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Comparative Proteomic Analysis of Differentially Expressed Proteins in *Amaranthus hybridus* L. Roots Under Cadmium Stress

Haijun Jin · Mingjian Xu · Hui Chen · Shirong Zhang · Xueyi Han · Zizhong Tang · Rong Sun

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Abstract *Amaranthus hybridus* L. has great potential for use in phytoremediation of soils contaminated with cadmium (Cd). In this study, we found higher absorption of Cd by the roots of *A. hybridus* than by its other organs. To understand the mechanism of Cd accumulation in *A. hybridus* roots, a comparative proteomic approach was used to differentiate the two-dimensional electrophoretic profiles of root proteins in Cd-free and Cd-treated plants. Twenty-eight differentially expressed proteins were successfully identified using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. Of these, 10 were specifically expressed under Cd stress, and another 11 were upregulated and 7 downregulated by >2.5-fold under Cd stress. We observed increased expression of proteins involved in energy metabolism, protein metabolism, stress and defense, and signal transduction. These changes likely enhanced Cd tolerance and enrichment in *A. hybridus*. The downregulated proteins were mainly involved in the synthesis of microRNAs, cell walls, and other structural components. These observations were further confirmed by quantitative fluorescence PCR. The resulting differences in protein expression patterns suggest that redirection of root cell metabolism might be an important survival mechanism for *A. hybridus* under Cd stress.

Keywords *Amaranthus hybridus* L. · Cadmium · Proteomics · Quantitative PCR

H. Jin · M. Xu · H. Chen (✉) · X. Han · Z. Tang · R. Sun
College of Life Science, Sichuan Agricultural University, Xinkang Road, Ya'an 625014, China
e-mail: anyone2394@aliyun.com

S. Zhang
College of Environmental Sciences, Sichuan Agricultural University, Huimin Road, Chengdu 611130, China

1 Introduction

With increasing industrialization and rapid urbanization, trace metal contamination of the terrestrial environment has become globally widespread (Lee et al. 2006). Cadmium (Cd) is a toxic pollutant that enters the environment mainly through mining, pesticides, and chemical fertilizers (Daud et al. 2013). Therefore, cleanup of Cd-contaminated soils is critical to minimizing their impact on the ecosystem.

In response to the negative effects of Cd, there has been ongoing development of a variety of technologies to remediate Cd-contaminated soils. These technologies include excavation, separation, extraction, electrokinesis, washing, oxidation, reduction, phytoextraction, phytovolatilization, solidification, and vitrification (Mitch 2002). Phytoremediation refers to the use of plants and associated soil microbes to reduce the concentrations or toxic effects of contaminants in the environment (Greipsson 2011). Phytoremediation of contaminated soils is seen as a cost-effective and environmentally friendly in situ remediation technique, which aims to maintain soil fertility and structure (Ali et al. 2013). Previous reports have suggested

Amaranthus hypochondriacus as a useful candidate for the phytoremediation of Cd owing to its high aboveground biomass, fast growth, and high Cd uptake (Li et al. 2012). Although researchers have also reported the accumulation of Cd in *Amaranthus hybridus* (Zhang et al. 2010), the molecular mechanisms governing Cd tolerance in this species are unknown.

Roots are a site of susceptibility and perception for many types of stress, including heavy metals, salinity, and nutrient deficiency (Jiang et al. 2007). In many cases, it is the roots' sensitivity to stress that limits the whole plant's productivity (Steppuhn and Raney 2005). Therefore, the molecular mechanisms governing the roots' response to Cd treatment need to be elucidated. Over the last decade, proteomics has become an important approach to studying biotic and abiotic stresses. Proteome analysis that includes two-dimensional electrophoresis and mass spectrometry (MS) is a useful method for determining proteomic alterations and differential protein expression as a result of the stress-response mechanism (Singh and Jwa 2013). In recent years, research on protein responses to Cd stress has made some progress in understanding heavy metal hyperaccumulation in plants. Proteins involved in glutathione synthesis, ATP metabolism, response to oxidative stress, and protein folding are upregulated in *Chlamydomonas reinhardtii* under Cd stress (Gillet et al. 2006). Zhao et al. (2011) reported major changes in the proteins involved in the photosynthetic pathway in *Phytolacca americana* under Cd stress.

In the present study, comparative proteomics of *A. hybridus* roots under Cd stress vs. Cd-free conditions was investigated. The aims were to (1) identify proteins potentially involved in Cd tolerance, Cd accumulation, or the regulation of Cd responses in *A. hybridus* roots; (2) explore possible Cd tolerance/accumulation mechanisms in the *A. hybridus* root; (3) gain insight into metabolic changes induced by Cd toxicity; and (4) contribute to establishing reference datasets on plant proteome changes due to heavy metal stress. This is the first report of proteomic changes in *A. hybridus* roots under Cd stress.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

In a previous study, we found an average Cd concentration of 60 mg/kg in the soil of Fuquan lead–zinc mine in Hanyuan, Sichuan Province, China (29°5′N, 102°16′E). Thus, in the present experiments, we used a Cd concentration of 60 mg/kg.

