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Comparative proteomic analysis of *Typha angustifolia* leaf under chromium, cadmium and lead stress

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ABSTRACT

The present study investigated *Typha angustifolia* leaf proteome in response to Cr, Cd and Pb stress. *T. angustifolia* of 90 (D90) and 130 d (D130) old plants were subjected to 1 mM Cr, Cd and Pb and samples were collected 30 d after treatment. 2-DE coupled with MS (mass spectrometry) was used to analyze and identify Cr, Cd and Pb-responsive proteins. More than 1600 protein spots were reproducibly detected on each gel, wherein 44, 46, 66 and 33, 26, 62 spots in D90 and D130 samples were differentially expressed by Cr, Cd, Pb over the control, respectively. Of these differentially expressed proteins, 3, 1, 8 overlapped in D90 and D130; while 5, 8, 5 with regulation factors above 3 in one of D90 or D130 samples. Total of 22 and 4 up- and down-regulated proteins were identified using MS and data bank analysis. Cr-induced expression of ATP synthase, RuBisCO small subunit and coproporphyrinogen III oxidase; Cd-induced RuBisCO large subunit; Pb up-regulated carbohydrate metabolic pathway enzymes of fructokinase, and improved RuBisCO activase and large subunit, Mg-protoporphyrin IX chelatase. Contrarily, eIF4F was inhibited by Cr/Pb, chloroplast FtsZ-like protein and GF14omega impeded by Cd and Pb, respectively.

1. Introduction

Heavy metal contamination in soil is a widespread phenomenon around the world and mainly originates from increasing industrial pollution, urban activities and agricultural practices [1–4]. In China nearly 20 MHa arable lands were contaminated by heavy metals, which account for about 1/5 the total area of arable land [5]. Among the heavy metals, cadmium (Cd) and lead (Pb) in particular causes increasingly international concern [6]. Yet, chromium (Cr, especially Cr(VI)), unlike Cd and Pb, has received little attention from plant scientists [7]. With no biological function but extremely toxic, excessive Cr(VI), Cd and Pb concentrations in the contaminated soils can result into a variety of problems such as poor quality of agricultural products, loss of yield, ground water contamination, and ultimately metal toxicity to animals and humans [8]. Furthermore, unlike organic pollutants, heavy metals cannot be degraded through any known biological processes [9], thus there is an urgent need for remediation of contaminated sites adopting an effective and environment friendly technology such as phytoremediation using green plants to extract, sequester and detoxify the pollutants [1,10]. Consequently, selection and screening of plant species which are tolerant to toxic levels of toxic heavy metals has grabbed attention in the treatment of metal polluted soils.

Typha angustifolia (narrow-leaved cattail), a perennial macrophyte, is characterized by its fast growing, high biomass accumulation and remarkable resistance to heavy metal stress [11,12]. However, a lack of understanding of the basic physiological, biochemical, and molecular mechanisms involved in heavy metal hypertolerance and detoxification prevents its optimization of the phytoextraction technique and its further commercial application. Therefore, a research priority is to gain basic information on the proteome changes in exposure to Cr, Cd and Pb in *T. angustifolia*.

Abiotic stress such as heavy metal, high/low temperature, drought and salt stress induces changes of protein expression in plants [13,14]. It has been reported [15] that several proteins, with a function in the protection and repair of proteins such as the heat shock proteins (Hsp), are expressed under Cd stress. Pathogenesis-related (PR) proteins have also been reported to be induced by heavy metals [16]. Recent developments in mass spectrometry including MALDI–TOF–TOF MS (matrix assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry), IPG (immobilized pH gradients) strips and bioinformatics tools have made proteomic analysis more sensitive, reliable and powerful to characterize and quantify plant responses to environmental stress. Salekdeh et al. [17] identified three salt stress-responsive proteins in rice by 2-DE and MS analysis. Cuypers et al. [18] found Cu stress induced PvPR1 and PvPR2 specific protein in bean. Up-regulation of the MRP-type ABC transporter AtMRP3 has been observed upon

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Cd treatment [19]. Amme et al. [20] analyzed cold response proteins in Arabidopsis leaves and found 22 spots with at least 2-fold altered expression under 6 °C cold treatment compared to the control (20 °C). Ndimba et al. [21] studied the effects of salinity and hyperosmotic stress on plant cellular proteins extracted from *Arabidopsis thaliana* cell suspension cultures, and detected 266 protein spots significantly changed in abundance after NaCl and sorbitol treatments. A study on the changes in rice leaf protein pattern in response to Cd exposure has shown a drastic effect on the photosynthetic apparatus [22]. Nevertheless, compared to the analysis of the transcriptome, the analysis of the plant proteome in response to abiotic stresses is still limited. Moreover, up to now there is no comparative proteomic report on the changes in protein pattern of *T. angustifolia* under Cr, Cd and Pb stress.

In the present work, the specific changes in leaf protein pattern of *T. angustifolia* induced by Cr, Cd and Pb were detected. Proteins were separated by 2-DE, quantified by digital analysis and identified by MALDI–TOF–TOF MS, to gain new insights into the molecular mechanisms governing Cr, Cd and Pb tolerance. We asked two questions: (1) how many leaf proteins of *T. angustifolia* changed reproducibly in response to Cr, Cd and Pb stress? (2) do plants adapted to Cr, Cd and Pb tolerance show different protein responses? Still remained unclear and needed to make easy to understand. Here, differences in expression levels in the proteome of *T. angustifolia* between the two application dates of 90 and 130 d after sowing for Cr, Cd and Pb were also examined. This work further facilitates an understanding of the biochemical mechanisms of *T. angustifolia* tolerance to Cr, Cd and Pb stress on the impact of protein spectrum, which will lay important basis for its phytoremediation of heavy metal-contaminated soils.

2. Materials and methods

2.1. Plant materials and Cr, Cd and Pb stress treatments

The pot experiment was carried out May–September, 2008. Agricultural soil was collected from the experimental farm (depth 0–15 cm) on Huajiachi Campus of Zhejiang University, Hangzhou, China. The soil was air-dried and mixed daily until 8% water content was reached. Air-dried soil of 4 kg was weighed and loaded into a plastic pot (5 L, 20 cm height). The soil used in this investigation had a pH of 6.8, with the available heavy metal concentrations (EDTA-soluble) of Cr, Cd, and Pb 1.67, 0.15, and 9.63 mg kg^{-1}, respectively. The textural analysis showed the following composition: sand 65.0%, silt 28.8%, clay 6.2%, which indicated that this soil could be classified as silt loam. Seeds of *T. angustifolia* were scattered in the above mentioned pots, and irrigated with tap water to maintain moist. Pots were kept in a greenhouse under natural light condition during 60 d after sowing. At the day 60 seedlings were thinned to leave 15 uniform, healthy seedlings per pot, and then pots were transferred to a growth incubator with light intensity of 300 $\mu m\, m^{-2}\, s^{-1}$ and day/night temperature of 25 ± 0.5 °C/22 ± 0.5 °C with 14 h of day time. There were two application dates for heavy metal treatments: D90 and D130, in which seedlings were allowed to grow for another 30, and 70 d (i.e. 90 = 60 + 30 and 130 = 60 + 70 d after sowing), respectively, before Cd, Cr(VI) and Pb application. During this period (day 60–90 and day 60–130 after sowing for the D90 and D130 application dates, respectively), soils in the pots were kept moist (90–100% water holding capacity) for the first 20 and 60 d (day 60–80 and day 60–120 after sowing for the D90 and D130), respectively, and then irrigation was stopped when the water holding capacity reached about 50%. When the soil becomes to be drought (90, and 130 d after sowing), 500 ml of distilled water (control, no addition of heavy metal), 1 mM $K_2Cr_2O_7$, 1 mM $CdCl_2$, and 1 mM Pb $(NO_3)_2$ solution were added to each pot, and 10 d later the corresponding solution was added again to form four treatments of control, Cr, Cd and Pb for the two different growth period plants of 90 and 130 d after sowing (denoted as control-D90, Cr-D90, Cd-D90, Pb-D90, and control-D130, Cr-D130, Cd-D130, Pb-D130, respectively). The soil was kept moist thereafter. The experiment was arranged in a randomized block design with two replicates. After 30 d of the first heavy metal application (the two different growth period plants of 120 = 60 + 30 + 30 and 160 = 60 + 30 + 70 + 30 d after sowing) plants were sampled, flash frozen in liquid nitrogen, and stored at −80 °C. Two biologically independent replicates were prepared. All reagents were analytical grade and all stock solutions were prepared with deionized water.

