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ORIGINAL ARTICLE

Comparative proteome analysis of high and low cadmium accumulating soybeans under cadmium stress

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Abstract A comparative proteomic study was performed to unravel the protein networks involved in cadmium stress response in soybean. Ten-day-old seedlings of contrasting cadmium accumulating soybean cultivars—Harosoy (high cadmium accumulator), Fukuyutaka (low cadmium accumulator), and their recombinant inbred line CDH-80 (high cadmium accumulator) were exposed to 100 μM $CdCl_2$ treatment for 3 days. Root growth was found to be affected under cadmium stress in all. Varietal differences at root protein level were evaluated. NADP-dependent alkenal double bond reductase P1 was found to be more abundant in low cadmium accumulating Fukuyutaka. Leaf proteome analysis revealed that differentially expressed proteins were primarily involved in metabolism and energy production. The results indicate that both high and low cadmium accumulating cultivars and CDH-80 share some common defense strategies to cope with the cadmium stress. High abundance of enzymes involved in glycolysis and TCA cycle might help cadmium challenged cells to produce more energy necessary to meet the high energy demand. Moreover, enhanced expressions of photosynthesis related proteins indicate quick utilization of photoassimilates in energy generation. Increased abundance of glutamine synthetase in all might be involved in phytochelatin mediated detoxification of cadmium ions. In addition, increased abundance of antioxidant enzymes, namely superoxide dismutase, ascorbate peroxidase, catalase, ensures cellular protection from reactive oxygen species mediated damages under cadmium stress. Enhanced expression of molecular chaperones in high cadmium accumulating cultivar might be another additional defense mechanism for refolding of misfolded proteins and to stabilize protein structure and function, thus maintain cellular homeostasis.

Keywords Cadmium · Soybean · Proteomics · Heavy metal accumulation

Abbreviations

2-DE	Two-dimensional polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
MS	Mass spectrometry
p*I*	Isoelectric point
IEF	Isoelectric focusing
ROS	Reactive oxygen species
LC	Liquid chromatography
GS	Glutamine synthetase
GSH	Glutathione
SOD	Superoxide dismutase

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Introduction

Bioaccumulation of toxic heavy metals in food chain has become a global health concern. Cadmium, the highly toxic non-essential heavy metal, when present in the

environment in excessive amount can cause serious damages to all organisms including plants (Benavides et al. 2005). Surface soil gets contaminated with cadmium primarily due to various anthropogenic activities. Indiscriminate use of phosphate fertilizers, zinc smelting, coal burning, by-products released by the power stations, metal industries and cement factories are the major contributors of cadmium pollution (Sanita di Toppi and Gabrielli 1999).

Uptake of the Cd^{2+} by the root generally exhibits two phases: apoplastic binding and symplastic uptake (Hart et al. 1998, 2002). Higher level of apoplastic binding as well as increased Cd^{2+} influx into the symplast through plasma membrane bound transport proteins results in high accumulation of Cd^{2+} in the root. Due to the lack of specificity, the transporters that are primarily responsible for the up take of essential elements such as Zn^{2+}, Fe^{2+} and Ca^{2+} often take up Cd^{2+} (Welch and Norvell 1999). One low-affinity cadmium transporter (OsLCT1), involved in cadmium loading to phloem and grain cadmium accumulation has recently been identified in rice (Uraguchi et al. 2011). In contrast to low cadmium accumulating cultivars, the hyper-accumulators usually have a greater capacity to translocate Cd^{2+} from root to aerial part. Higher rate of xylem loading is probably the reason of enhanced root-to-shoot translocation of Cd^{2+} (Lu et al. 2008).

Once Cd^{2+} enters the cell, it causes disturbance in the cellular functions by replacing zinc, calcium or iron from the proteins, as these elements exhibit chemical similarity with that of cadmium (Verbruggen et al. 2009). In addition, Cd^{2+} has higher affinity to bind with the sulfhydryl groups of proteins and thus causes enzyme inactivation (Nocito et al. 2007). These interferences in turn affect plant's vital biochemical pathways related to carbohydrate metabolism (Sanita di Toppi and Gabrielli 1999), nitrate absorption (Hernández et al. 1996) and photosynthesis (Vassilev et al. 1995).

