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Full Length Research Paper

Identification of differentially expressed proteins in response to Pb stress in *Catharanthus roseus*

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Lead (Pb) is a widespread nonessential heavy metal in cells and causes molecular damage to plants through the formation of reactive oxygen species (ROS) which can be alleviated by enzymatic and nonenzymatic antioxidant machinery of the plant. In the present study, proteomic studies at 3, 6, 12 and 24 days demonstrated that the effect of 150 µM Pb on *Catharanthus roseus*. In response to Pb, a total of 76 proteins, out of the 95 differentially expressed proteins, were subjected to MALDI-TOF-MS Of these, 46 identities were identified by PMF and 19 identities were identified by microsequencing. Basic metabolisms such as photosynthesis, photorespiration and protein biosynthesis in *C. roseus* leaves were without exception strongly inhibited at the beginning of Pb treatment. On the other hand, tricarboxylic acid (TCA) cycle, glycolysis, shikimate pathway, phytochelatin synthesis, redox homeostasis and signaling proteins were induced during recovery period. Such defense systems play an important role in maintaining the survival and growth of *C. roseus* under strong and sustained oxidative stress.

Key words: Antioxidants, chlorophyll, MALDI-TOF-MS, oxidative stress, protein identification.

INTRODUCTION

Defensive responses in plants to abiotic stresses like heavy metals have become a major part of the research in plant sciences which mainly concentrate on the elucidation of mechanisms playing role during plant–metal interactions (Verma et al., 2008). The effects of certain heavy metals such as mercury (Hg), cadmium (Cd) and lead (Pb) on cell systems have received attention in recent decades due to the increasing exposure of living organisms to these metals in the environment (Cavallini et al., 1999). Pb is a widespread nonessential heavy metal, which enters the ecosystems from natural as well as anthropogenic sources. Pb exists in many forms in natural sources throughout the world. According to the environmental protection agency (EPA), Pb is the most common heavy metal contaminant in the environment (Watanabe, 1997). Considerable importance has been attached to the problems of Pb pollution with the development of modern industries and agriculture. Many reports are available on the accumulation and toxicity of Pb in crops and animals (Mochizuki et al., 2002; Wu et al., 2008).

Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress (Hall, 2002). It is known that excess Pb in cells cause molecular damage to plants either directly or indirectly through the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$) (Lin and Kao, 2000). These species react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, and inactivation of enzymes, thus affecting cell viability. The deleterious effects resulting from the cellular oxidative state may be alleviated by enzymatic and nonenzymatic antioxidant machinery of the plant that vary at various cellular and sub cellular levels in different plants. Plants use a diverse array of enzymes like antioxidants as well as low molecular weight antioxidants like cysteine, nonprotein thiol, and ascorbic acid to scavenge different types of ROS, thereby protecting potential cell injury against tissue dysfunction

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(Halliwell, 1987).

Recently, it has been underlined that metabolic changes triggered in order to improve cell fitness under specific conditions are the result of a network of different stimuli, rather than a response to a unique stimulus (Trewavas, 2005). The signal transduction pathways are themselves a network of reactions in which thousands of second messengers, protein kinases and other molecules generate an information flow that diverges, adapts, synergizes and integrates in order to optimize the final response (Taylor and McAinsh, 2004). Therefore, the effects of metals on model plant systems need to be investigated critically at the phytotoxicity and interactive aspects.

MATERIALS AND METHODS

Plant material, growth conditions and Pb treatment

C. roseus seeds were germinated and grown under ambient conditions at temperatures ranging from 18 to 24°C (night and day, respectively). When plants reached the desired size (15-17 cm), they were divided in to two sets followed by transfer and acclimation to hydroponic culture in a modified 1/4- strength Hoagland's solution. The first one acted as control, while in the second set, the nutritive solution was enriched with Pb $(NO_3)_2$ up to a final concentration of 150 µM. Sampling started at day 3, 6, 12 and 24. Leaves from the same foliar stage were used for proteomic and chlorophyll content analysis.

Determination of chlorophyll

For the chlorophyll determination leaves were ground with a mortar and pestle. Aliquots of 100 mg were extracted in 3 ml of 80% acetone and incubated for overnight at 4°C, centrifuged at 5000 *g* for 3 min and absorbance measured at 647 and 664 nm on a spectrophotometer. Chlorophyll (mmol l^{-1}) contents were calculated according to Graan and Ort (1984).