2.2. Protein extraction and quantification

Total leaf protein extracts were prepared essentially according to the phenol extraction method described by Carpentier et al. [23] with minor modification. Leaf samples (3 g) of control and Cr, Cd, Pb-treated plants were grounded in a mortar separately to a fine powder in liquid nitrogen and homogenized in an extraction buffer containing 30 mg PVPP. The homogenate was suspended in 7 ml ice-cold phenol extraction buffer (0.7 M sucrose; 0.1 M KCl; 50 mM EDTA, 0.5 M Tris–HCl, 1% (w/v) DTT, pH 7.5; complete protease inhibitor cocktail (Roche Applied Science)) and immediately added 7 ml ice-cold Tris buffered phenol and vortexed for 15 s. The sample was then vortexed for 10 s every 5 min and repeated for six times at 4 °C. After centrifugation (30 min, 5000 × *g*, 4 °C) the phenolic phase was collected, and the sample was re-extracted with 14 ml of extraction buffer (added with the same volume of phenol extraction as collected items), and vortexed for 10 s every 5 min and repeated for six times at 4 °C. After centrifugation (30 min, 5000 × *g*, 4C) the phenolic phase was collected and precipitated overnight with five volumes 100 mM ammonium acetate in methanol at −20 °C. After centrifugation at 5000 × *g* for 30 min at 4 °C, the supernatant was removed and the pellet was rinsed twice in ice-cold acetone/0.2% DTT. Between the two rinsing steps, the sample was incubated for 60 min at −20 °C. The pellet was air-dried, resuspended in 200 μl lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris–HCl, pH 7.4, containing 1% (w/v) DTT; Amersham Biosciences, USA), and vortexed for 1 h at room temperature. Protein concentration was determined by standard Bradford assay using bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA).

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

2.3. Two-dimensional gel electrophoresis analysis

Proteins were separated by two-dimensional gel electrophoresis (2-DE). The first dimension separates proteins according to their isoelectric point. The sample was diluted with a rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHARPS, 0.002% bromophenol blue, 0.5% IPG-buffer, 0.28% DTT, pH 8.5) to 0.5–1 mg protein per 100 μl, 100 μg of proteins were loaded onto pH 4–7 IPG strips (24 cm, linear, Amersham Biosciences) and was applied via anodic cup loading. The gel strips were rehydrated for at least 8 h in 450 ml rehydration buffer. Isoelectrofocusing (IEF) was carried out on the Ettan IPGphor (Amersham Biosciences, USA) at 20 °C with current limit 50 μA/strip: 10–12 h at 30 V, 1 h at 500 V, 1 h at 1000 V (gradient), and 10 h at 8000 V to give a total of about 100 kVh. Prior to second dimension analysis, the individual strips were equilibrated for two times in order to resolubilise proteins and reduce disulphur bonds: 15 min in 10 ml denaturing solution (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 50 mM Tris (pH 8.8) containing 1% (w/v) DTT and subsequently for 15 min in 10 ml equilibra-

tion buffer containing 2.5% (w/v) IAA (iodoacetamide). The second dimension provided protein separation according to molecular weight. After equilibration, the separation in the second dimension SDS-PAGE was performed with 12.5% 1.5 mm SDS-polyacrylamide gels using Ettan DALT System (Amersham Biosciences, USA). A denaturing solution (0.5% agarose in running buffer) was loaded onto the gel strips and electrophoresis was performed in a Laemmli running buffer (25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS). The gels were run at 2–2.5 W per gel for the first 40 min and followed by 17 W per gel for 6 h until the dye front reached the bottom of the gel. The protein spots in analytical gels were visualized by silver staining [24]. For each sample, at least three 2-DE analyses each protein extract were performed. Acrylamide and protein standard (bovine serum albumin) were purchased from Bio-Rad (Hercules, CA, USA).

2.4. Protein visualization, image analysis and quantification

To analyze the expressed protein patterns, stained gels were scanned and calibrated using a PowerLook1100 scanner (UMAX), followed by analysis of protein spots using GE HealthCare Software (Amersham Biosciences). Spot detection was realized without spot editing. The protein spots were quantified using the vol.% criterion. Only those with significant and reproducible changes ($p < 0.05$) were considered to be differentially accumulated proteins in relative abundance in Cr, Cd or Pb-treated plants compared to controls. The target protein spots were automatically excised from the stained gels and digested with trypsin using a Spot Handling Workstation (Amersham Biosciences). Peptides gel pieces were placed into the EP tube and washed with 1:1 mixture of 50 μl of 30 mM $K_3Fe(CN)_6$ and 100 mM NaS_2O_3 for 10–15 min until completely discolored then washed with 200 μl bi-distilled water (two times for 5 min each). The washed solution was drained and washed with 50% ACN (acetonitrile, Fisher A/0626/17) and 100% ACN rotationally, and then incubated in 25 mM NH_4HCO_3 (ammonium bicarbonate, Sigma A6141) for 5 min at 37 °C. After leaching out of the incubation solvent, 50% ACN and 100% ACN were rotationally added and dried at 40 °C for 5 min, respectively. Trypsin digestion was carried out as follows: sequencing-grade porcine trypsin (Promega, Madison, WI, USA) was suspended in 25 mM NH_4HCO_3 at a concentration of 12.5 ng/μl to rehydrate the dried gel pieces. The trypsin digestion was carried out for 16 h at 37 °C. Peptides were extracted from the digest as follows for three times: 10 μl of 50% ACN containing 0.1% TFA (trifluoroacetic acid, GE HealthCare) was added to each tube and incubated for 5 min at 37 °C and the supernatants were transferred to new EP tube. The extracts were pooled and then vacuum concentrated for about 2 h. A solution of peptides was filtrated via Millipore (Millipore ZTC18M096) and mixed with the same volume of a matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA. After the peptides were co-crystallized with CHCA by evaporating organic solvents, tryptic-digested peptide masses were measured using a MALDI–TOF–TOF mass spectrometer (ABI4700 System, USA). All mass spectra were recorded in