In course of time, plants have developed complex mechanisms to regulate the uptake, mobilization and intracellular concentration of Cd^{2+}. Apart from the plasma membrane exclusion method, the most common way to protect the cell from the adverse effects of Cd^{2+} includes synthesis of phytochelatins and their compartmentalization into vacuole (Sanita di Toppi and Gabrielli 1999). Identification of proteins involved in the cadmium stress sensing and signal transduction is thus a fundamental step of understanding plants response to cadmium stress.

Soybean is widely cultivated throughout the world for its high content of seed oil and protein. Plants readily absorb cadmium from soil through their root system. However, the translocation rate of Cd^{2+} from root to aerial part varies within species or even at variety level. Considerable genetic variations among the soybean cultivars in Cd^{2+} uptake, accumulation and translocation to aerial part have been reported (Arao et al. 2003). Cultivar like Enrei accumulates high amount of Cd^{2+} in their roots, however, rate of translocation to aerial portions is quite low. Interestingly, Harosoy is more efficient in translocating Cd^{2+} from root to shoot. Thus, the aerial parts of these contrasting cadmium accumulating soybean cultivars experience different levels of cadmium stress as compared to roots. Unraveling the target proteins that play pivotal roles in Cd^{2+} translocation and detoxification would help us for better understanding the cadmium tolerance mechanism.

Different physiological and biochemical aspects of cadmium stress response have been studied in *Bacopa* (Mishra et al. 2006), *Brassica* (Baryla et al. 2001), *Pisum* (Dixit et al. 2001), *Hordeum* (Vassilev et al. 1995). Advancement in the mass spectrometry (MS) technology has opened new avenues for deeper exploration of plant response to cadmium stress at the functional level and thus refines our knowledge about plant heavy metal stress related signaling pathways. Over the last decade, proteomic techniques have been well exploited by several researchers to get a better picture about the cadmium stress induced proteome changes in *Arabidopsis* (Semane et al. 2010; Roth et al. 2006; Sarry et al. 2006), *Populus* (Durand et al. 2010; Kieffer et al. 2009a, b, 2008), rice (Lee et al. 2010; Aina et al. 2007; Ahsan et al. 2007; Hajduch et al. 2001), tomato (Rodriguez-Celma et al. 2010). A recent review by Villiers et al. (2011) pointed out that only a few proteomic studies have been carried out so far on soybean in relation to cadmium stress response. Differential protein expression in cultured cells of soybean in response to cadmium was studied in detail by Sobkowiak and Deckert (2006). Ahsan et al. (2012) investigated differential responses of root microsomal proteins in contrasting cadmium accumulating soybean cultivars under cadmium stress. In precise, this present investigation would be the first report of organ-specific proteome changes under cadmium stress in soybean.

Although, the molecular and cellular responses of soybean to other abiotic stresses have been extensively analyzed, the genetic basis of soybean stress responses is not well understood (Tran and Mochida 2010). In the present study, a comparative proteomic approach was used to ascertain the cadmium stress induced changes of leaf proteome in two contrasting cadmium accumulating soybean cultivars and their recombinant inbred line. In addition, for better understanding the varietal differences at root protein level, root proteomics was also carried out in control plants. Emphasis was given to find out target proteins that play crucial role in protecting cellular metabolism from the cadmium induced oxidative stress damages.