Plant seeds were collected from the farm at Sichuan Agricultural University in Sichuan Province (30°0′N, 103°1′E). Soil culture of the seedlings was performed according to the method described by Zhang et al. (2010). Each plastic pot was filled with 6.0 kg of ground soil with or without (as a control) 60 mg/kg Cd (Cd solution was prepared by dissolving analytical grade $CdCl_2 \cdot 2.5\ H_2O$). The soil was equilibrated for more than 4 weeks. Each pot had six seedlings, and each treatment had three replicates. The pots were maintained in a greenhouse at ambient temperature with natural illumination (light intensity 2500–50,000 lx and a photoperiod of 15–16 h light/9–8 h dark). To ensure sufficient nutrients for growth in the soil, the same base fertilizer was applied with aperiodic watering. Roots, stems, leaves, and fruit were harvested after 90 days. A portion of the materials under Cd stress were used to assay Cd content. The remainder of the samples were frozen in liquid nitrogen and stored at −80 °C; proteins were extracted from these samples.

2.2 Determination of Cd Concentration

The roots were immersed in EDTA–2Na solution (20 mM) for 3 h to remove surface ions. Then, the samples (roots, stems, leaves, and fruit) were washed with distilled water, dried at 80 °C for 48 h, and pulverized. The Cd concentration was determined using an atomic absorption spectrometer (AA6300, Shimadzu, Tokyo, Japan).

2.3 Statistical Analysis

The Cd bioaccumulation results were subjected to analysis of variance (ANOVA) and Student's *t* test for comparisons. Significance was determined at $P < 0.05$.

2.4 Protein Extraction

Cd-free and Cd-treated roots (3 g) were ground to a fine powder in liquid nitrogen and transferred into 10-ml tubes; 10 ml of 10 % (*w*/*v*) trichloroacetic acid/0.07 % (*v*/*v*) 2-mercaptoethanol in acetone was added to each tube and incubated at −20 °C for 3 h with regular shaking at 15-min intervals. Then, the tubes were centrifuged at 15,000×*g* for 15 min at 4 °C and the

supernatants were discarded. The pellet was washed three times with 0.07 % 2-mercaptoethanol in acetone. Finally, the protein pellet was air-dried and resuspended in 300-μl lysis buffer (8 M urea, 4 % *w*/*v* CHAPS, 1 % *w*/*v* DTT, 2 % *v*/*v* IPG buffer, pH 3–10 NL). Bradford's method was used for protein quantification.

2.5 Two-Dimensional Gel Electrophoresis

For a 17-cm (pH 3–10) linear-gradient IPG strip (Bio-Rad Laboratories, Hercules, CA), each sample included 800-μg protein in 350-μl rehydration buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 0.01 % *w*/*v* bromophenol blue) containing 65 mM DTT and 0.2 % IPG buffer (pH 3–10; GE Healthcare, Uppsala, Sweden). Two-dimensional gel electrophoresis and Coomassie Brilliant blue staining were performed following the method of Wei et al. (2014). Isoelectric focusing (IEF) was performed using a Bio-Rad PROTEAN IEF cell with the following program: 50-V active rehydration for 12 h, 200 V (line ramp) for 1 h, 500 V (line ramp) for 1 h, 1000 V (rapid ramp) for 1 h, 10,000 V (line ramp) for 5 h, 10,000 V (rapid ramp) to 60,000 Vh, and 500 V (rapid ramp) for 24 h at 20 °C. The strips were then equilibrated in standard equilibration solution (375 mM Tris–HCl pH 8.8, 6 M urea, 20 % *w*/*v* glycerol, 2 % *w*/*v* SDS) containing 2 % DTT for 15 min, then in the same equilibration buffer containing 2.5 % (*w*/*v*) iodoacetamide for an additional 15 min. The equilibrated strips were subjected to SDS-PAGE in a 12.5 % Tris–HCl criterion gel, set at 15 mA for 30 min and then 30 mA until bromophenol blue stops along 0.5 cm; cold-cycle setting temperature was 10 °C. The gels were stained with a nontoxic-type rapid protein staining kit (Sangon Biotech Co., Ltd., Shanghai, China) and destained with distilled water until background staining was negligible. The gels were scanned with a GSC-8000 scanner (Bio-Rad) and analyzed by PDQuest 8.0 software. Only those spots with over 2.5-fold changes in volume after normalization between Cd-free and Cd-treated roots were defined as altered. All experiments were performed in triplicate to ensure reproducibility.

2.6 Mass Spectrometry and Protein Identification

The selected spots were excised from the gel and washed twice with distilled water. Protein samples were trypsin-digested and the resulting peptides were extracted according to standard techniques (Bringans et al. 2008). The digested peptides were analyzed on an ABI 4800 Proteomics Analyzer with TOF/TOF™ optics (Applied Biosystems, Foster City, CA, USA). The proteins were identified by searching in the National Center for Biotechnology Information nonredundant (NCBI nr) database using the MASCOT program (http://www.matrixscience.com). Protein identification was assigned when the protein score confidence interval (CI) was >95 %.