Two-dimensional gel electrophoresis and image analysis

Total soluble proteins were extracted following the tricarboxylic acid (TCA) /Acetone method (Dai et al., 2007). We performed a 2-DE analysis using 24 cm IPG strips (p*I* 3–10, Bio-Rad) according to the manufacturer's instructions. The IPG strips were rehydrated with 250 µg of protein and isoelectric focused. The isoelectric focused strips were incubated for 15 min in an equilibration buffer I (6 M urea, 30% glycerol, 2% SDS, and 0.375 M Tris, pH 8.8) containing 1% DTT and then incubated for 15 min in an equilibration buffer II containing 2.5% iodoacetamide. The equilibrated IPG strips were further resolved by 12.5% SDS-PAGE. The protein spots in gels were visualized by modified Coomassie brilliant blue stain (Wang et al., 2007). 2-DE experiments were repeated thrice for each protein sample and the images were obtained. 2-DE gels were analyzed using the Delta 2-D software version 3.1 (Decodon Germany) and spot detection parameters were set according to the manufacturer's instructions. The replicate gels were used for making the first level match set for averaged gels. The averaged and reference gels were then recreated to reflect the amended spot detection. The result for each spot is expressed as the ratio between Pb spot intensity and control spot intensity for up-regulated proteins and as the opposite ratio for down-regulated proteins.

In-gel digestion

The gel spots of interest were excised manually and subjected to in-gel digestion with trypsin (Promega, Madison, WI), according to Sarry et al. (2006).

Mass spectrometry and protein identification

All MALDI-TOF mass spectra were collected with an Ultraflex mass spectrometer and analyzed by the peak list-generating FlexControlTM 2.2 software (Bruker Daltonics, Germany). The TOF spectra were recorded in the positive ion reflector mode with a mass range from 600 to 4000 Da. Ten subspectra with 30 shots per subspectrum were accumulated to generate one main TOF spectrum. Afterward, the search results were assessed by the MASCOT software (www.matrix-science.com). The criteria for considering a protein to be successfully identified were: (1) at least two peptides were matched to the sequence of the homolog in the NCBI database and (2) the overall protein MOWSE score was significant at $P < 0.05$.

Statistical analysis

Values are means ± standard deviation (SD) of three different experiments with at least three replicated measurements. Differences among treatments were analyzed by one-way analysis of variance (ANOVA), taking $P < 0.05$ as significant according to student t test.

RESULTS AND DISCUSSION

The effect of Pb on growth and integrity of *C. roseus*

The present study investigated the changes of the proteome patterns of *C. roseus* leaves under Pb stress. On the 3rd day of Pb treatment, no significant changes were observed but there was a slight wilt in treated plants. On the 6th day, visual symptoms like chlorosis appeared on the young leaves and lateral root development was significantly decreased. At this stage, about 25% of the Pb-treated plant showed with chlorosis. Older foliar stages also showed chlorosis symptoms and an accelerated senescence combined with an early abscission and root development, whereas in control plants upto 24 days, no similar shedding could be observed. In addition to the chlorosis and senescence, leaf area was reduced. Also more profound morphological changes like stunned growth and fragility touch, which could be explained by a deposit of lignin or cellulose in the cell walls of tissues observed. A lignification of cell walls have been described in roots and leaves of Pb-treated plants, probably resulting from elevated oxidative stress and peroxide content (Kieffer et al., 2009). After 12 days, Pb-treated plants showed acclimatization to the primary shock and gradually recovered from the stress, and in 24 days, 70% of the leaves recovered from growth inhibition, chlorosis and senescence.

Total chlorophyll measurements showed a constant

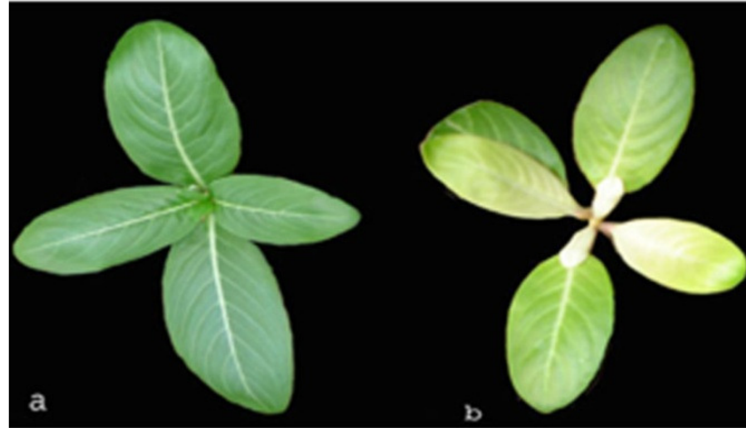

Figure 1. *C. roseus* leaves showing pb toxicity symptom of chlorosis. (a) Control, (b) 24 d after pb-treatment.