Materials and methods

Plant growth condition and cadmium treatment

Two contrasting cadmium accumulating soybean (*Glycine max* L.) cultivars Harosoy (high cadmium accumulator) and Fukuyutaka (low cadmium accumulator), and their recombinant inbred line (RIL F7 generation) CDH-80 (high cadmium accumulator) were used as plant materials for the present investigation. Seeds were first surface sterilized in sodium hypochlorite solution and allowed to germinate on silica sand. Seedlings were maintained in growth chamber at 25 °C with 16-h photoperiod (light intensity 600 μmol m^{-2} s^{-1}) and 70 % relative humidity. Ten-day-old plants were exposed to cadmium stress by immerging the roots in conical flask containing 100 μM $CdCl_2$ (Wako, Osaka, Japan) solution. A parallel set of 10-day-old plants were maintained in water without $CdCl_2$ for 3 days and considered as control. Morphological parameters like root length, root weight, shoot length and shoot weight were recorded after completion of 3 days of stress. Three independent biological experiments were performed for each cultivar. During the stress period, cadmium solution was replaced daily with freshly prepared one of the same strength to avoid low oxygen stress condition. Uni-foliate leaves and roots were collected and stored at −80 °C for proteomic analysis.

Protein extraction

Five hundred milligram of plant organ was ground to fine powder with a mortar and pestle in liquid nitrogen. The powder was immediately transferred to 10 % trichloroacetic acid and 0.07 % 2-mercaptoethanol in acetone. The mixture was vortexed and sonicated for 5 min and incubated at −20 °C for 1 h with regular shaking at 15 min intervals. After incubation, the suspension was centrifuged at 9,000×*g* for 20 min at 4 °C. The supernatant was discarded and the resulting pellet was washed twice with 0.07 % 2-mercaptoethanol in acetone. The resulting pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and re-suspended in lysis buffer (8 M urea, 2 M thiourea, 5 % CHAPS, and 2 mM tributylphosphine) by vortexing for 1 h at 25 °C. The suspension was centrifuged at 20,000×*g* for 20 min at 25 °C. The supernatant was finally collected as protein extract. Protein concentration was determined using the Bradford method (Bradford 1976) with bovine serum albumin as the standard. The protein extract was subjected to two-dimensional polyacrylamide gel electrophoresis (2-DE).

Two-dimensional polyacrylamide gel electrophoresis

Four hundred and fifty microgram of protein sample in a final volume of 200 μL of lysis buffer containing 0.4 % ampholytes pH 3–10 (Bio-Lyte, Bio-Rad, Hercules, CA, USA) was loaded into a focusing tray. Immobilized pH gradient strips (3–10 NL, 11 cm, Bio-Rad) were passively rehydrated for 2.5 h and then actively rehydrated for 14 h at 50 V. Isoelectric focusing (IEF) was carried out using a Protean IEF Cell system (Bio-Rad) under the following conditions: 250 V for 15 min with a linear ramp, 8,000 V for 1 h with a linear ramp, and finally 8,000 V at 35,000 V/h with a rapid ramp at 20 °C.

After IEF, the strips were first incubated in equilibration buffer I (6 M urea, 2 % SDS, 0.375 M Tris–HCl pH 8.8, 20 % glycerol, and 130 mM dithiothreitol) for 15 min and then in equilibration buffer II (6 M urea, 2 % SDS, 0.375 M Tris–HCl pH 8.8, 20 % glycerol, and 135 mM iodoacetamide) for another 15 min. SDS-PAGE in the second dimension was carried out using 15 % separation gel with 5 % stacking gel at 35 mA for ∼2 h and 30 min until the dye line reached to the end of the gel. After electrophoresis, the gels were stained with coomassie brilliant blue (CBB) (Phast Gel Blue R, GE Healthcare, Piscataway, NJ, USA) containing 35 % methanol and 10 % acetic acid for 1 h, and then destained with 35 % methanol and 10 % acetic acid for 12 h.

Gel image analysis

CBB stained gels were scanned using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer; Bio-Rad). Protein spots were detected and quantified on the basis of relative intensity using PDQuest software (version 8.0.1, Bio-Rad). The intensity of a given protein spot was expressed in terms of its volume, which was defined as the sum of the intensities of all pixels constituting the spot in the image. To compensate for subtle differences in sample loading, gel staining, and destaining, the volume of each spot was normalized as a percentage of the total volume of all the spots present in the gel. Manual editing was carried out after automated detection and matching. The p*I* and M_r of each protein were determined using 2-DE standard markers (Bio-Rad). Three replicates from three independent biological extracts were used for analysis.