2.7 Quantitative PCR Detection

Total RNA was isolated from frozen samples using Column Plant RNAout (Tiangen, Beijing, China) according to the manufacturer's instructions. RNA purification and complementary DNA (cDNA) synthesis were conducted with the PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed on a CFX96 Real-Time PCR instrument (Bio-Rad) using SYBR Green I for detection. The qRT-PCR mixture contained 7.5 μl 2× SYBR®Premix Ex Taq™ (TaKaRa), 1 μl of tenfold-diluted cDNA, 1 μl of 100 nM of each sense and antisense primer, and 4.5 μl of double-distilled water. The *18sRNA* gene was used as the internal control. Primer sequences for *18sRNA*, *ClpC*, *SRTG152-II*, *Pgh1b*, *rbcL*, and *FBA* are listed in Table 1.

The PCR conditions were as follows: 95 °C for 2 min, 40 cycles at 95 °C for 20 s, 54 °C for 15 s, and 68 °C for 15 s (Wei et al. 2014). The relative messenger RNA (mRNA) expression of the target gene was calculated using the CT method (Livak and Schmittgen 2001). All experiments were performed in triplicate.

3 Results

3.1 Analysis of Cd Content in *A. hybridus* Under Cd Stress

Cd content in the roots was higher than in other parts of *A. hybridus* under Cd stress ($P<0.05$; Fig. 1). We inferred that more Cd accumulated in the roots under Cd stress and less Cd was transported to the aboveground parts, thereby preventing damage to the latter. The Cd content in the roots suggested that Cd stress results in large amounts of heavy metal accumulation in the roots.

Table 1 Genes and primers used for quantitative PCR

Gene	Protein	Forward/reverse primer sequence (5′ → 3′)
18sRNA		Forward primer AGCAGATTGACCAGCGAACA Reverse primer CAGAAAGGAGCACCACCC
ClpC	ClpC protease (chloroplast)	Forward primer GGTGAGTTGCGTGACAGAGA Reverse primer ACCTATCCTACGCCCACCTT
SRTG152-II	Salt tolerance protein II	Forward primer ATTTTGGGTCGGCACTCTGG Reverse primer GCTTCATTGCTCCAAAGGGTG
Pgh1b	2-phospho-D-glycerate hydrolase	Forward primer TGTTTCCCTTGCTGTCTG Reverse primer ATCCACCATTGATGACGT
rbcL	Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit	Forward primer TTGACTTATTATACTCCTGAGTATG Reverse primer CATACTCAGGAGTATAATAAGTCAA
FBA	Fructose-biphosphate aldolase	Forward primer CTCGCTCGCTACGCCATCA Reverse primer TCCTCCTCACTCTGCCCACC

3.2 Two-Dimensional Gel Comparison

Twenty-five spots were differentially expressed, with an over 2.5-fold change in volume after normalization between Cd-free and Cd-treated roots being defined as altered (Fig. 2). In the 25 excised gel plugs, 28 proteins were successfully identified (Table 2). Ten proteins were specifically expressed under Cd stress, while 11 and 7 proteins were upregulated and downregulated, respectively. In these differentially expressed proteins, isoforms of one protein were identified in multiple spots, such as 2-phospho-D-glycerate hydrolase (also known as enolase), which was found in two spots (4610 and 4617; Table 2). It is possible that the different protein spots were different forms of the same protein with different translation modifications, such as phosphorylation and glycosylation, or indicative of protein degradation. We also found that the following two proteins were identified in one spot: flavoprotein WrbA-like and glutathione S-transferase were found in one spot (1310, 4507, and 5101; Fig. 2). This might be due to different proteins having a similar molecular weight and isoelectric point.

The identified proteins were analyzed for functional classification. The specifically expressed and upregulated proteins were mainly involved in energy metabolism (spots 2413, 2213, 4617, 4610, and 6401), carbohydrate metabolism (spot 4305), protein metabolism (spots 3808, 1108, and 4607), amino acid metabolism (spots 4507a, 4507b, 5101a, 4504, and 2314), stress and defense (spots 5101b, 1310a, 1310b, and 5307), and signal transduction and mRNA synthesis (spots 1410, 6801, and 7303; Table 2). The downregulated proteins were mainly involved in cell-wall-related metabolism (spots

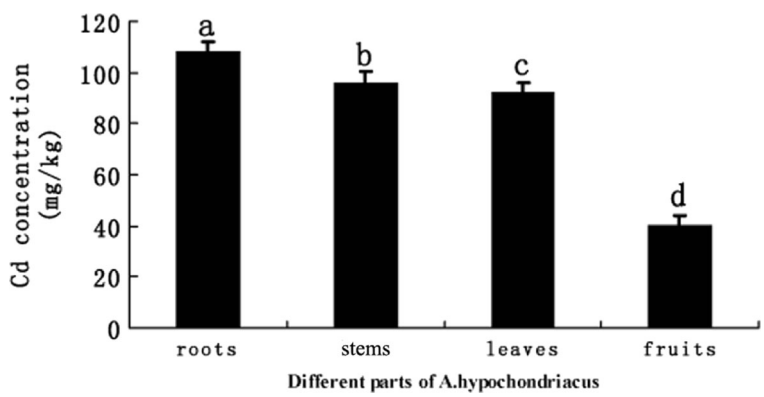

Fig. 1 Cd concentration in roots, stems, leaves, and fruit of Cd-exposed plants. Averages of three replicates are shown for control and Cd-treated plants ($P < 0.05$)