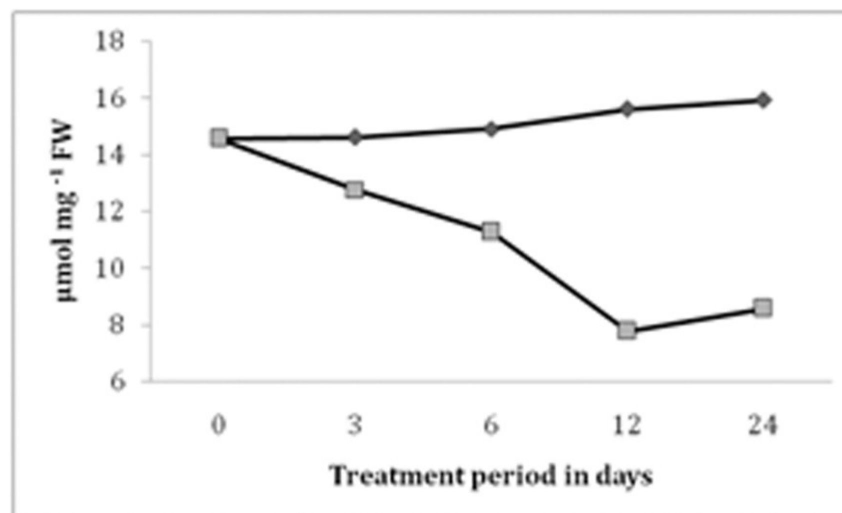

Figure 2. Total chlorophyll (a+b) contents in leaves from control (♦) and 150 µM of pb-treated *C. roseus* (■). Each point represents the mean of three determinations.

marginal increase during the experiment period in control plants. In Pb treatment, chlorophyll level was decreased by 12, 25, 23 and 15% at 3, 6, 12 and 24 days respectively. Chlorophyll content was by increased 2 and 10% at 12 and 24 days, respectively, than 6 days Pb treatment (Figure 1). Similar results were observed in maize plants (Lagriffoul et al., 1998), and in Mentha (Bekiaroglou and Karataglis, 2002). The effect of Pb on PS II has been described as directly on the donor side, accompanied by a decrease in efficiency of PS II and a decrease in chlorophyll and carotenoids. In a study on spinach, chlorophyll a and b content, as well as the ratio, were strongly diminished with increasing concentration of Pb (Xiao et al., 2008).

Total soluble proteins were extracted from leaves of control and Pb stressed plants and separated by 2-DE gel. The average number of detectable spots in this study reached ~600 on each 2-DE gel (Figure 2). The intensity of each spot found on 2-DE gel was analyzed with the Decodon, Delta 2D Software (Figure 3). Three replicate 2-DE gels were run for each sample that were then computationally combined into a representative standard gel by image analyzing software at 3, 6, 12 and 24 days. In order to reduce huge data, each representative gels of different time course were computationally combined into a master gel of control and Pb treatment. From a spot-to-spot comparison and statistical analysis between control and treatment, a total of 95 stained spots were found to have significant changes ($p \leq 0.05$). Of these, 57 spots were up-regulated and 38 spots were down-regulated in response to Pb induced stress. Most of these spots had a greater than 1.5-fold change in abundance under at least one time course of treatment and 31 of these spots exhibited a more than 2-fold change. Of the 57