Peptide preparation for mass spectrometry analysis

Protein spots were excised from CBB stained 2-DE gels and destained in 50 mM ammonium bicarbonate for 1 h at 40 °C. Proteins were reduced within the gel pieces by

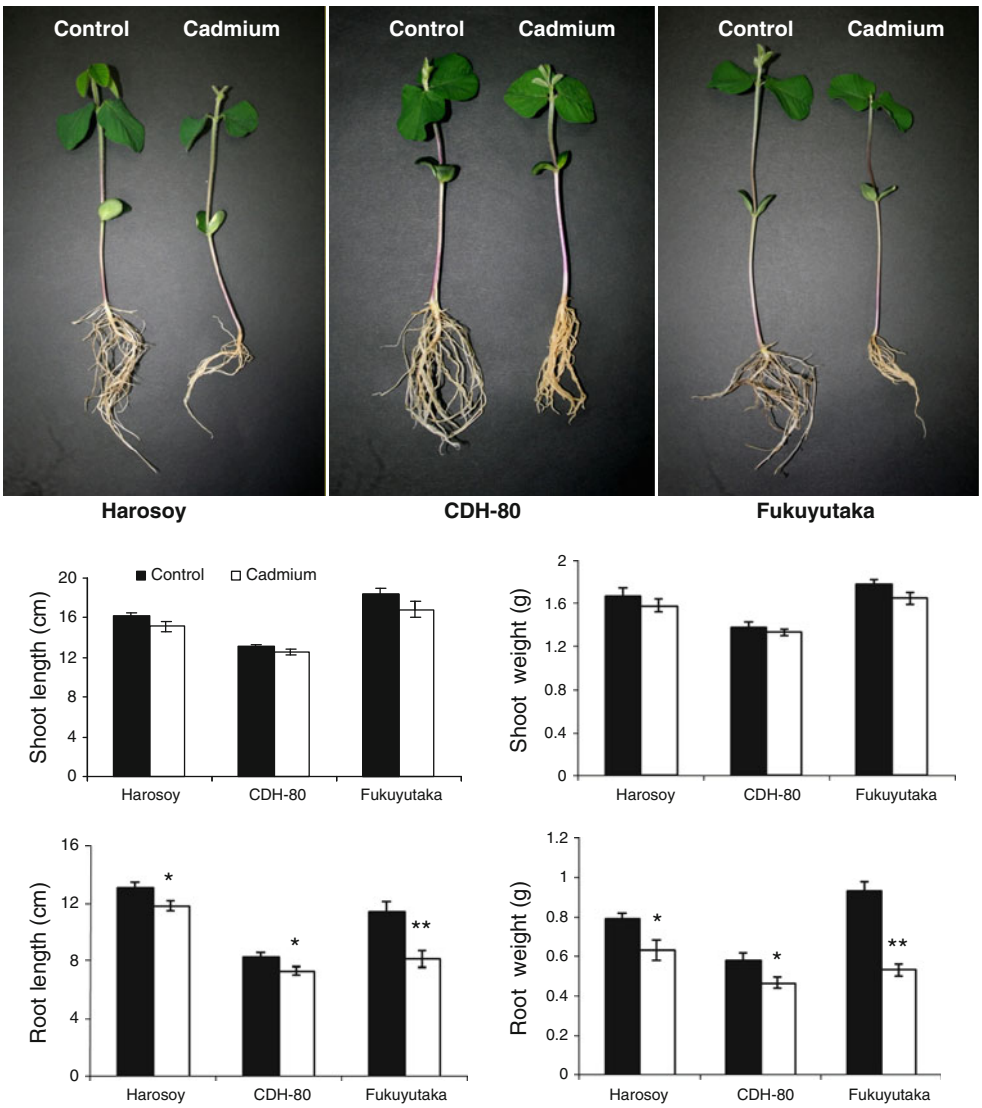


Fig. 1 Effects of cadmium stress on growth performance of soybean cultivars Harosoy, and Fukuyutaka and their RIL CDH-80. Ten-day-old seedlings of Harosoy, CDH-80 and Fukuyutaka were treated with 100 μM $CdCl_2$ solution for 3 days. *Columns* represent mean ± SE ($n = 9$). The symbols *asterisk* and *double asterisk* indicate significant differences at 5 and 1 % level, respectively. Significant differences between the control and cadmium treated seedlings were determined by performing Student *t* test

incubation in 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 1 h at 60 °C, followed by incubation for 30 min in 40 mM iodoacetamide in 100 mM ammonium bicarbonate. Proteins were digested at 37 °C in 100 mM ammonium bicarbonate containing 1 pM trypsin (Sigma-Aldrich, St. Louis, MO, USA). The resulting tryptic peptides were extracted from the gel grains 3 times using 0.1 % trifluoroacetic acid in 50 % acetonitrile. The procedure described above was performed using an automated robotic system (DigestPro, Intavis Bioanalytical Instruments AG, Cologne, Germany). The final peptide solution was concentrated and desalted using NuTip C-18 pipet tips (Glygen, Columbia, MD, USA). The desalted peptide solution was finally analyzed using MS.

Protein identification by nano-liquid chromatography-tandem mass spectrometry

Peptides were analyzed on a nanospray LTQ XL Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA) operated in data-dependent acquisition mode controlled by Xcalibur software (Thermo Fisher Scientific). Peptides in 0.1 % formic acid were loaded onto a 300 μm ID × 5 mm C18 PepMap trap column using an Ultimate 3000 nano liquid chromatography (LC) system (Dionex, Germering, Germany). Peptides eluted from the trap column were further separated on a 75 μm ID × 15 cm, 3-μm C18 PepMap100 nano column with 0.1 % formic acid in acetonitrile at a flow rate of 200 nL/min. Samples were sprayed into the