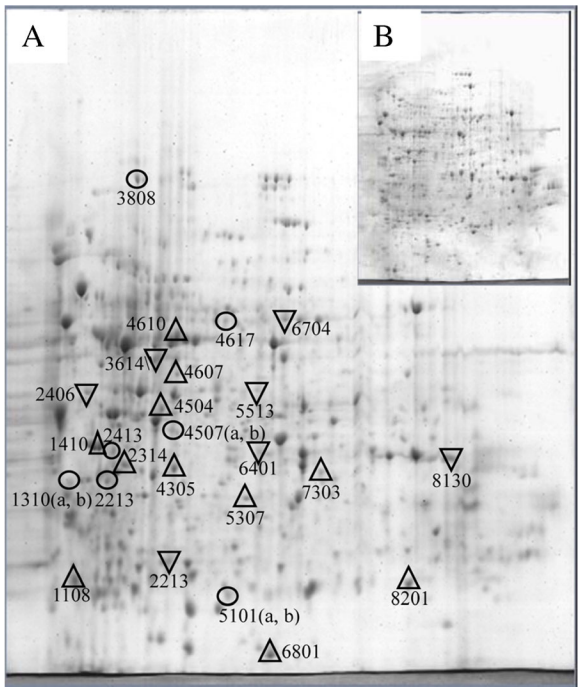

Fig. 2 Two-dimensional map of the proteins of Cd-treated (**a**) and Cd-free (**b**) roots. *Circles* designate proteins that are specifically expressed in the Cd-treated group, and upfacing and downfacing *arrowheads* represent upregulated and downregulated proteins in the Cd-treated vs. Cd-free group. **a**, **b** Two proteins identified in one spot

6704, 8130, and 5513), microRNA (miRNA) synthesis (spot 2213), and other structural components (spots 3614 and 2406; Table 2).

3.3 Verification of Transcriptional Expression of Genes Encoding the Five Proteins

ClpC, *SRTG152-II*, *Pgh1b*, and *rbcL* demonstrated significantly higher mRNA transcript levels in plants under Cd stress vs. controls (2.00, 1.87, 1.42, and 1.62, respectively, with expression in control plants arbitrarily set to 1; Fig. 3). *FBA* had significantly lower transcript levels (0.50) under Cd stress. From the qPCR analysis, the transcription levels of these genes were correlated with the expression of their respective proteins, further validating our comparative proteomic study.

4 Discussion

Although suggested to be a useful resource for the phytoremediation of Cd (Li et al. 2012), the molecular mechanism governing Cd accumulation in *A. hybridus* is unknown. In our study, we found higher Cd content in *A. hybridus* roots than in its other organs; therefore, we chose *A. hybridus* root as the experimental material. Through comparative proteomic analysis, we identified some proteins that were differentially expressed under Cd stress, which might help unravel the molecular mechanisms governing Cd accumulation in *A. hybridus* roots. The differentially expressed proteins were mainly involved in energy and carbohydrate metabolism, protein metabolism, amino acid metabolism, cell-wall-related and other structural components, stress and defense, signal transduction, and RNA metabolism.

4.1 Energy and Carbohydrate Metabolism

Plant stress responses require a large amount of ATP to provide sufficient energy. Golldack et al. (2014) found that the expression of genes related to energy metabolism is enhanced when plants are stressed. Some key enzymes in glycolysis and ATP synthesis play an important role. In our study, fructokinase (spot 2213), enolase (spot 4617), 2-phospho-D-glycerate hydrolase (spot 4610), fructose-biphosphate aldolase (spot 6401), triosephosphate isomerase (spot 8201), and ATP synthase subunit beta (spot 2413) were upregulated under Cd stress. Increased levels of fructokinase may be beneficial for the reestablishment of carbohydrate-metabolism homeostasis and may participate in the protection and restoration of damaged proteins and membranes (Bah et al. 2010). Enolase accumulates in rice roots in response to salt stress (Lee et al. 2009). ATP synthase subunit beta is an important component of ATP synthase responsible for catalytic ATP synthesis or hydrolysis. Cd has been shown to induce expression of ATP synthase in *Typha angustifolia* (Bah et al. 2011). Recent studies have shown a close connection between fructose-biphosphate aldolase and various abiotic stresses (Jiang et al. 2007; Sarry et al. 2006). Triosephosphate isomerase plays an important role in glycolysis and is essential for efficient energy production (Trujillo et al. 2014). The above results suggest that the plant's energy metabolism is strongly affected by Cd stress and the changes in these proteins may reconfigure the system to combat Cd stress. Kieffer et al. (2008) also reported that upregulation of mitochondrial respiration provides for the Cd-exposed plants' energy needs. A high abundance of enzymes involved in glycolysis and the TCA cycle might help Cd-challenged cells produce the