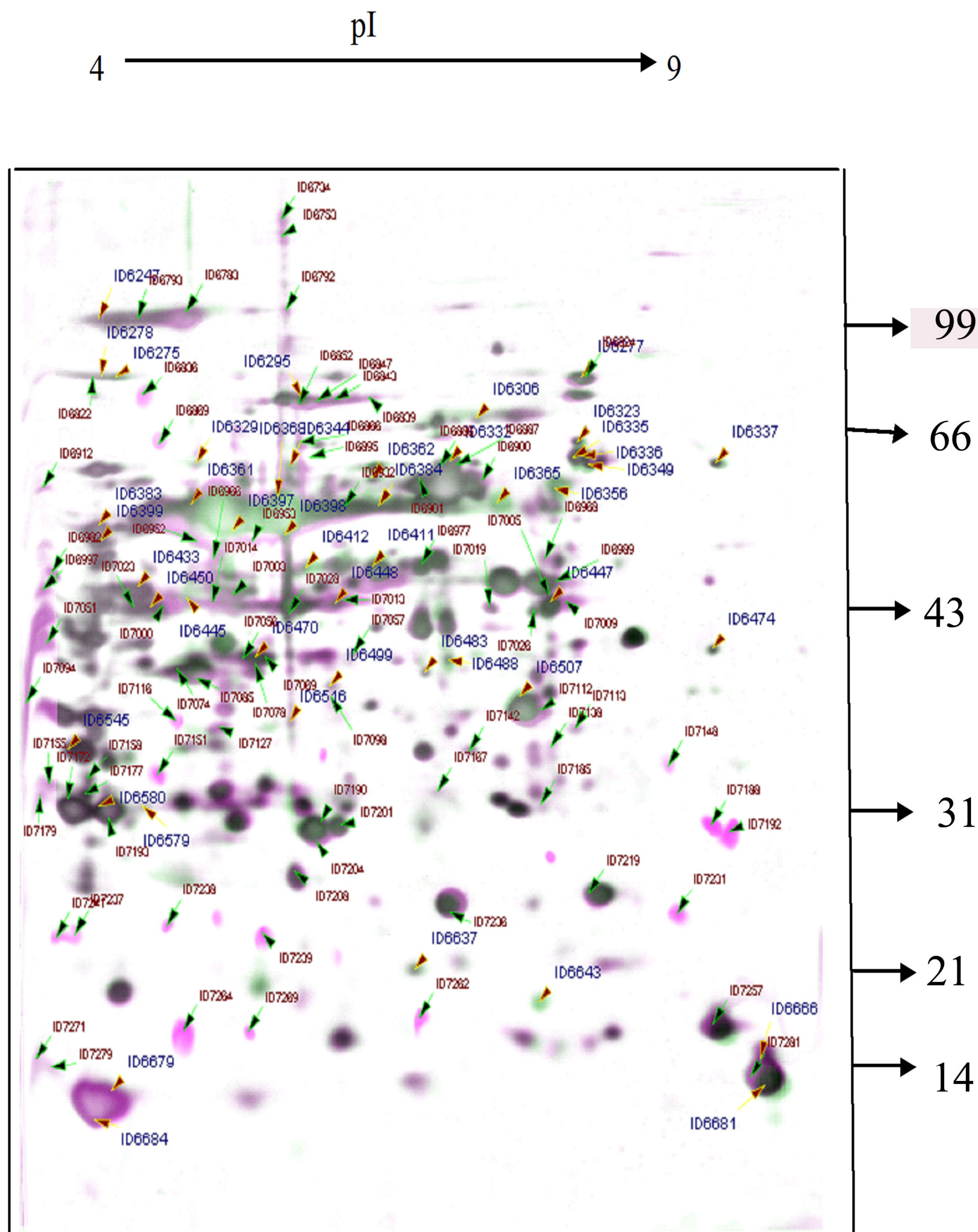

Figure 3. Changes in protein abundance in leaves of *C. roseus* during cadmium stress at 25 µM pd-treatment. Total leaf soluble proteins were separated by isoelectric focusing followed by SDS–PAGE. Proteins were stained with CBB and analyzed using Delta 2D software. Two replicate gels, each with protein isolated from an independent biological replicates were run that were then computationally combined into a representative standard gel by Delta 2D.

up-regulated spots, 27 spots were up-regulated under all four time course of treatment and 16 spots showed increase in abundance under any three time course of treatment. Among the 38 down-regulated spots, 11 spots were down-regulated under all four time course of treatment and 9 spots were down-regulated at any three time course of treatment. Taken together, spots for more than 86% of differentially expressed proteins exhibited co-regulation under at least two time course of treatment.

In response to Pb, a total of 76 proteins, out of the 95 differentially expressed proteins, were subjected to MALDI-TOF-MS Of these, 46 identities were identified by PMF and 19 identities were identified by microsequencing as listed in Table 1. Among these, 40 up-regulated and 24 down-regulated proteins were identified and based on the metabolic and functional features, the identified proteins were classified into 13 major categories namely photosynthesis (17%), CO2 assimilation (6%), carbohydrate metabolism (11%), protein folding and assembly (14%), protein biosynthesis (6%), redox homeostasis (9%), cell rescue/defense (9%), metabolisms of amino acids (6%) and lipids (3%), protein degradation (2%), energy (3%), signal transduction (6%) and RNA processing (2%).

Pb induced changes in expression of photosynthetic proteins

Exposure of Pb stress led to impairment of the photosynthetic function of *C. roseus*. Meanwhile 11 differentially expressed identities were found to be associated with the photosynthetic process (Table 1) and their dynamics showed the effects of Pb stress on photosynthesis at the protein level. These proteins are implicated in three functional subgroups. In the first subgroup involved in PS I and II, two precursors of chlorophyll a/b binding proteins (CP26 in PS II and type III in PS I) and one PS II reaction center psb28 protein were down-regulated following Pb stress. PS II stability factor HCF136 and PS I light-harvesting chlorophyll a/b-binding protein were up-regulated. CP26 is a component of the light-harvesting complex of PS II in plants and facilitates light absorption and transfer of the excitation energy to reaction centers for charge separation (Takahashi et al., 2006). Type III in PS I is a pigment-protein subunit component of the antenna system in plants and combines chlorophyll molecules (Mozzo et al., 2006). The expression of these two proteins was gradually increased after a primary shock (Table 1).