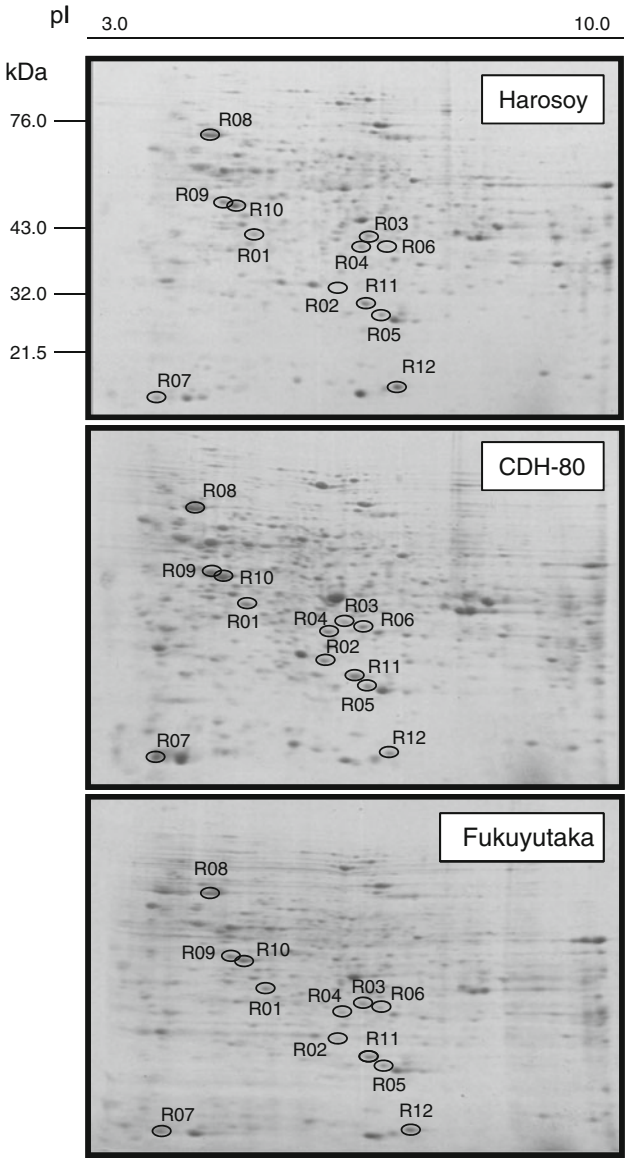


Fig. 2 2-DE gel images of soybean root proteins showing differential expression patterns in Harosoy, CDH-80 and Fukuyutaka under unstressed condition. Proteins were extracted from 13-day-old seedlings, separated by 2-DE and stained with CBB. *Circles* mark the position of differentially expressed proteins among the Harosoy, CDH-80 and Fukuyutaka

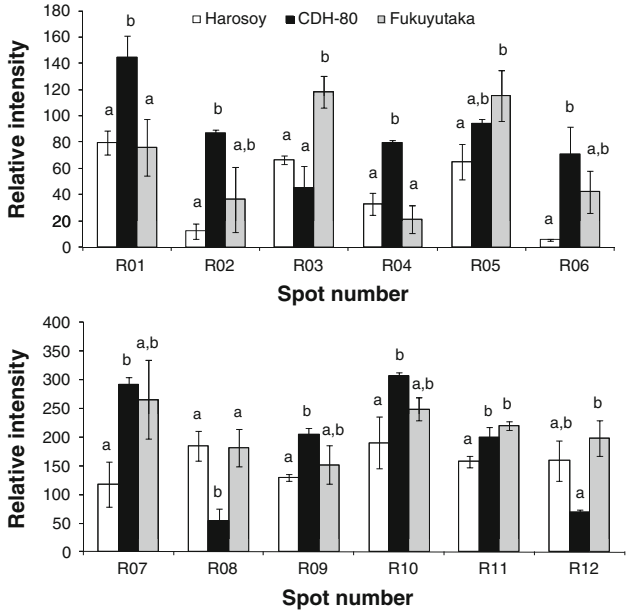


Fig. 3 Expression levels of proteins that were differentially expressed in Harosoy, CDH-80 and Fukuyutaka under unstressed condition. Each *value* represents the mean ± SE of relative intensity determined from gels of three biological replicates. *Values in columns* with *different letters* are significantly different at 5 % level according to Duncan's Multiple Range Test

MS using a PicoTip emitter (20 μm ID, 10 μm tip ID, New Objective, Woburn, MA, USA) at a spray voltage of 1.8 kV. Full-scan mass spectra were acquired in the Orbitrap over a mass range of 150–2,000 *m/z*, with a resolution of 15,000. The three most intense ions above an intensity threshold of 1,000 units were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35 % after accumulation to a target value of 1,000 intensity units. Dynamic exclusion was employed within 30 s to prevent repetitive selection of peptides. DTA files were generated from acquired MS/MS spectra files and then converted to Mascot generic files using BioWorks software (version 3.3.1, Thermo Fisher Scientific).

The following parameters were used to create the peak list: parent ions in the mass range with no limitation, one grouping of MS/MS scans, and a threshold of 100. To estimate false-discovery rate (FDR) at the protein level, the resulting peptide sequence data were searched in the database obtained from the soybean genome database (Phytozome, version 6.0, http://www.phytozome.net/soybean) using the MASCOT search engine (version 2.2.04, Matrix Science, London, UK). Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. The search parameters were: peptide mass tolerance of 10 ppm, fragment mass tolerance of 0.2 Da, and peptide charges of +1, +2, and +3.

For protein identification, only peptides that appeared for the first time (marked in red bold in the Mascot report) contributed to the identification. The minimum requirements for the identification of a protein were at least two peptide sequence matches above the identity threshold with more than 13 % sequence coverage. A homology search of the amino acid sequences of identified proteins was performed against the NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) non-redundant sequence database using BLASTP to assign protein identities.