Table 2 Protein spots identified by MALDI-TOF/TOF-MS

Spot number	Protein name	Accession number	Protein MW	Protein PI	Protein score CI (%)	Expression type	Function
2413	ATP synthase subunit beta, mitochondria (*Aegilops tauschii*)	gi|475548007	59,152.9	5.85	100	Upregulated	Energy metabolism
2213	Fructokinase (*Beta vulgaris*)	gi|1052973	35,594.3	5.38	100	Upregulated	Energy metabolism
4617	Enolase (*Phytolacca americana*)	gi|376372996	48,418.5	5.39	100	Upregulated	Energy metabolism
4610	2-Phospho-D-glycerate hydrolase (*Mesembryanthemum crystallinum*)	gi|1087071	48,660.6	5.62	99.995	Upregulated	Energy metabolism
6401	Fructose-biphosphate aldolase (*Sesuvium portulacastrum*)	gi|196051521	38,685.2	6.49	100	Upregulated	Energy metabolism
8201	Triosephosphate isomerase (*Pediocactus simpsonii*)	gi|315468807	3,173.6	4.95	100	Upregulated	Energy metabolism
4305	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, chloroplast (*Ofaiston monandrum*)	gi|34576703	50,163.2	6.6	100	Upregulated	Carbohydrate metabolism
3808	ClpC protease (chloroplast; *Spinacia oleracea*)	gi|4105131	99,588.4	8.78	99.842	Specifically expressed	Protein metabolism
1108	Proteasome subunit alpha type, partial (*Genlisea aurea*)	gi|527202041	26,022.0	4.81	100	Upregulated	Protein metabolism
4607	Eukaryotic elongation factor 1A (*Suaeda japonica*)	gi|24371057	49,741.9	9.20	100	Upregulated	Protein metabolism
4507a	Methionine adenosyl transferase (*Mesembryanthemum crystallinum*)	gi|1724104	43,314.8	5.43	100	Specifically expressed	Amino acid metabolism
4507b	S-adenosyl-L-methionine synthetase (*Beta vulgaris*)	gi|71000461	43,645.0	5.57	100	Specifically expressed	Amino acid metabolism
5101a	Flavoprotein WrbA-like (*Cicer arietinum*)	gi|525313835	21,708.0	6.51	100	Specifically expressed	Amino acid metabolism
4504	S-adenosyl-L-homocysteine hydrolase (*Mesembryanthemum crystallinum*)	gi|1724102	53,771.2	5.75	100	Upregulated	Amino acid metabolism
2314	O-acetylserine(thiol)lyase (*Knorringia sibirica*)	gi|186688080	40,759.2	6.40	100	Upregulated	Amino acid metabolism
6704	UDP-glucose 6-dehydrogenase (*Silene latifolia*)	gi|343172202	13,030.6	5.58	99.99	Downregulated	Cell-wall-related
8130	UDP-glucuronic acid decarboxylase, partial (*Silene latifolia*)	gi|343173219	38,755.9	6.84	100	Downregulated	Cell-wall-related
5513	Lignin-forming anionic peroxidase precursor, putative (*Ricinus communis*)	gi|223549629	35,427.5	8.45	99.986	Downregulated	Cell-wall-related
3614	Chloroplast inner envelope protein (*Silene latifolia*)	gi|343172030	110,072.4	5.75	99.585	Downregulated	Structural components
2406	Actin (*Celosia argentea*)	gi|322422113	41,941.1	5.31	100	Downregulated	Structural components
5101b	Glutathione S-transferase (*Ginkgo biloba*)	gi|66736578	25,828.0	6.24	100	Specifically expressed	Stress and defense
1310a	Salt tolerance protein II (*Sesuvium portulacastrum*)	gi|217039109	38,340.9	5.00	100	Specifically expressed	Stress and defense
1310b	Salt tolerance protein I (*Sesuvium portulacastrum*)	gi|217039107	38,360.9	5.15	100	Specifically expressed	Stress and defense
5307	Plastid-lipid-associated protein, chloroplast precursor, putative (*Ricinus communis*)	gi|223536371	35,150.2	4.84	100	Upregulated	Stress and defense
1410	Phytochrome C, partial (*Talinum portulacifolium*)	gi|377823304	42,648.7	6.84	99.693	Upregulated	Signal transduction

Table 2 (continued)

Spot number	Protein name	Accession number	Protein MW	Protein PI	Protein score CI (%)	Expression type	Function
6801	Wall-associated receptor kinase-like 14 (*Solanum lycopersicum*)	gi|724601449	7,612.55	6.39	100	Upregulated	Signal transduction
7303	Maturase K (chloroplast; *Acantholimon lycopodioides*)	gi|29737640	61,710.7	9.54	95.128	Upregulated	mRNA synthesis
2213	Dicer-1, putative (*Ricinus communis*)	gi|223545577	173,940.7	6.02	96.625	Downregulated	miRNA synthesis

extra energy required to meet the high-energy demand (Hossain et al. 2012).

One protein related to carbohydrate metabolism (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, spot 4305) was upregulated. This protein may belong to a family of phytoremediation-related proteins. Ribulose-1,5-bisphosphate carboxylase/oxygenase is the enzyme that assimilates CO_2; it catalyzes the initial step in the Calvin cycle and is the major soluble leaf protein in plants. Ali and Komatsu (2006) suggested that increased ribulose-1,5-bisphosphate carboxylase expression can enhance plant resistance under drought stress. Therefore, upregulation of ribulose-1,5-bisphosphate carboxylase/oxygenase may protect carbon assimilation from Cd toxicity in *A. hybridus*, as evidenced by the gain in Cd-exposed plants' height and biomass in our study.