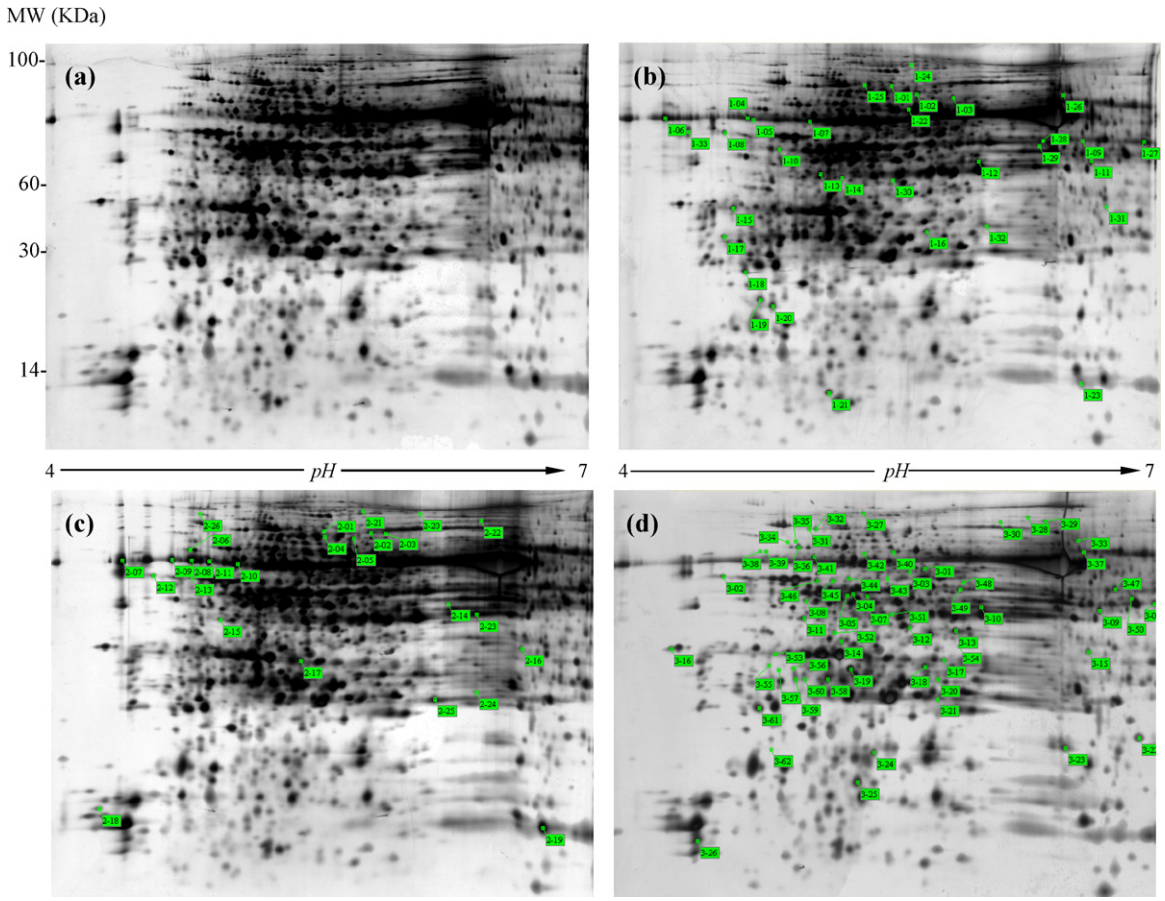

Fig. 1. Representative 2-DE maps comparing *T. Angustifolia* leaf proteins isolated from normal (a) and 1 mM Cr (b), Cd (c) or Pb (d) treated plants (D130). Total leaf proteins were extracted and separated by 2-DE. In IEF, 100 μg of proteins were loaded onto pH 4–7 IPG strips (24 cm, linear). SDS-PAGE was performed with 12.5% gels. The spots were visualized by silver staining. Differentially accumulated protein spots are indicated by green sashes. Twenty-three up-regulated spots (1-01–1-23) and 10 down-regulated spots (1-24–1-33) are indicated on the map of Cr-treated sample (b); 19 up-regulated spots (2-01–2-19) and 7 down-regulated spots (2-20–2-26) indicated on the map of Cd-treated sample (c); 26 up-regulated spots (3-01–3-26) and 36 down-regulated spots (3-27–3-62) indicated on the map of Pb-treated sample (d). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

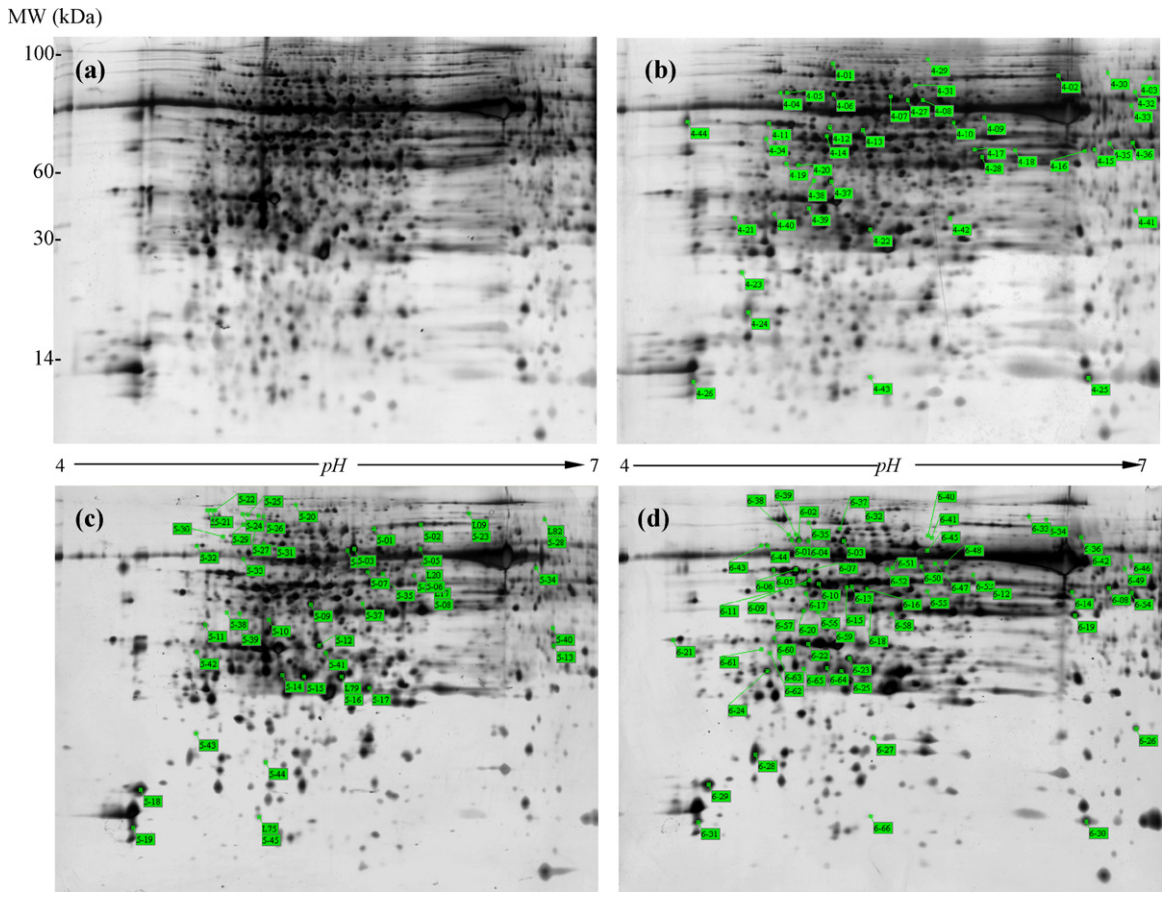

Fig. 2. Representative 2-DE maps comparing *T. Angustifolia* leaf proteins isolated from normal (a) and 1 mM Cr (b), Cd (c) or Pb (d) treated plants (D90). Differentially accumulated protein spots are indicated by green sashes. Twenty-eight up-regulated spots (4-01–4-28) and 16 down-regulated spots (4-29–4-44) are indicated on the map of Cr-treated sample (b); 19 up-regulated spots (5-01–5-19) and 27 down-regulated spots (5-20–5-46) are indicated on the map of Cd-treated sample (c); 31 up-regulated spots (6-01–6-31) and 35 down-regulated spots (6-27–6-66) are indicated on the map of Pb-treated sample (d). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

positive reflector mode and generated by accumulating data from 1000 laser shots. The following threshold criteria and settings were used: detected mass range of 700–3200 Da (optimal resolution for the quality of 1500 Da), using a standard peptide mixture (des-Argl-Bradykinin Mr904.468, Angiotensin I Mr1296.685, Glul-Fihrinopeptide B Mr1570.677, ACTH (1–17) Mr2093.087, ACTH (18–39) Mr2465.199; ACTH (7–38) Mr3657.929) as an external standard calibration, with laser frequency of 50 Hz, repetition rate of 200 Hz, UV wavelength of 355 nm, and accelerated voltage of 20,000 V. Peptide mass fingerprint data were matched to the NCBInr database using Profound program under 50 ppm mass tolerance.

2.5. Peptide and protein identification by database search

Data were processed via the Data Explorer software and proteins were unambiguously identified by searching against a comprehensive non-redundant sequence database (NCBInr) using the MASCOT software search engine (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS). The search parameters were as follows: (1) peptide quality of 800–4000 Da, mass tolerance for the fragment ion of 0.25 Da; (2) a minimum of seven matching peptides; (3) one missed cleavage; (4) taxonomy: Viridiplantae (green plants); and (5) allowed modifications, alkylation of cysteine by carbamidomethylation of Cys (complete) acetylation of the N-terminus and oxidation of methionine (partial). Moreover, in order to evaluate protein identification, we considered the percentage of sequence coverage, the observation of distribution of matching peptides (authentic hit is often characterized by peptides that are adjacent to one another in the sequence and that overlap), the distribution of error (distributed around zero), the gap in probability and score distribution from the first to other candidate; only matches with over 90% sequence identity and a maximum *e*-value of 10^{-10} were considered.