Table 1 Differentially expressed proteins in roots of Harosoy, CDH-80 and Fukuyutaka under unstressed condition

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	M_r (kDa)/p*I*		MS[i]	Func.[j]	Local.[k]
							Theo.[g]	Exp.[h]			
R01	Isoflavone 7-O-methyltransferase	XP_003556407.1	347	23	6	721	40.1/5.4	42.5/5.2	MS/MS	Met	Cysk
R02	L-Ascorbate peroxidase 2	NP_001235587.1	181	23	5	442	27.1/5.6	33.2/5.6	MS/MS	Dis/Def	Cyto
R03	NADP-dependent alkenal double bond reductase P1	XP_003521022.1	149	22	6	563	38.0/5.9	40.7/5.7	MS/MS	Dis/Def	Cyto
R04	Dihydroflavonol-4-reductase	XP_003551848.1	495	43	14	498	38.0/8.0	39.5/5.6	MS/MS	Met	Cyto
R05a	Chalcone-flavonone isomerase 1A	NP_001235219.1	395	46	9	394	23.3/6.2	28.4/5.8	MS/MS	Met	Cyto
R05b	P34 probable thiol protease	XP_003547166.1	184	19	4	723	41.4/5.4	28.4/5.8	MS/MS	ProtDes	Extr
R06	Isoflavone reductase homolog A622	XP_003537595.1	248	45	11	610	34.0/6.1	39.3/5.8	MS/MS	Met	Cyto
R07a	Protein LlR18B	XP_003546343.1	171	64	6	271	16.5/4.6	18.3/4.2	MS/MS	Met	Cyto
R07b	Uncharacterized protein	NP_001235125.1	106	32	4	284	15.1/10.4	18.3/4.2	MS/MS	Unclassi	Cyto
R08	Heat shock 70 kDa	XP_003554320.1	455	47	25	1165	71.8/5.0	72.3/4.6	MS/MS	Dis/Def	Cyto
R09a	Actin	XP_003552700.1	248	39	12	756	42.0/5.2	56.1/5.0	MS/MS	CellSt	Cysk
R09b	Transaldolase	XP_003532330.1	192	15	4	829	48.1/5.8	56.1/5.0	MS/MS	Ene	Cyto
R10a	Actin	XP_003547582.1	638	60	17	754	41.7/5.3	54.8/5.1	MS/MS	CellSt	Cysk
R10b	NAD-dependent dihydropyrimidine dehydrogenase subunit PreA	XP_003528976.1	100	21	6	851	46.5/5.9	54.8/5.1	MS/MS	Met	Cyto
R11	Triosephosphate isomerase	XP_003547334.1	164	59	10	451	27.4/5.8	30.2/5.7	MS/MS	Ene	Cyto
R12	Ripening related protein	NP_001238076.1	111	34	6	272	21.7/6.2	18.9/5.9	MS/MS	Uncl	Extr

[a] Spot number as given in Fig. 2

[b] Accession number according to the NCBI database

[c] Ions score of identified protein using soybean genome sequence database

[d] Sequence coverage; proteins with <13 % sequence coverage were excluded from the result

[e] Number of query matched peptides; proteins with at least 2 matched peptides were considered

[f] The score of the high-scoring segment pair from that database sequence

[g] Theo. theoretical, M_r molecular weight, p*I* isoelectric point

[h] Exp. experimental, M_r molecular weight, p*I* isoelectric point

[i] MS MALDI-TOF MS, MS/MS nanoLC MS/MS

[j] Func. function category using functional classification: ProtDes protein destination/storage, Met metabolism, Dis/Def disease/defense, Ene energy, CellSt cell structure, Sig/Trans signal transduction, Unclassi unclassified, Uncl. unclear classification

[k] Local. localization category using local classification, Mito mitochondria, Cyto cytoplasm, Vacu vacular, Pero peroxisome