The second subgroup is composed of three down-regulated proteins related to pigment biosynthesis including Magnesium protophorphyrin IX methyltransferase (MgPMT), glutamate-1-semialdehyde 2.1 aminomutase (GSA) and plastid lipid-associated protein (PAP). MgPMT is essential for the formation of chlorophyll and subsequently for the formation of photosystem I and II and cytochrome b6f complexes (Pontier et al., 2007). Low Mg PMT activity leads to reduced activity of Mg chelatase and 5-aminolevulinate synthesis and, correlates with decreased transcript level of GSA. In a similar study by (Langenkämper et al., 2001), the expression chromoplastogenesis protein which involves the dramatic over accumulation of carotenoids sequestered into structures containing lipids and proteins called plastid lipid-associated proteins, allowing sequestration and stabilization of the carotenoids, was studied under oxidative stress. The third subgroup consists of two oxygen-evolving complex proteins (OECP) and a thylakoid lumenal protein. Of them, the two intact proteins, including a 33-kDa OECP of PS II and a thylakoid lumenal protein were up-regulated, whereas the one OECP decreased in abundance. Remarkably similar results are also observed in proteome analysis of poplar and rice in response to abiotic stresses, suggesting that abiotic stresses can enhance the expression of OECP of PS II (Yan et al., 2006).

RCA is acting as Rubisco molecular chaperone, controlling the correct assembly of subunits into active complex and proper conformation of the active center, as well as removing blocking sugars and binding the correct substrate (Siedlecka and Krupa, 2004).

Changes in protein biosynthesis, folding and assembly in response to P

A total of 14 identified proteins in response to Pb induced stress were found to be attributed to protein metabolism (Table 1) and could be divided into three functional groups. The first group consists of four identities functioning in protein biosynthesis with two down-regulated proteins, including putative 50S ribosomal protein L12 and EF-Tu (elongation factor), which are directly involved in initiation and elongation of the newly growing peptide chains. Two up-regulated identities are polyadenylate-binding protein (PABP) and seryl-tRNA synthetase. The second group consists of one identity of degradation-related protein Oligopeptidase A-like (OPA) which was up-regulated in response to Pb induced stress. OPA is the major soluble enzyme able to hydrolyze the lipoprotein signal peptide and further degrades the partially degraded portions of the signal peptide (Novak and Dev, 1988). Thus, the up-regulation of this protein indicates that protein degradation is enhanced in Pb treatment. The third group consists of nine identities related to protein folding and assembly, among these the seven up-regulated identities includ two HSP70s, HSP20, HSP90, DnaK type molecular chaperone and two protein disulfide isomerase (PDI) and two down-regulated identities, are nascent polypeptide - associated complex and 20 kDa chaperonin. It has been reported that Pb stress could induce changes in tertiary structure of proteins and/or increased degradation, thus

Table 1. List of the differentially expressed proteins were identified by MALDI-TOF-MS and Tandem MS in *C. roseus*, in response to Pb-treatment.

Protein	NCBI accession no.	Spot ID	Average fold change [a]				C [b]	Peptides identified
			Day 3	Day 6	Day 12	Day 24		
Photosynthesis								
Putative chlorophyll a/b-binding protein type III (PSI) precursor	gi|159138875	ID6679	-3.76	-3.40	-2.29	-1.85		LQDWYNPGSMGK FAMLGAAGAIAPEIFGK
Chlorophyll a/b-binding protein CP26 precursor	gi|62733871	ID6545	-2.56	-2.71	-1.11	ns	52	--------
Putative 33-kDa oxygen-evolving protein of photosystem II	gi|15235478	ID7057	ns	1.83	2.74	0.92	34	--------
Thylakoid lumenal 17.4 kDa protein, chloroplast precursor	gi|220976774	ID6412	ns	-1.83	-2.31	-1.28	49	--------
Probable photosystem II oxygen-evolving complex protein 2 precursor	gi|24059930	ID6365	-0.88	-1.81	-2.01	-0.86	57	--------
Photosystem II reaction center psb28 protein	gi|166989935	ID6643	-2.43	-1.04	-0.31	ns		IQFYEGTDEPVVPEIR
PSII stability factor HCF136	gi|75272890	ID6979	ns	0.29	1.93	2.71	64	--------
Photosystem I light-harvesting chlorophyll a/b-binding protein	gi|493723	ID7151	ns	0.71	2.60	3.48	14	--------
Probable plastid lipid-associated protein	gi|15235575	ID6336	-0.79	-2.22	-1.58	ns	67	--------
Magnesium protoporphyrin IX methyltransferase	gi|15234905	ID6483	-1.37	-2.04	ns	ns		AQLPSENLPK TVENTMLMLTEDR
Glutamate-1-semialdehyde 2,1-aminomutase 2	gi|15229018	ID6356	ns	-1.96	-0.94	-0.68	49	--------
CO_2 assimilation								
Rubisco large subunit	gi|89280643	ID6445	-3.27	-3.14	-1.86	-1.51	48	--------
Rubisco activase 2	gi|71834884	ID7005	ns	1.70	2.81	2.81	39	--------
Ribulose 1,5-bisphosphate carboxylase	gi|12019640	ID6398	-2.64	-2.71	-1.72	-1.68		EVEYLLR LPMFGTTDASQVLK
Carbonic anhydrases	gi|8096277	ID6349	ns	-2.98	ns	-0.67	71	--------
Carbohydrate metabolism								
Glyceraldehyde 3-phosphate dehydrogenase	gi|11994300	ID7019	2.28	3.17	2.08	1.98		VPTPNVSVVDLVVQVSK
Transketolase	gi|2894445	ID6866	ns	2.87	2.56	2.71	56	--------
Triosephosphate isomerase, cytosolic	gi|38112662	ID6637	-1.47	-1.93	-0.38	ns	42	--------
Citrate synthase	gi|21553775	ID6900	ns	1.80	1.52	0.84		VLPTAQSGAEPLPEGLLWLLLTGK
Putative inorganic pyrophosphatase	gi|47775656	ID7014	0.27	0.66	1.27	1.76	47	--------
Sucrose-6F-phosphate phosphohydrolase	gi|51968932	ID6448	ns	-0.42	-1.20	-1.94	77	--------
2-phospho-D-glycerate hydroylase (Enolase)	gi|21672003	ID6966	3.32	3.16	2.76	1.97	59	--------