Fold increase and decrease in Cr, Cd, and Pb exposed leaves vs. unexposed plants were calculated as treated/control and −control/treated for up- and down-regulated proteins, respectively. For single-peptide identified proteins, positive/negative regulation was assigned when it was shown that the regulation factors were above 1.5 ($p < 0.05$).

3. Results

3.1. 2-DE analysis of leaf proteins in Cr, Cd or Pb -stressed T. angustifolia

The total proteins from leaves were resolved into more than 1600 spots (ranging 1634–2112) in each reproducible SDS-polyacrylamide gels (isoelectric focusing pH range, 4–7; size, 24 cm). Protein spots were visualized by silver staining (Figs. 1 and 2). The total proteins from leaves were resolved into more than 1600 spots (ranging 1634–2112) in each reproducible SDS-polyacrylamide gels (isoelectric focusing pH range, 4–7; size, 24 cm). Figs. 1 and 2 show the entire image of the silver Blue-stained 2-DE gels of total extracted proteins from *T. angustifolia* leaves under normal and Cr, Cd or Pb-stressed condition of D90

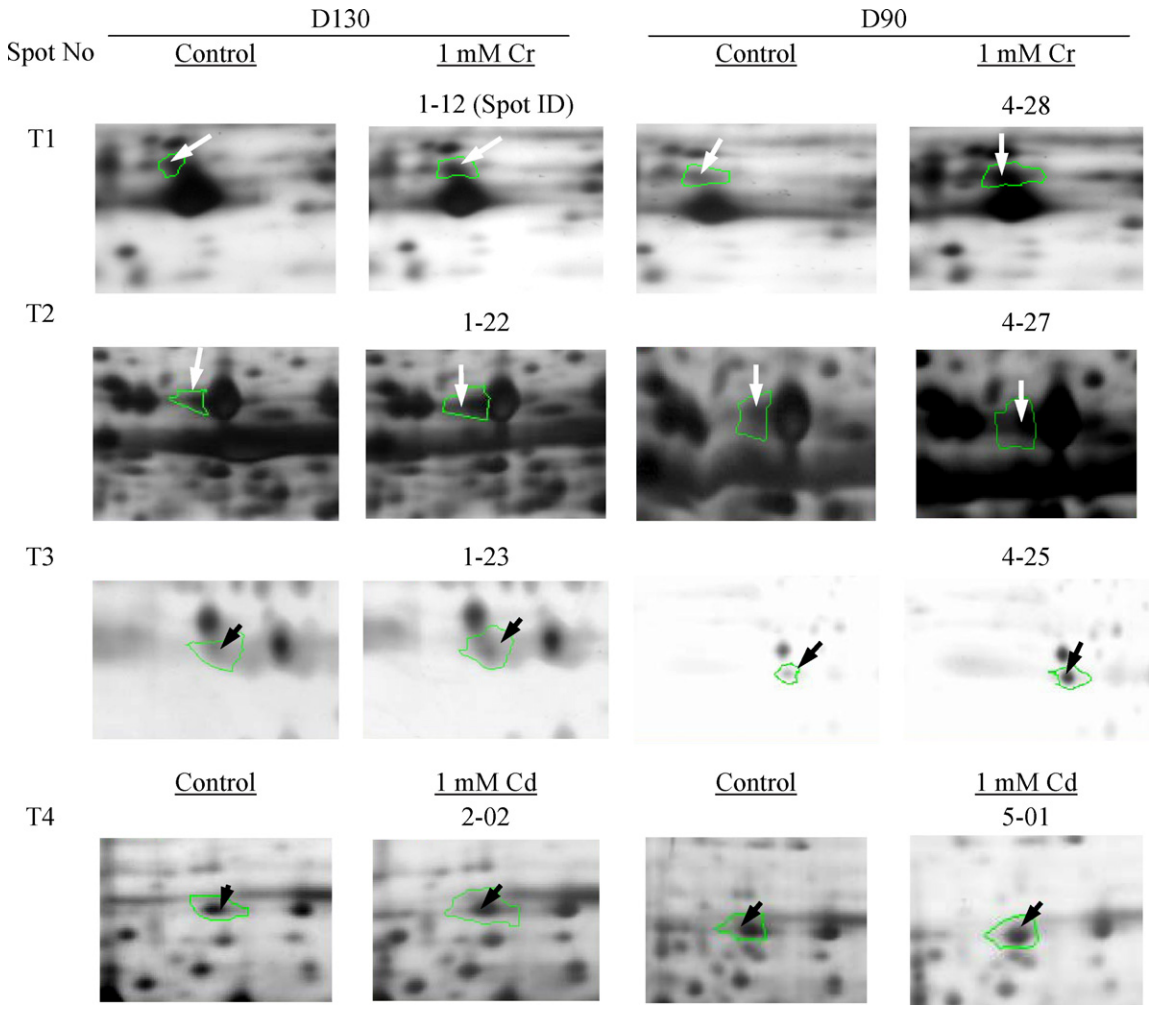

Fig. 3. The 'spot view' of proteins up-regulated by Cr or Cd in the both application dates (D90 and D130). The areas in the arbitrary polygon of *T. Angustifolia* leaf proteins (indicated with arrows) from control plants have been enlarged and placed side by side with the corresponding areas of gel obtained from plants treated with 1 mM Cr or Cd. Protein spot ID refers to numbers in Figs. 1(B and C) and 2(B and C).

and D130, respectively (D90 and D130: 500 ml of 1 mM Cr, Cd or Pb applied 90 and 130 d after sowing, respectively, and 10 d later the corresponding solution was added again, for details, refer to experimental procedures). Comparing 2-DE gels from the control and from 1 mM Cr, Cd, or Pb-treated samples showed many differences in protein presence. Overall, the protein levels of 44, 46 and 66 spots were found to be altered by Cr, Cd and Pb for D90, respectively. Of these alerted proteins, 28, 19 and 31 were up-regulated by Cr, Cd and Pb, respectively, while 16, 27 and 35 were down-regulated. As to D130 samples, the protein levels of 33, 26 and 62 spots were found to be altered by Cr, Cd and Pb, respectively, with 23, 19 and 26 being up-regulated, while 10, 7 and 36 down-regulated (Supplementary Fig. S1).

Further comparing the data of the two experiments of D90 and D130, 3, 1 and 8 proteins were found in both experiments to have been up-regulated by Cr, Cd and Pb, respectively. Representative samples of 12 (3 + 1 + 8) spots of up-regulated proteins were enlarged and placed side by side with corresponding areas of gels obtained from plants of the control and treated with 1 mM Cr, Cd and Pb (Figs. 3 and 4). These up-regulated proteins were changed in a similar manner in the both experiments, appear to be related to Cr, Cd and Pb stress and detoxification. Therefore, 12 spots of proteins were analyzed by MALDI–TOF–TOF MS for identification. In addition, MALDI–TOF–TOF MS analysis was also conducted on the proteins of 5, 8, 5 spots (c.f. 3, 3, 2 spots in D90 and 2, 5, 3 in D130, respectively), that the positive/negative regulation factors were above 3 between control and Cr, Cd or Pb-treated samples. Therefore, total 30 spots (c.f. 12 spots up-regulated by Cr, Cd and Pb in the both of D90 and D130, 8 and 10 with relatively high abundance in D90 and D130) were selected for MALDI–TOF–TOF MS analysis. All theses 30 protein spots identified as having significant changes expression levels and present in sufficient amounts to be visible on a coomassie-stained preparative gel were excised for mass spectrometric analysis. The resulting spectra of 30 protein spots were used to query databases for protein identification.