4.2 Protein Metabolism

Proteins are the major players in life's activities, and environmental stress can alter protein metabolism. In our study, ClpC protease (spot 3808) and proteasome subunit alpha type (spot 1108), which are involved in protein degradation, were specifically expressed and upregulated, respectively. Eukaryotic elongation factor 1A (eEF1A, spot 4607), which is involved in protein synthesis, was upregulated. ClpC protease belongs to the Clp protease system, which plays a central role in plastid development and function, through selective removal of misfolded, aggregated, or otherwise unwanted proteins (Nishimura and van Wijk 2015). The *clpC* gene of *Bacillus subtilis* is induced in response to various stresses, including Cd stress (Krüger et al. 1994). Proteasome subunit alpha type is an alpha-type subunit of the 20S proteasome core complex that participates in degrading proteins through the ubiquitin–proteasome pathway (Li et al. 2011). Pena et al. (2007) found a threshold response of the 20S proteasome system to Cd stress mediated through oxidative modification of the proteasome itself, which prevented the accumulation of oxidatively damaged protein in the cell. In this experiment, we suggest that proteins damaged by Cd stress are degraded or removed by ClpC protease and the 20S proteasome. Pena et al. (2006) also found this protein-degradation mechanism in sunflower under Cd stress. eEF1A is a GTP-binding protein that targets

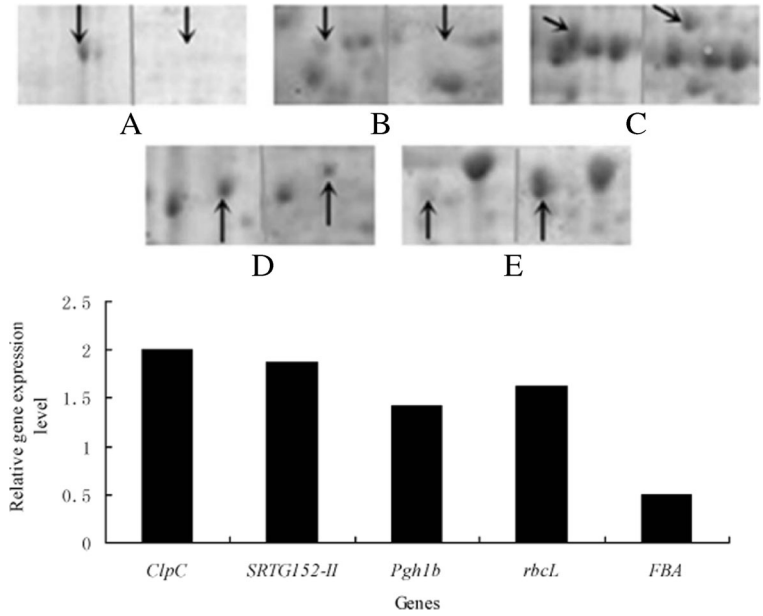

Fig. 3 Comparison of protein-synthesis patterns in Cd-treated (*left*) and Cd-free (*right*) and qPCR analyses of the five gene transcripts. **a** *ClpC* (spot 3808, ClpC protease), **b** *SRTG152-II* (spot 1310b, salt tolerance protein II), **c** *Pgh1b* (spot 4610, 2-phospho-D-glycerate hydrolase), **d** *rbcL* (spot 4305, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), **e** *FBA* (spot 6401, fructose-biphosphate aldolase), and **f** qPCR analysis of the five gene transcripts

aminoacylated tRNAs to the ribosome. Induction of *eEF1A* expression by environmental stresses has been suggested to reflect the general adaptive response of rice plants to adverse circumstances (Li and Chen 1998). eEF1A is known to be involved in several cellular process, including embryogenesis, cell proliferation, senescence, oncogenic transformation, and cytoskeletal organization (Gangwani et al. 1998). In addition, eEF1A has been proposed to recognize damaged proteins and shuttle them to the proteosome for degradation (Sasikumar et al. 2012). Because of the key role of eEF1A in cell metabolism, this finding might provide new insight into Cd-tolerance strategies.

4.3 Amino Acid Metabolism

Five protein spots were involved in amino acid metabolism. Methionine adenosyl transferase (spot 4507), S-adenosyl-L-methionine synthetase (spot 4507), and flavoprotein WrbA-like (spot 5101) were specifically expressed, while S-adenosyl-L-homocysteine hydrolase (SAH-hydrolase, spot 4504) and O-acetylserine(thiol)lyase (spot 2314) were upregulated in the Cd-exposed roots. Methionine adenosyl transferase, also known as S-adenosyl-L-methionine synthetase, catalyzes the synthesis of one of the most important biological molecules, S-adenosyl-methionine. S-adenosyl-methionine serves as a precursor of nicotianamine, for which a role in metal ion homeostasis through chelation mechanisms has been reported (Aloui et al. 2011). Therefore, the specific expression of S-adenosyl-L-methionine synthetase in *A. hybridus* could be an attribute of its hypertolerance when exposed to Cd-induced stress, as no patches or necrosis was seen in these plants. Aloui et al. (2011) reported that S-adenosyl-L-methionine plays an important role in anti-Cd stress in *Medicago truncatula*. The flavoprotein WrbA-like was derived according to its reported effect on the binding interaction between DNA and the tryptophan repressor, which regulates the biosynthesis of tryptophan in prokaryotes (Wolfova et al. 2005). However, growing evidence implicates proteins of the WrbA family in the cellular response to oxidative stress (Jensen et al. 2002). SAH hydrolase catalyzes the reversible hydrolysis of SAH to adenosine and L-homocysteine to maintain low cellular activity of SAH. The accumulation of SAH is cytotoxic due to inhibition of DNA and RNA methylation (Rocha et al. 2005). Genetic inhibition of SAH-hydrolase activity is either lethal or induces severe developmental defects (Rocha et al. 2005). It is possible that SAH hydrolase has an important role in the development of *A. hybridus* under Cd stress. O-acetylserine(thiol)lyase converts O-acetylserine to L-cysteine in the presence of free or bound sulfide. Cysteine biosynthesis enhances plants' sequestration of toxic metals, which may be useful for the phytoremediation of heavy-metal-contaminated environments.