Table 1. Contd.

Protein biosynthesis								
50 S ribosomal protein L12	gi|109940141	ID6684	-0.85	-2.51	-0.92	ns	73	--------
Translation elongation factor Tu	gi|37222953	ID6516	ns	-0.73	-181	-0.48	54	--------
Polyadenylate-binding protein	gi|15238390	ID7237	ns	ns	1.76	1.84	59	--------
Seryl-tRNA synthetase	gi|1359497	ID6932	0.91	1.50	1.84	ns		GFTGLQPPFFMR
Protein folding and assembly								
HSP70	gi|27362885	ID6852	0.51	1.83	1.70	ns		TPSYVAFTDTEK
DnaK-type molecular chaperone hsc 70.1	gi|21539505	ID6783	0.72	2.03	1.26	0.58	61	--------
26.5 kDa class P-related heat shock protein	gi|15234627	ID7239	ns	1.73	0.53	ns	78	--------
Protein disulfide isomerase	gi|77999357	ID6912	1.01	1.63	1.20	0.64	52	--------
Protein disulfide isomerase	gi|99991	ID7023	ns	0.97	1.63	ns	52	--------
20 kDa chaperonin, chloroplastic	gi|399241	ID6681	ns	-1.42	-2.11	ns	67	--------
Hsp70	gi|129852295	ID6836	2.71	2.83	2.88	0.84		VAMNPINTVFDAK
Hsp90	gi|170456	ID6793	0.70	1.84	1.73	ns	66	--------
Nascent polypeptide -associated complex	gi|223529884	ID6399	-1.18	-2.01	ns	-0.68	69	--------
Protein degradation								
Oligopeptidase A-like	gi|221124450	ID6972	3.61	2.83	1.67	1.57	45	--------
Cell rescue/defense								
Catalase 3	gi|7385207	ID6886	0.75	1.81	1.84	1.20		LNVRPNY APGVQTPTIVR
Ascorbate peroxidase	gi|310561	ID7190	ns	3.72	3.61	1.89	56	--------
Cytoplasmic Cu/Zn-SOD	gi|63259317	ID7269	1.82	2.04	1.09	0.58	36	--------
Class III Peroxidase	gi|167533	ID7078	ns	0.71	1.81	ns		LDGVVSR YLSHGGVDFPVPAGRLVCFVVVVFMAAAAA MAGADR
Putative 32 kDa myrosinase binding protein, chain A	gi|168177034	ID6433	-2.70	-0.82	ns	ns	48	--------
2-Cys peroxiredoxin BAS1	gi|7242491	ID7264	ns	0.46	1.11	1.73	53	--------
Redox homeostasis								
Glutathione S-transferase tau family	gi|15218301	ID7085	ns	1.82	2.09	2.45	76	--------
Glutathione synthase	gi|145358475	ID7204	0.61	1.73	ns	ns		DEVIVNEQSGYLMR HQAISELGVYGAYLR
Chalcone synthase	gi|1246019	ID7013	ns	0.77	1.73	1.59	45	--------
Glutamate dehydrogenase	gi|20873461	ID7003	1.92	2.03	0.66	ns	48	--------

Table 1. Contd.