3.2. MALDI–TOF–TOF MS analysis and identification of differentially expressed proteins by Cr, Cd and Pb stress

From the excised protein spots, a total of 30 proteins with significantly differential expression levels were analyzed by MALDI–TOF–TOF MS. Four of them had no MS/MS data, although they could be identified by PMF data, their theoretical MW and *p*I did not fit well with the experimental ones. Their identities need to be further confirmed. Only 22 and 4 up- and down-regulated proteins were unambiguously identified by MS and data bank analysis (Tables 1–3). Although the genomic sequence of *T. angustifolia* has not yet been characterized, proteins were able to be identified via homologous proteins primarily from other green plants by the

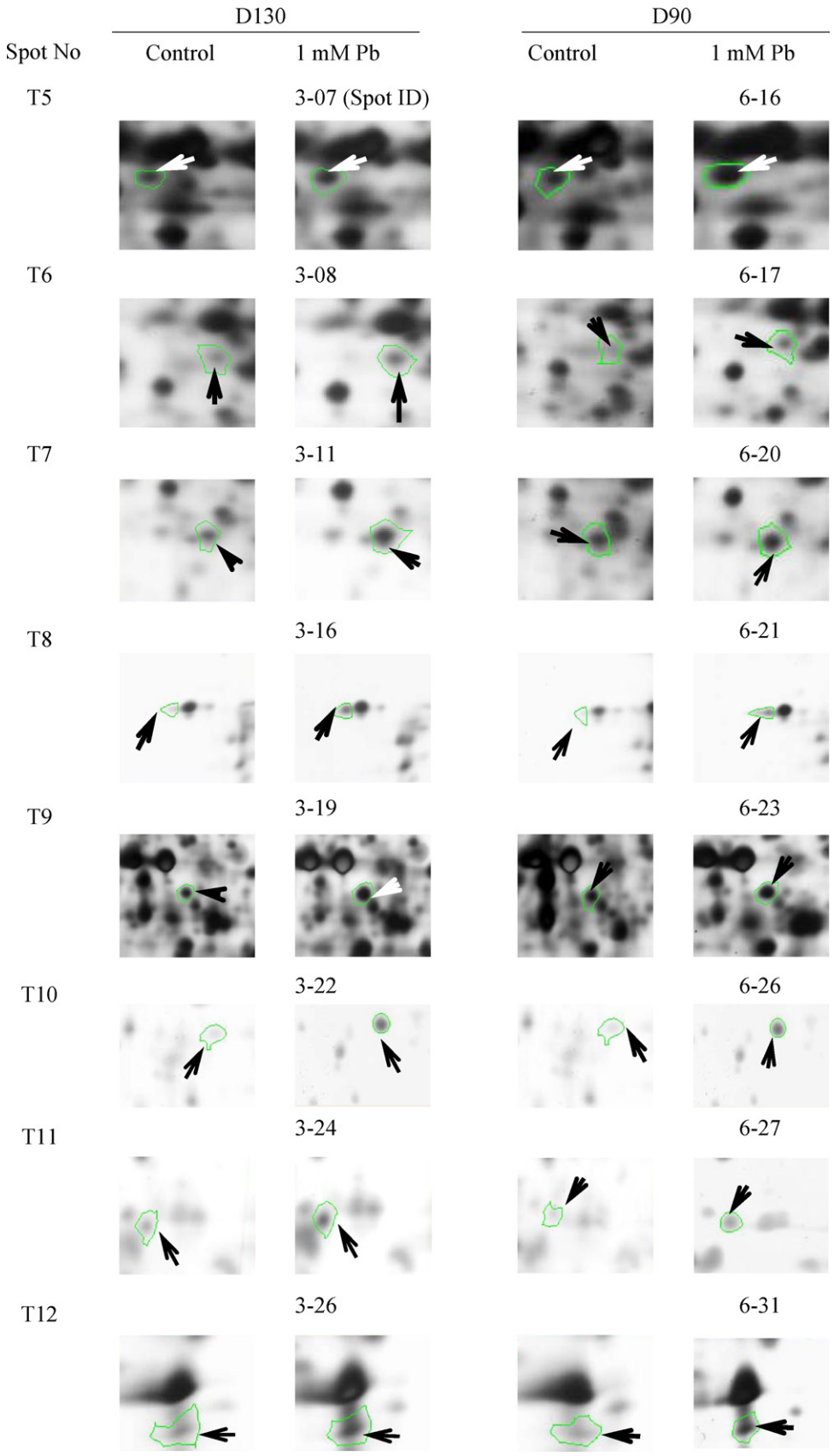

Fig. 4. The 'spot view' of proteins up-regulated by Pb in the both of D90 and D130. The areas in the arbitrary polygon of *T. Angustifolia* leaf proteins (indicated with arrows) from control plants have been enlarged and placed side by side with the corresponding areas of gel obtained from plants treated with 1 mM Pb. Protein spot ID refers to numbers in Figs. 1D and 2D.

Table 1
Proteins whose expression was significantly induced in Cr or Cd -treated *T. Angustifolia* leaves in the both of Cr/Cd application dates.

Spot no.	Spot ID		Protein name	Accession number	MW (Da)	pI	Protein score C.I. %	Amino acid sequence coverage %	Number of peptides matched	Fold increase	
	D130	D90								D130	D90
Cr-induced protein											
T1	1-12	4-28	Coproporphyrinogen III oxidase [*Oryza sativa*]	GI:29367603	36450.1	6.0	100	51.23	14	3.5	2.3
T2	1-22	4-27	ATP synthase CF1 alpha subunit [*Lotus japonicus*]	GI:13518443	55746.3	5.2	100	48.63	17	1.5	1.5
			ATP synthase CF1 alpha subunit [*Amborella trichopoda*]	GI:34500899	55329.0	5.2	100	49.51	19		
T3	1-23	4-25	RuBisCO small subunit (*Musa acuminate*)	gi|3914598	20497.2	9.2	99.99	48.33	9	2.6	2.6
			Ribulose bisphosphate carboxylase small chain,chloroplast precursor (RuBisCO small subunit) (*Glycine tomentella*)	gi|3914590	19936.0	8.9	99.99	55.62	7		
			Ribulose bisphosphate carboxylase small chain,chloroplast precursor (RuBisCO small subunit) (*Glycine tabacina*)	gi|3914591	20005.0	8.9	99.99	55.62	7		
Cd-induced protein											
T4	2-02	5-01	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit [*Anisophyllea corneri*]	gi|2653881	48558.6	6.6	100	46.36	15	1.9	1.7
			Ribulose-1,5-bisphosphate carboxylase large subunit [*Afrocarpus falcatus*]	gi|13548750	49220.7	6.2	100	48.76	15		
			Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Podocarpus reichei*]	gi|21427234	49307.9	6.6	99.99	52.81	16		

Protein spot ID refers to numbers in Figs. 1(B and C) and 2(B and C). Accession number of top database match from the NCBInr database. Protein induced in Cr or Cd exposed leaves vs. unexposed plants. Fold increase were calculated as treated/control for up-regulated proteins. All ratios shown are statistically significant ($p < 0.05$). D90 and D130 referred to the first application date of heavy metals: 90 and 130 d after sowing.

MASCOT software search engine, which combines de novo peptide sequencing with database identifications.

As to the proteins changed in a similar manner in both of D90 and D130 samples, as shown in Table 1, exposure to 1 mM Cr induced the expression of coproporphyrinogen III oxidase, ATP synthase CF1 alpha subunit, and RuBisCO small subunit by database match with homologous proteins from *Oryza sativa*, *Lotus japonicus*, *Amborella trichopoda*, *Musa acuminate*, *Glycine tomentella* and *Glycine tabacina* proteins. While 1 mM Cd-induced ribulose-1,5-bisphosphate carboxylase oxygenase large subunit by database match with homologous proteins from *Anisophyllea corneri*, *Afrocarpus falcatus*, and *Podocarpus reichei* (Table 1). Eight Pb-induced proteins in both of D90 and D130 samples were identified via matching to proteins from *O. sativa*, *A. thaliana*, *Lycopersicon esculentum*, *Brassica napus* and *Lilium longiflorum*. These proteins are ribulose-1,5-bisphosphate carboxylase/oxygenase activase, Mg-protoporphyrin IX chelatase, fructokinase, binding/catalytic/coenzyme binding, 30S ribosomal protein S10, putative peptidyl-prolyl cis-trans isomerase, GRSF and plastocyanin (Table 2).

Concerning regulation factors above 3 between control and Cr, Cd or Pb-treated samples of one of D90 and D130 (Table 3 and Fig. 5), Cr-induced ATP synthase CF1 alpha subunit by 3-fold in D90 and ribulose 1,5-bisphosphate carboxylase by 3.4-fold in D130 relative to control. Contrarily, the expression of eukaryotic initiation factor 4A (eIF4A) was inhibited by 3.1-fold in D90. Cd-induced ribulose bisphosphate carboxylase large subunit but depressed chloroplast FtsZ-like protein in D90; induced ribulose-1,5-bisphosphate carboxylase oxygenase large subunit and beta-tubulin in D130. Pb repressed GF14omega isoform in D90 and eIF4A in D130, but the expression of ribulose 1,5-bisphosphate carboxylase large subunit and ascorbate peroxidase (APX) in D130 were increased by 3.1- and 7.1-fold, respectively.