4.4 Cell-Wall-Related and Other Structural Components

In response to stress, cell-wall metabolism can be modulated (Robertson et al. 1995). We identified the following five downregulated proteins: UDP-glucose 6-dehydrogenase (spot 6704), UDP-glucuronic acid decarboxylase (spot 8130), lignin-forming anionic peroxidase precursor (spot 5513), chloroplast inner envelope protein (spot 3614), and actin (spot 2406) associated with the cell wall and other structural components. The plant cell wall contains large amounts of pectic polymers and hemicelluloses, which are predominantly derived from the precursor UDP-glucuronic acid (UDP-GlcA). The major enzyme for the formation of UDP-GlcA is UDP-glucose 6-dehydrogenase, which catalyzes the four-electron transfer enzyme responsible for the oxidation of the 6-hydroxyl group of UDP-α-D-glucose to produce UDP-α-D-GlcA (Wegrowski and Pitsillides 2014)., Inhibition of UDP-glucose dehydrogenase activity upon Cd exposure has been reported in *Cucumis sativus* (Kabała et al. 2008). UDP-glucuronic acid decarboxylase catalyzes the conversion of UDP-GlcA to UDP-xylose via a decarboxylation reaction. UDP-xylose is a nucleotide sugar required for the synthesis of diverse plant cell-wall polysaccharides, including xyloglucan (Pattathil et al. 2005). Lignin-forming anionic peroxidase precursor was identified at 7 hpi, supporting the idea of papilla formation and cell-wall enforcement through cross-linking between lignin precursors. Lignin-forming anionic peroxidase responds to wounding, pathogen attack, and oxidative stress (Egea et al. 2001). In this study, the downregulation of the three proteins (UDP-glucose 6-dehydrogenase, UDP-glucuronic acid decarboxylase, and the lignin-forming anionic peroxidase precursor) indicated that Cd stress affects formation of the root cell wall. The chloroplast inner envelope protein mediates the import of proteins into chloroplasts, which facilitates the exchange of metabolites or proteins between the cytosolic and stromal compartments (Koo and Ohlrogge 2002). Thus, downregulation of chloroplast inner envelope protein in the *A. hybridus* root might also inhibit the exchange of metabolites or proteins between the cytosolic and stromal compartments. Actin is a globular multifunctional protein that forms microfilaments. It is involved in the movement of organelles and in cellular morphogenesis, which involves cell division as well as cell elongation and differentiation (Higaki et al. 2007). In the present study, downregulation of actin may inhibit the growth of *A. hybridus* root under Cd stress as it was evident in cell division, elongation, and differentiation.

4.5 Stress and Defense

Plant cells can be induced to produce some stress- and defense-related proteins under Cd stress. We identified the following four different proteins involved in this metabolism: glutathione S-transferase (GST, spot 5101), salt tolerance protein I (spot 1310), and salt tolerance protein II (spot 1310) were specifically expressed under Cd stress, while the plastid-lipid-associated protein (spot 5307) was upregulated. GST can catalyze the conjugation of glutathione or homoglutathione to xenobiotic substrates for the purpose of detoxification. GST may also bind toxins and function as a transport protein, the latter function giving rise to the initial term for GST, ligandin (Gião et al. 2010). Compared with stems, rice roots had higher glutathione content and GST activity, indicating much higher ability of Cd detoxification in roots than in stems (Zhang and Ying 2008). In the current study, specific expression of GST under Cd exposure was probably due to the fact that it plays a corresponding role in Cd detoxification in the *A. hybridus* root. Salt tolerance protein I and salt tolerance protein II are associated with the response to salt stress, some of which presumably involves mediation of salt tolerance. We could not find any related reports on these salt tolerance proteins. In the current study, these proteins may have been related to Cd ion tolerance. Plastid-lipid-associated proteins, also termed fibrillin/CDSP34 proteins, are known to accumulate in fibrillar-type chromoplasts, such as those of the ripening pepper fruit, and in leaf chloroplasts of *Solanaceae* plants under abiotic stress conditions (Langenkämper et al. 2001). Singh and McNellis (2011) reported that plastid-lipid-associated proteins are involved in chromoplast pigment accumulation, hormonal responses, protection of the photosynthetic apparatus from photodamage, and plant resistance to a range of biotic and abiotic stresses. Therefore, the upregulation of plastid-lipid-associated proteins in the *A. hybridus* root suggests their participation in Cd detoxification.