Putative quinone reductase	gi|24030344	ID7238	ns	1.28	1.92	0.37	50	--------
γ-glutamylcysteine synthetase	gi|4262277	ID6839	0.94	2.71	1.57	0.68		EAGFLNAVDEVVR TCTVQVNLDFSSEADMIR
Signal transduction								
Mitogen-activated protein kinase	gi|27542952	ID6953	ns	1.21	1.75	0.53	61	--------
Oxo-phytodienoic acid reductase	gi|30140335	ID7158	1.06	ns	ns	0.92	76	--------
S-adenosyl L-methionine synthetase	gi|162462945	ID7026	2.11	2.03	0.63	ns		TFYTPDPVVGYTDYPFLGQPK
Probable annexin	gi|51896029	ID6070	ns	-0.69	-1.78	0.80	73	--------
Amino acid metabolism								
Glutamine synthetase	gi|1419094	ID6977	0.50	1.11	1.88	ns	53	--------
Isovaleryl-CoA dehydrogenase	gi|10129810	ID6450	-1.90	-0.61	ns	ns		SYLYSVAR VEGGYVLNGNK
Shikimate dehydrogenase	gi|535771	ID6852	ns	2.55	1.82	1.70	74	--------
Cysteine synthase	gi|4996620	ID7069	0.87	1.93	1.81	ns	59	--------
Lipid metabolism								
Lipoxygenase	gi|110740075	ID7201	ns	3.61	2.78	ns		LTIPDYPFANDGLILWDAIK
Allene oxide synthase	gi|33504426	ID6898	ns	1.05	2.66	ns	72	--------
Energy								
Putative H+-transporting ATP synthase	gi|58332	ID6734	1.69	1.27	2.80	1.02	51	--------
ATP synthase gamma chain	gi|17367084	ID6887	1.81	1.62	1.94	0.63		LTNQQWVTMALMGGFAR
RNA processing								
ATP-dependent RNA helicase, putative	gi|15231574	ID6277	-2.64	-0.89	ns	ns	56	--------
Others								
Unknown protein	gi|6706413	ID6361	-3.74	-2.81	-0.82	ns	47	--------
Hypothetical protein	gi|53689726	ID6247	ns	-0.85	-2.62	ns	51	--------
Unnamed protein	gi|164449265	ID6792	ns	0.24	0.73	2.33	39	--------

Putative protein identification and accession number of the closest match in the database are indicated. [a] Spot abundance is expressed as the ratio of intensities of up-regulated (plus value) or down-regulated (minus value) proteins between stressed and control plants. Fold changes had p values ≤ 0.05.[b]Sequence coverage. [=] ns indicate no significant change.

requirements for molecular chaperones would be relevant. Molecular chaperones assist other polypeptides in folding and prevent unproductive interactions without becoming part of a final structure (Mayer and Bukau, 1999). HSPs expression has been documented in response to various abiotic stresses in tomato (Wanglp et al.2008), Arabidopsis (Sarry et al., 2006), and rice (Wan and Liu, 2008).

Proteins involved in protection against oxidative stress

A total of 22 identified proteins in response to Pb induced stress were obviously related to leaf antioxidative reactions and could be classified into five functional categories, namely cell rescue/defense, redox homeostasis, signal transduction, amino acid and lipid metabolism. Of the six cell rescue/defense-related proteins, five (2-Cys peroxiredoxin BAS1, catalase, ascorbate peroxidase, class III peroxidase and Cu/Zn-SOD) were increased, and myrosinase binding protein was highly decreased in the Pb treatment. SOD dismutates O_2^- to H_2O_2 and ascorbate peroxidase is considered to be the key enzyme in the detoxification of H_2O_2 (Asada, 1999). Reduction of H_2O_2 by peroxiredoxin is another mechanism employed to inactivate reactive oxygen species at PS I and constitutes an alternative water-water-cycle in photosynthetic electron transport. In addition to their role in antioxidant defense in photosynthesis, respiration, and stress response, they may also be involved in modulating redox signaling during development and adaptation (Dietz, 2003).

A total of six identities implicated in redox homeostasis, were up-regulated in response to Pb stress, including glutathione synthase (GSH2), glutathione S-transferase (GST), chalcone synthase (CS), glutamate dehydrogenase (GSH1), quinone reductase and γ-glutamylcysteine synthetase (Table 1). GST is an antioxidative protein, and its expression can be strongly enhanced by abiotic and biotic stresses (Dixon et al., 2002).

CS is a key enzyme in cysteine biosynthesis, indicating that it may increase the cellular cysteine level, which is one of the main factors limiting GSH biosynthesis in plants (Vierling, 1991). Taken together, these results suggest that a marked up-regulation of CS may play a pivotal role in enhancing cellular GSH levels or other GSH-involved metabolites, such as phytochelatin (PC), in *C. roseus* during Pb stress. Furthermore, two lipid metabolism related identities, lipoxygenase and allene oxide synthase (AOS) increased in response to Pb stress. Induction of AOS resulted in formation of jasmonate precursor 12-oxophytodienoic acid (PDA) and Jasmonic acid (JA) using hydroperoixed and JA acts as a major signal in stress induced gene expression (Sobhana et al., 2000).