4. Discussion

Some plants have evolved naturally selected metal hypertolerance and thus are able to thrive in metal-contaminated soil [11]. These traits and their potential exploitation for the phytoremediation of soil–metal contamination have stimulated intensive research on plant systems. However not much is known about proteins response to elevated metal levels in plants. *T. angustifolia*, a fast-growing plant with high biomass and remarkable tolerance to heavy metals, is one of the desirable candidates for the induced phytoextraction of metal polluted soils [11,9]. Therefore, *T. angustifolia* is an interesting plant species for analyzing molecular basis of heavy metal tolerance mechanisms. In this study, plants of *T. angustifolia* showed high tolerance to heavy metal toxicity with no visual toxic symptom when exposed to 1 mM Cr, Cd or Pb stress, although Cr-stress reduced plant height and dry weight per plant by 10.2%, 33.5% on average of D90 and D130. Moreover, Cd and Pb addition also increased plant height and biomass (c.f. on average of D90 and D130, plant height and dry weight increased by 11.3% and 12.3% over the control). This observation is consistent with our previous reports [9]. Under metal stress (e.g. Cd and Pb), tolerant species and genotypes in plant kingdom could reduce toxic metal activity to alleviate or eliminate its toxicity through regulating the physiological and biochemical metabolism. In order to survive, plants have to develop efficient and specific heavy metal detoxification mechanism in different plant species [25]. The processes of heavy metal detoxification have been studied in a wide range of crop and herbaceous species [26], and also investigated in perennials, which some of them are reported to be notably tolerant against heavy metals [27]. Several sequestration and detoxification strategies are known to occur in a number of herbaceous taxa. However, depending on the metal and its concentration, diverse plant

Table 2
Proteins whose expression was significantly induced in Pb-treated *T. Angustifolia* leaves in the both of Pb application dates.

Spot no.	Spot ID		Protein name	Accession number	MW (Da)	p*I*	Protein score C.I. %	Amino acid sequence coverage %	Number of peptides matched	Fold increase	
	D130	D90								D130	D90
T5	3-07	6-16	Os11g0707000 [*O. sativa* (japonica cultivar-group)]	gi|115486823	47899.8	5.9	100	48.04	14	1.5	2.6
			Ribulose-1,5-bisphosphate carboxylase/oxygenase activase [*O. sativa*]	gi|1778414	47826.8	5.9	100	42.13	12		
			Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor (RuBisCO activase)	gi|109940135	51421.4	5.4	100	43.78	14		
T6	3-08	6-17	Magnesium-chelatase subunit chli, chloroplast precursor (Mg-protoporphyrin IX chelatase)	gi|3334150	45842.9	5.5	100	49.88	15	1.5	1.9
			Magnesium-chelatase subunit chli, chloroplast precursor (Mg-protoporphyrin IX chelatase)	gi|3334149	46598.6	6.6	100	44.60	13		
			Sulphur [*Nicotiana tabacum*] a subunit of the magnesium-chelatase	gi|11493393	46244.2	6.0	100	40.09	12		
T7	3-11	6-20	Pfkb-type carbohydrate kinase family protein [*Arabidopsis thaliana*]	gi|22330456	41445.3	5.5	100	21.61	7	1.8	1.7
			Putative fructokinase [*O. sativa* Japonica Group]	gi|51535181	43427.5	6.0	100	32.27	9		
			Fructokinase [*Lycopersicon esculentum*]	gi|23476263	40164.3	5.4	100	25.07	7		
T8	3-16	6-21	GRSF [*Lilium longiflorum*]	gi|99029149	23605.2	10.4	99.98	26.57	4	2.0	3.8
T9	3-19	6-23	Binding/catalytic/coenzyme binding [*Arabidopsis thaliana*]	gi|18404496	34858.2	8.4	100	20	5	1.7	1.5
T10	3-22	6-26	30S ribosomal protein S10, chloroplast precursor	gi|75274488	21592.1	8.6	100	30.96	5	1.6	2.3
			30S ribosomal protein S10, chloroplast, putative [*Arabidopsis thaliana*]	gi|15231154	20824.0	9.0	100	28.27	4		
			30S ribosomal protein S10-like [*Brassica napus*]	gi|56693625	21637.2	8.7	100	27.55	4		
T11	3-24	6-27	Putative peptidyl-prolyl cis-trans isomerase, chloroplast precursor [*O. sativa* Japonica]	gi|13486733	24702.3	8.1	98.46	42.42	6	1.9	2.1
T12	3-26	6-31	Plastocyanin	gi|130270	10353.0	4.3	99.98	22.22	1	1.6	1.8

Protein spot ID refers to numbers in Figs. 1D and 2D. Accession number of top database match from the NCBInr database. Protein induced in Pb exposed leaves vs. unexposed plants. Fold increase were calculated as treated/control for up-regulated proteins. All ratios shown are statistically significant ($p < 0.05$). D90 and D130 referred to the first application date of heavy metals: 90 and 130 d after sowing.

