increase in methionine level, resulting in compromising the supply of donor methyl groups required for methylation reactions in various biosynthetic pathways in *P. americana* coping with Cd cytoxicity.

Furthermore, we identified glutathione-S-transferase (GST) isoforms (spot 7, 66) on the 2-D gels (Fig. 3). The level of the GSTs was found increased up to 4.6-fold under Cd treatment (Table 1), supporting the suggested roles of GST in metal sequestration/detoxification (Marrs, 1996). It is tempting to further clarify the metal specificity of the GST isoforms identified in *P. americana*. Sulfotransferase (SOT) represents the enzyme catalyzing the transfer reaction of sulfate group (SO_4^{2+}) from 3′-phosphoadenosyl 5′-phosphosulphate (PAPS) to various metabolites (Klein and Papenbrock, 2004). Earlier studies using DNA microarray analysis revealed clear up-regulation of *SOT* gene in the hyperaccumulator *T. caerulescens* exposed to Cd (De Mortel et al., 2008). In this study, we found that the protein level of sulfotransferase was increased more than 5-fold in *P. americana* under Cd treatment (spot 5). On the other hand, we found Thi1 protein involved in thiamine biosynthesis reduced apparently under Cd treatment (Fig. 3, Table 1).

3.6. Transcription, translation and chaperones

Dramatic changes were also observed for a number of proteins associated with transcription and translation. Sig1, the principal sigma factor of the plastidic RNA polymerase, was identified on the 2D-gels (spot 149) and found induced/enhanced under Cd treatment (Fig. 3). The apparent isoelectric point (pI) of the Sig1, however, is estimated at pH 5.0 under our experimental condition (Fig. 3B). This is in discrepancy with the predicted or experimentally determined *pIs* for Sig1 (>9.0) in plant (Kestermann et al., 1998) and in cyanobacteria (Welsh et al., 2008). The acidic shift in *pI* of Sig1 isoform observed on the 2D-gels indicates that post-translational modifications, e.g. phosphorylation, may have occurred during the Cd treatment. A recent work by Shimizu et al. (2010), showed that phosphorylation of Sig1 occurred *in vivo* in *Arabidopsis*. In terms of transcription/translation-related proteins, we found that the NAC domain protein (spot 135) and elongation factor 2 (spot 122) reduced significantly under Cd treatment. NAC domain proteins are plant-specific transcriptional factors that regulate numerous gene expressions under both normal and various stress conditions (Souer et al., 1996). More recently, a NAC protein was found to be involved in a novel abscisic acid (ABA)-dependent stress-signaling pathway in *Arabidopsis* (Fujita et al., 2004). Notably, a heat shock cognate 70

(spot 27), a well-known molecular chaperone protecting proteins under various stress conditions (Nelson et al., 1992), and a calreticulin family protein (spot 58) were found enhanced by Cd treatment. Considering that Cd is chemically similar to Ca, and calreticulin (CRT) is a highly conserved Ca^{2+}-binding protein in eukaryotic cells (Roth et al., 2006; Michalak et al., 2009), we suggest that the elevated level of the calreticulin protein may serve as an efficient way to protect the *P. americana* cells against Cd cytoxicity.

3.7. Antioxidative enzymes and oxidoreductases

A number of proteins known as antioxidative enzyme or oxidoreductases were also identified among the differentially expressed proteins. Regarding to the proteins related to antioxidant reactions, we found that the precursor of 2-Cys-peroxiredoxin (spot 79) was enhanced under Cd treatment. Based on TargetP program (http://www.cbs.dtu.dk/services/TargetP/) prediction, the mature protein is targeted to the chloroplast. Considering its possible chloroplastic location as well as its general role in protection of plant cells by reducing reactive oxygen species (ROS) such as hydrogen peroxide (Møller et al., 2007), we suggest that the increased level of 2-Cys-peroxiredoxin precursor observed in the present experiments may imply that hydrogen peroxide was a major ROS species accumulated in the chloroplast of *P. americana* under such toxic conditions. In terms of oxidoreductases, we observed that benzoquinone reductase (spot 63) increased more than 2-fold under Cd treatment (Fig. 3, Table 1). Since the enzyme catalyzes bivalent redox reactions without production of free radical intermediates (Matvienko et al., 2001), the elevated level of the benzoquinone reductase observed under Cd treatment suggests that it may serve as one of the modules of ROS avoidance in coping with Cd toxicity. In addition, a putative auxin-induced protein (spot 154) belonging to aldo/keto reductase family was enhanced/induced significantly upon Cd treatment (Fig. 3). Since elevated levels of aldo/keto reductases were also observed in plants under diverse stress conditions (Nikiforova et al., 2003; Bona et al., 2007), we propose that the up-regulation of the protein observed in this work could be a general stress response in *P. americana*.

4. Conclusions

The present work revealed, for the first time, the major changes in the leaf proteome of the hyperaccumulator *P. americana* in response to Cd treatment. In total, 32 differentially expressed protein spots were identified using highly reproducible 2D-gels in combination of MALDI-TOF/TOF mass spectrometry. Our experimental data clearly demonstrated that proteins related to sulfur metabolism, particularly the key enzymes involved in the regeneration of methionine and methyl cycle, were up-regulated during the Cd treatment. Conversely, several key enzymes related to Calvin–Benson cycle for CO_2-fixation were down-regulated, suggesting that photosynthetic pathways are also susceptible to Cd in a hyperaccumulator plant. Furthermore, we observed that a number of proteins associated with transcription/translation, antioxidant and oxidoreduction reactions were among the Cd-responsive proteins, suggesting that a substantial rearrangement in the proteome occurred in *P. americana* exposed to Cd stress. These results provide fundamental information on molecular networks in leaf tissue of *P. americana* in cope with Cd toxicity. Moreover, this work also provides a good number of candidate proteins/genes for in-depth studies toward the molecular mechanisms of plant resistance to Cd stress. Keep in mind that roots are the first tissue in contact with the high level of Cd applied, and efficient translocation of the toxic metal from roots to shoot appears crucial in hyperaccumulation physiology (Krämer, 2010), further investigations are initiated towards understanding the specific roles of the candidate proteins mentioned above, as well as molecular networks in root tissue of *P. americana* in cope with Cd toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.06.029.

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