A possible Pb-responsive protein network

C. roseus leaves strongly affect the protein metabolism-related protein and also on carbohydrate metabolism-associated proteins. It is clear that the abundances of Rubisco, RLS and MgPMT involved in the Calvin cycle declined under oxidative stress. As a result, the protein degradation system provoked by oxidative stress might accelerate the partial degradation of these key enzymes participating in the Calvin cycle. The photosynthetic rate is impaired by Pb induced stress (Figure 1) by inhibiting the expression of light-harvesting antenna proteins such as CP26 and Type III. The down-regulation of CP26 and Type III, together with the up-regulation of 33-kDa OECP and OECP2, leads to the reduced light harvesting capability and eventually impaired photosynthesis up to 20 days.

In this study, increased level of GST in *C. roseus* may reduce the availability of ROS, resulting in Pb tolerance. These results correlated with the initiation of production of GST and decline in the ROS after 6 days. Among the metabolic pathways that seemed to be stimulated were the GSH pathways, via the upregulation of glutamate and cysteine biosynthesis, to resupply the GSH bound to Pb. Also six important enzymes involved in GSH, R-S-GSH complexes and phytochelations synthesis, involved in metal sequestration were up-regulated in response to Pb induced stress. SAMS, a signaling protein involved in synthesis of homocysteine as well as ethylene synthesis and 2-Cys Prx, an antioxidant enzyme of chloroplast also were strongly induced during recovery period. The activated antioxidative systems in Pb-treated cells of *C. roseus* possess a stronger capability for removing ROS, which can attenuate the oxidative damage and increased level of PCs ultimately establishing a new redox homeostasis. In the present study, the slight increase SOD, POD and CAT was noted, SOD activity under Cd exposure continued to increase and reached a maximum at 6-day and remained constant up to end of the experiment, but over expression was observed between 2 and 4 days. CAT activity in the leaves of control and 25 mM CdCl2 treated plants exhibited a similar pattern up to 2 days of experiment, while there was a tremendous increase in activity at 4 and 6 days and there was no changes at 12 days of treatment. Similar activity is observed in POD also. This indicated that *C. roseus* had a predominant ability to cope or acclimate to stress by more rapidly developing an antioxidative defense system. Taken together, these basic metabolisms such as photosynthesis, photorespiration, aerobic respiration and protein biosynthesis in *C. roseus* leaves were without exception strongly inhibited at the beginning of Pb treatment and notably diminished at recovery stage. It is thus believed that plants must be required to make economical use of their substance and energy to adequately deal with severely adverse environments. On the other hand, ATP synthase, H+-transporting ATP

synthase, TCA cycle, glycolysis, pentose phosphate pathway, shikimate pathway, PC synthesis, redox homeostasis and signaling proteins were strongly induced after primary shock. Such a defense systems play an important role in maintaining the survival and growth of *C. roseus* under strong and sustained oxidative stress and sequestration. It is also expected that this network can mirror the management of cellular activities in plants under oxidative stress and provide a basis for further functional research of each member of this network in intracellular redox homeostasis and antioxidant metabolisms.

Conclusion

In response to Pb, a total of 76 proteins, out of the 95 differentially expressed proteins, were subjected to MALDI-TOF-MS Of these, 46 identities were identified by PMF and 19 identities were identified by microsequencing as listed in Table 1. Among these, 40 up-regulated and 24 down-regulated proteins were identified and based on the metabolic and functional features, identified proteins were classified into 13 major categories namely photosynthesis (17%), CO2 assimilation (6%), carbohydrate metabolism (11%), protein folding and assembly (14%), protein biosynthesis (6%), redox homeostasis (9%), cell rescue/defense (9%), metabolisms of amino acids (6%) and lipids (3%), protein degradation (2%), energy (3%), signal transduction (6%) and RNA processing (2%).

Abbreviations: ROS, Reactive oxygen species; **EPA,** environmental protection agency; **Hg,** mercury; **Cd,** cadmium; **Pb.** lead; **H_2O_2,** hydrogen peroxide; **$O_2^{\cdot -}$,** superoxide; **MgPMT,** magnesium protophorphyrin IX methyltransferase; **GSA,** glutamate-1-semialdehyde 2.1 aminomutase; **PAP,** plastid lipid-associated protein; **OECP,** oxygen-evolving complex proteins; **PABP,** polyadenylate-binding protein; **OPA,** Oligopeptidase A-like; **PDI,** protein disulfide isomerase; **GSH2,** glutathione synthase; **GST,** glutathione S-transferase; **GSH1,** glutamate dehydrogenase; **PC,** phytochelatin; **AOS,** allene oxide synthase; **SOD,** superoxide dismutase; **TCA,** tricarboxylic acid; **POD,** Peroxidase; **CAT,** Catalase; **ATP,** adenosine triphosphate.

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