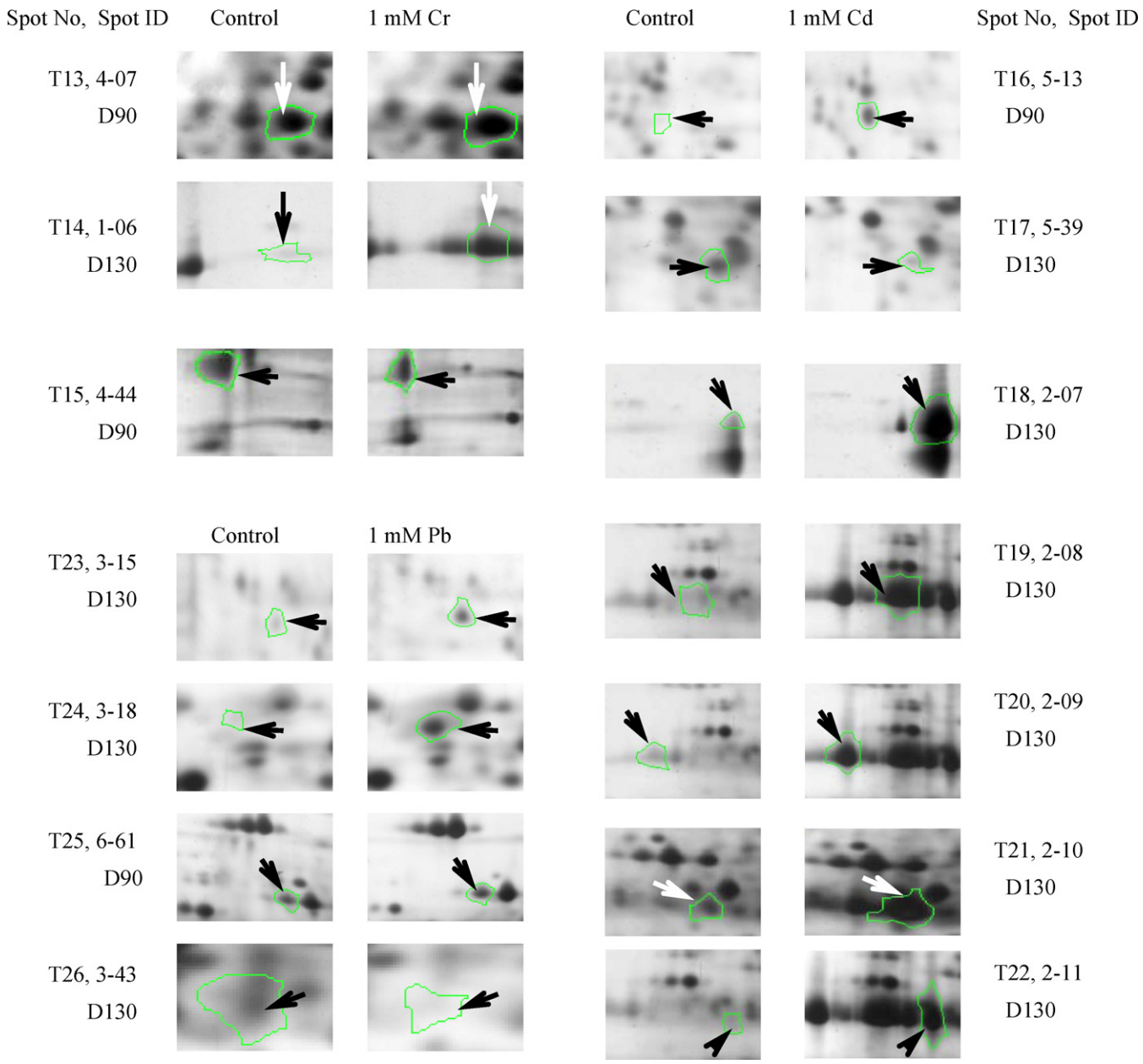

Fig. 5. The 'spot view' of proteins up/down-regulated by Cr, Cd or Pb in one of the D90 or D130 application date. The areas in the green arbitrary polygon of *T. Angustifolia* leaf proteins (indicated with arrows) from control plants have been enlarged and placed side by side with the corresponding areas of gel obtained from plants treated with 1 mM Cr, Cd or Pb. Protein spot ID refers to numbers in Figs 1 and 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

species exhibit a variety of stress-alleviating mechanisms. Moreover, little research has been carried out on the physiochemical responses of *T. angustifolia* under high levels of Cr, Cd and Pb stress, and the mechanisms responsible for its hypertolerance to heavy metal stress and detoxification on the protein level to the best of our knowledge remain unknown. In this work, proteins response to Cr, Cd and Pb stress were investigated for the first time by 2-DE and MALDI–TOF–TOF MS analyses with high confidence, in spite of the lack of genomic information of *T. angustifolia*. The quantitative proteomic analysis of differential protein expression profiles utilized in this work was effective in identifying both comprehensive and biologically significant response to Cr, Cd and Pb stress.

Specific impacts of various metals on photosynthesis have been demonstrated by Krupa and Baszynski [28], and the impacts of heavy metals on photosynthesis have been reviewed by Prasad and Strzalka [29], who described negative effects on pigment, photosystem, enzyme, and gas exchange on the protein level to the best of our knowledge which still remains unknown. In the present study, we identified Cr-induced three proteins expression in the both of D90 and D130: ATP synthase CF1 alpha subunit (c.f. up-regulated 1.5- and 1.5-fold, respectively, in D90 and D130 compared with control), RuBisCO small subunit (c.f. 2.6- and 2.6-fold) and coproporphyrinogen III oxidase (CPO, EC 1.3.3.3) (2.3- and 3.5-fold) (Table 1). In plants ATP synthase is present in chloroplasts and integrated into thylakoid membrane; the CF1-part sticks into stroma, where dark reactions of photosynthesis and ATP synthesis take place. ATP-dependent synthase/protease plays essential roles in controlling the availability of short-lived regulatory proteins and in removing abnormal or damaged proteins. It has been reported [30] that these enzymes play a critical role in the removal of damaged proteins and in the fine control of some key cellular components, combining a peptidase and a chaperone activity.

Table 3
Proteins whose expression was significantly induced (+) or repressed (−) in Cr, Cd or Pb -treated *T. Angustifolia* leaves by a factor >3 in one of the D90 or D130 samples.

Spot no.	Spot ID		Protein name	Accession number	MW (Da)	p*I*	Protein score C.I. %	Amino acid sequence coverage %	Number of peptides matched	Fold increase (+) or decrease (−)	
	D130	D90								D130	D90
Cr-induced/repressed proteins											
T13		4-07	ATP synthase CF1 alpha subunit [*Amborella trichopoda*]	GI:34500899	55329.0	5.2	100	35.98	13		+3.0
T14	1-06		Ribulose 1,5-bisphosphate carboxylase	gi|343628	51881.9	6.1	100	69.10	25	+3.4	
T15		4-44	Eukaryotic initiation factor 4A (eif4a)[*O. sativa* Japonica Group]	gi|303844	46901.9	5.3	100	55.93	17		−3.1
Cd-induced/repressed proteins											
T16		5-13	Ribulose bisphosphate carboxylase large subunit [*Bentinckia nicobarica*]	gi|13242831	52856.4	6.0	99.99	44.75	17		+6.7
T17		5-39	Chloroplast ftsz-like protein [*Nicotiana tabacum*]	gi|7672159	42863.2	5.9	91.92	39.95	12		−3.1
T18	2-07		Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit [*Crypteronia paniculata*]	gi|6691139	50582.6	6.3	99.96	41.89	15	+11.7	
			Ribulose bisphosphate carboxylase large chain	gi|6093933	51907.3	6.4	99.95	38.68	13		
			Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit [*Mayaca fluviatilis*]	gi|21684951	51650.0	6.2	99.94	38.06	14		
T19	2-08		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Gironniera subaequalis*]	gi|2274826	47573.1	6.3	100	50	15	+6.0	
			Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Hemiptelea davidii*]	gi|2281013	47635.1	6.3	100	45.81	14		
			Ribulose 1,5-bisphosphate carboxylase large subunit [*Licania tomentosa*]	gi|7240309	48604.6	6.6	100	47.95	15		
T20	2-09		Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit)	gi|2500666	50217.4	6.6	100	53.86	17	+4.7	
			Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit)	gi|2500662	50245.4	6.6	100	52.32	17		
			Ribulose 1,5-bisphosphate carboxylase large subunit [*Cleome hassleriana*]	gi|7240142	51615.0	6.0	100	41.97	15		
T21	2-10		Beta-tubulin R2242 – rice	gi|1076738	49831.6	4.7	100	55.18	17	+3.9	
			Tubulin beta-3 chain (beta-3-tubulin)	gi|74053562	50088.8	4.7	100	49.10	16		
			Beta-tubulin [*O. sativa* (japonica cultivar-group)]	gi|493725	50075.7	4.7	100	53.02	16		
T22	2-11		Ribulose-1,5-bisphosphate carboxylase large subunit [*Phyllocladus trichomanoides*]	gi|13548880	49616.9	6.3	99.93	39.20	13	+6.8	
Pb-induced/repressed proteins											
T23	3-15		Ribulose 1,5-bisphosphate carboxylase large subunit[*Caryopteris bicolor*]	gi|1695931	50498.5	6.1	100	51.43	14	+3.1	
T24	3-18		Ascorbate peroxidase (APX) [*Solanum tuberosum*]	gi|24636598	27394.8	5.7	98.94	32.20	6	+7.1	
T25		6-61	GF14omega isoform [*Arabidopsis thaliana*]	gi|487791	29109.5	4.7	99.15	52.12	10		−4.1
T26	3-43		Eukaryotic initiation factor 4A (EIF4a)[*O. sativa* Japonica Group]	gi|303844	46901.9	5.3	100	54.00	16	−3.7	

Protein spot ID refers to numbers in Figs. 1 and 2. Accession number of top database match from the NCBInr database. Protein induced in Cr, Cd, Pb exposed leaves vs. unexposed plants. Fold increase and decrease were calculated as treated/control and −control/treated for up and down-regulated proteins, respectively. All ratios shown are statistically significant ($p < 0.05$).