4.6 Signal Transduction and RNA Metabolism

In this experiment, we found upregulation of two proteins, phytochrome C (spot 1410) and wall-associated receptor kinase-like 14 (spot 6801), which are associated with signal transduction under Cd stress.

Phytochrome C is one of the phytochromes that mediates plant growth and development in response to varying light conditions in the red/far-red spectrum (Smith 1995). Phytochrome has been implicated in the regulation of hypocotyl elongation in response to blue light in *Arabidopsis thaliana* (Kunihiro et al. 2010). We know that the hypocotyl is associated with the formation of plant roots. Zhang et al. (2010) reported an increase in root length and biomass of *A. hybridus* under Cd (60 mg/kg) stress. In our experiment, phytochrome C was upregulated, which suggests that Cd stress can promote the growth and development of *A. hybridus* roots. Wall-associated receptor kinase-like 14 is a cytoplasmic serine/threonine kinase. Previous reports have suggested the involvement of a wall-associated receptor kinase in plant defense and heavy metal responses (Verica et al. 2003). The following two RNA synthetases were identified in our study: maturase K (spot 7303) is an intron maturase, a protein that participates in the intron splicing of RNA II, and dicer-1 (spot 2213), a key enzyme in miRNA biogenesis, is involved in transforming miRNA precursors into mature miRNAs. Several miRNAs have been identified using new technologies to have roles in plant stress responses (Budak et al. 2015). Plant miRNAs usually show near-perfect pairing with their mRNA targets, which induces gene repression through cleavage of the target transcripts (Jones-Rhoades et al. 2006). In our study, maturase K was upregulated, and dicer-1 was downregulated, indicating that transcription may be higher in *A. hybridus* root cells under Cd stress.

5 Conclusions

In the present study, we found Cd enrichment to be higher in *A. hybridus* roots than in its other organs. A total of 28 differentially expressed proteins were identified in response to Cd stress. We found the following tolerance and enrichment strategies to cope with Cd stress (Fig. 4): (1) increased expression of enzymes involved in energy metabolism (ATP synthase subunit beta, fructokinase, enolase, 2-phospho-D-glycerate hydrolase, and fructose-biphosphate aldolase) to produce the energy needed to meet the high-energy demand of Cd-challenged cells; (2) a higher abundance of proteins involved in protein metabolism (ClpC protease and proteasome subunit alpha type) to scavenge the proteins damaged by Cd; (3) increased expression of eEF1A, involved in protein metabolism, to produce more proteins to resist Cd stress and maintain metabolic balance; (4) increased expression of proteins involved in stress and defense (GST, salt tolerance protein I, and salt tolerance protein II to detoxify Cd^{2+}, which in turn protects the cellular components from abiotic stress damage; and (5) increased abundance of proteins involved in signal transduction (phytochrome C and wall-associated receptor kinase-like 14) to mediate plant

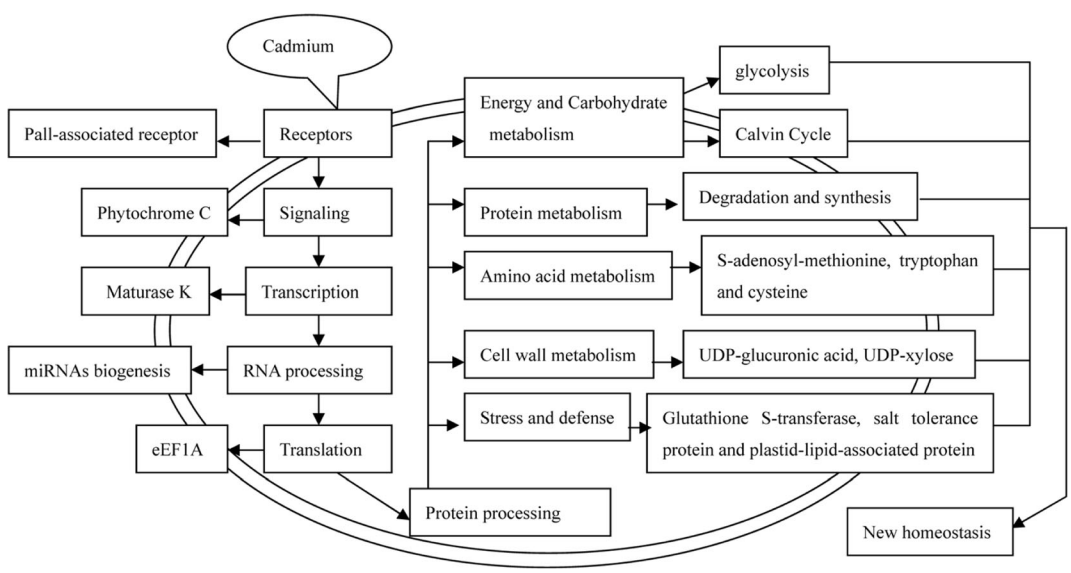

Fig. 4 A simple model of the responses to Cd stress in the root cell of *A. hybridus*

growth and development. Despite an increased understanding of the mechanisms that allow *A. hybridus* root to combat Cd stress, the Cd-tolerance mechanisms are still only partially understood. In addition to providing insight into Cd-stress responses, these Cd-responsive protein data provide a good starting point for further study of the Cd-tolerance mechanisms in *A. hybridus* using genetic and proteomic approaches.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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