Therefore the expressed up-regulation of ATP synthase in *T. angustifolia* could be an attribute of it hypertolerance when exposed to Cr-induced stress as no patches or necrosis were seen in this plants. Leaf proteins contain one of the purest and highly nutritive components: RuBisCO (ribulose 1,5-bisphosphate carboxylase). RuBisCO, also called Fraction-I protein, accounts for up to 30–70% of soluble leaf proteins (SLP) [31] and plays a part in photosynthesis. Ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, RuBisCO) catalyzes the initial step of carbon metabolism, the fixation of carbon dioxide, in photosynthetic eukaryotes. RuBisCO is an extremely slow catalyst and moreover its carboxylation activity is compromised by competing side-reactions, the most notable with another atmospheric gas, O_2, which attacks the same enediol intermediate of RuBP. The opposing oxygenase activity of RuBisCO results in the synthesis of phosphoglycolate, a molecule of limited use to most organisms. Phosphoglycolate is re-circulated by photorespiration, an energy-requiring salvage pathway. This causes a constant drain on the pool of the sugar substrate (RuBP) and results in a decrease of the efficiency of carbon fixation by up to 50%. Thus, the key to the efficiency of any particular RuBisCO enzyme should be to enhance it with the ultimate aim to suppress oxygenation and improve carboxylation by RuBisCO as a means to improve plant height and biomass a required characteristics of a possible candidate for phytoremediation of soil–metal-contaminated sites. CPO, a key enzyme in the biosynthetic pathway of chlorophyll, catalyzes the oxidative decarboxylation of coproporphyrinogen III to proto-porhyrinogen IX in the chlorophyll biosynthetic pathways in plants [32]. Plant cDNA sequences encoding CPO were obtained from soybean [33], tobacco and barley [34]. Ishikawa et al. [32] found a deficiency of coproporphyrinogen III oxidase causes lesion formation in Arabidopsis. The fact that ATP synthase, RuBisCO small subunit and CPO up-regulated, under Cr-stress, might indicate that they might play an important role in Cr-detoxification by energization of the chloroplasts, and maintaining normal or even high level of biosynthesis of chlorophyll and carbon metabolism.

Under Cd stress, 19/27 (up/down-regulated) in D90 and 19/7 in D130 spots showed a Cd-dependent alteration in the protein ratio (Table 2). However, only one spot was observed to change in a similar manner (up-regulated 1.9- and 1.7-fold) in both of D90 and D130 samples, i.e. ribulose-1,5-bisphosphate carboxylase oxygenase large subunit. In addition, we found five and one spots in D130 and D90 samples with up-regulated factors over 3-fold, and these spots were excised and analyzed by MALDI–TOF–TOF MS for identification. Surprisingly, except for beta-tubulin, five of them represented the same protein of ribulose-1,5-bisphosphate carboxylase oxygenase large subunit, (Table 3). Concerning the four protein spots on 2D gel of 2-07, 2-08, 2-09 and 2-11 (spot no: T18, T19, T20, T22), post-translational modification with small-molecule modification may affect their isoelectric point of the same protein, and glycosylation and other molecular modification will affect the molecular weight (spot no: T16; spot ID: 5–13). Therefore, up-regulation of modified ribulose-1,5-bisphosphate carboxylase oxygenase large subunit may be beneficial to protect photosynthesis against Cd-toxicity in *T. angustifolia* as it was evidence in the increase in plant height and biomas in our study. However, detailed studies on these post-translational modification may facilitate a better understanding of the mechanisms involved in Cd-tolerance of *T. angustifolia*.

Under Pb stress, more spots showed a Pb-dependent alteration in the protein ratio (Figs. 1 and 2; Tables 2 and 3) than that of Cr and Cd. Pb-induced 31 and 26 protein spots up-regulation in D90 and D130 samples, respectively; 35 and 36 down-regulation. Meanwhile, 8 spots were changed in a similar manner (up-regulated) in both of D90 and D130 samples (Table 2, Fig. 4). Protein identification showed that they can be categorized as three groups according to their functions: photosynthesis-related, carbohydrate metabolism-associated and defense response proteins. Five of them are proteins involved in photosynthesis. They are ribulose-1,5-bisphosphate carboxylase/oxygenase activase, Mg-protoporphyrin IX chelatase, 30S ribosomal protein S10, putative peptidyl-prolyl cis-trans isomerase (chloroplast precursor), and plastocyanin (participates in electron transfer between P700 and the cytochrome b6-f complex in photosystem I). Envelope membranes play a significant role in chlorophyll biosynthesis and chloroplast biogenesis, which includes protoporphyrinogen oxidase [35], certain subunits of Mg-chelatase [36], Mg-protoporphyrin IX:S-adenosyl methionine methyl transferase [37], and protochlorophyllide oxidoreductase [38]. Plastocyanin is a small (Mr = 10,500) electron-transfer protein containing a single blue or type 1 copper ion [39]. It plays a central role in photosynthesis where it carries electrons from the cytochrome b6/f complex to photosystem I [40]. Their elevated expression of these protein spots identified after exposure to Pb-induced stress in our study may be in favor of efficaciously protection on photosynthesis against Pb-toxicity. One of the protein spots expressed is carbohydrate metabolism-associated proteins of putative fructokinase. The main role of fructokinase is in carbohydrate metabolism, more specifically, sucrose and fructose metabolism. Increased fructokinase may be beneficial for the re-establishment of homeostasis of carbohydrate metabolism and participate in the protection and restoration of damaged proteins and membranes. Furthermore, another two protein spots identified are defense response binding/catalytic/coenzyme binding and GRSF (RNA-binding protein G-rich sequence factor). The germline restrictive silencing factor (GRSF) is a 24-kDa protein that is essential for the unique functions of male germline cells in fertilization. GRSF recognizes silencer sequences in promoters of genes specific to the germline. Our result revealed that sequestration of repressor by the excess silencer sequences can lead to the enhancement of the plant to survive under Pb-induced stress. Concerning regulation factors above 3 between control and Pb-treated samples of one of D90 and D130 (Table 3), the expression of ribulose 1,5-bisphosphate carboxylase large subunit and ascorbate peroxidase (APX) in D130 were improved significantly. APX exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants. APX in combination with the effective AsA–GSH cycle functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms [41]. Therefore, these up-regulated proteins may be involved in Pb-detoxification mainly via efficaciously protection on photosynthesis and improvement of carbohydrate metabolism and antioxidant capacity.

Contrarily, the expression of eukaryotic initiation factor 4A (eIF4A) was inhibited by Cr-stress of D90 and by Pb stress of D130. Pb also repressed GF14omega isoform 4.1-fold in D90. Chloroplast FtsZ-like protein was down-regulated 3.1-fold by Cd stress in D90. Plant FtsZ (filamentous temperature-sensitive Z) proteins is involved in chloroplast division and plastid division [42]. Plant GF14omega was implicated, as a constituent of a protein/G box complex, to be involved in regulation of gene transcription [43] and some primary metabolism [44]. For example, nitrate reductase and Suc phosphate synthase, which are key enzymes in nitrogen and carbon metabolism, respectively, are both inhibited by binding of GF14 [45,46]. Therefore, more detailed study is necessary for the description of the relationship between these proteins and metal tolerance in *T. angustifolia.*

5. Conclusions

In the present study, protein profiles were used to analyze Cr, Cd or Pb stress-responsive proteins in leaves of *T. angustifolia.* A total of 26 differentially expressed proteins were identified in response to Cr, Cd and Pb stress by using 2-DE coupled with the MALDI-

TOF and data bank analyses. Of these, we identified up-regulated photosynthesis-related proteins with metal specificity, i.e. RuBisCO small subunit and CPO under Cr-stress; modified RuBisCO large subunit under Cd stress; RuBisCO activase, Mg-protoporphyrin IX chelatase, 30S ribosomal protein S10, putative peptidyl-prolyl cis-trans isomerase (chloroplast precursor), and plastocyanin under Pb stress. Their elevated expression may be in favor of efficaciously protection on photosynthesis against metal toxicity. These results suggest that the effective protection of photosynthesis has a crucial role regarding hyper-metal-tolerance in *T. angustifolia*.

In addition, Cr-induced expression of ATP synthase and Pb-up-regulation of carbohydrate metabolic pathway enzymes of fructokinase suggests that Cr/Pb stress is highly expressed in energy and metabolic pathways, and that plants may require high energy to cope with Cr-stress. Furthermore, Pb-up-regulation of defense response binding, APX and GRSF may also be involved in Pb-detoxification for its Pb-hypertolerance. Thus, the results of this study suggest that plants of *T. angustifolia* cope with heavy metal stress in a complex manner, where protection on photosynthesis may play a pivotal role in this complex cellular network. Although, this study is an initial proteomic investigation into *T. angustifolia* leaf response to Cr, Cd and Pb stress. It is our belief that this kind of study provides a good starting point in understanding the overall metal responses of plants and the identification of proteins in response to Cr, Cd and Pb stress and provides new insights that can lead to a better understanding of the molecular basis of hyper-metal-tolerance in *T. angustifolia*. In any event, additional studies focused on these genes and comparative analyses may facilitate a better understanding of the mechanisms involved in *T. angustifolia* hyper-metal-tolerance.

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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.jhazmat.2010.08.023.

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