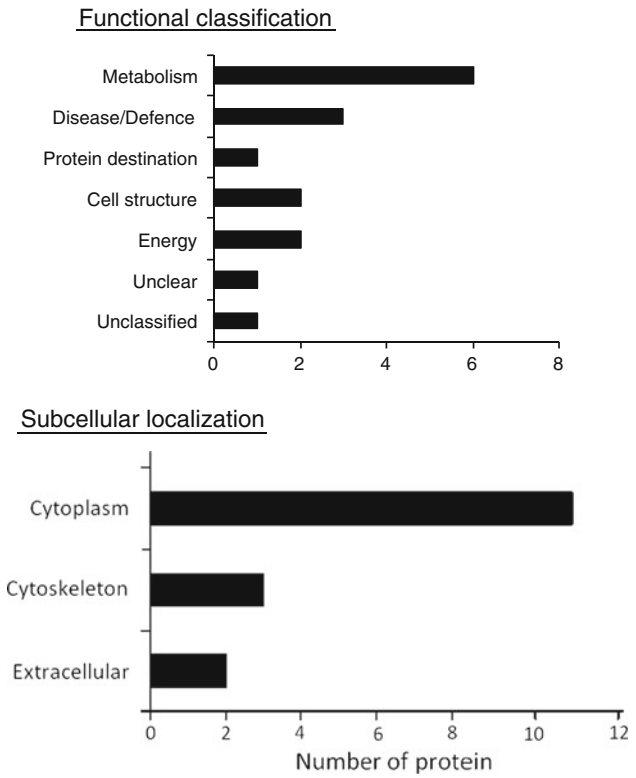


Fig. 4 Functional classification and subcellular localization of differentially expressed root proteins among Harosoy, CDH-80 and Fukuyutaka. Detail explanation of functional classification and subcellular localization are presented in Table 1

Protein identification by matrix assisted laser desorption ionization time-of-flight mass spectrometry

Peptides were mixed with α-cyano-4-hydroxycinamic acid matrix and dried onto a plate specific for the analysis of matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS using a Voyager-DE RP (Applied Biosystems, Foster City, CA, USA). Calibration was external and data were collected in the reflector mode. The pick list of peptide mass was generated by Perspective-GRAMS/386 (version 3.04) software (Galactic Industries, Salem, NH, USA). The obtained peptide mass spectra were searched using an in-house-licensed MASCOT search engine and compared with the soybean genome database.

Search parameters used the fixed cysteine carbamidomethylation and variable methionine oxidation as modifications, peptide mass tolerance ±0.5 Da, fragments ions 1 Da, one missed cleavage, and trypsin was specified as the proteolytic enzyme. Identified proteins with a peptide mass fingerprint were denoted as having an unambiguous identification by the following criteria: (1) the deviation between the experimental and theoretical peptide masses needed to be <50 ppm; (2) at least 7 different predicted peptide masses were needed to match the observed masses for an identification to be considered valid; (3) the matching peptides needed to cover at least 28 % of the known protein sequence, and (4) protein scores needed to have >60 identity for soybean genome database.

Functional assignment of proteins and cellular localization

Identified proteins were annotated to their biological function according to Bevan et al. (1998). Information of the identified proteins obtained from WoLF PSORT prediction (http://wolfpsort.org/), ESLpred (http://www.imtech.res.in/raghava/eslpred), and SubLoc (http://www.bioinfo.tsinghua.edu.cn/SubLoc) was used to determine their subcellular localization.

Results

Effect of cadmium stress on growth of soybean seedlings

Cadmium stress had adverse effects on plant growth. Significant decreases in root length and root weight were recorded after 3 days of stress in both cultivars, as well as in their RIL line CDH-80 (Fig. 1). However, the magnitudes of the decreases were more in Fukuyutaka (1.4-fold in root length and 1.7-fold in root weight), as compared to Harosoy and CDH-80 (Fig. 1). No significant variations in shoot length and shoot weight were observed in either of them under 3 days of stress.

Differences in protein expression in roots of Harosoy, CDH-80 and Fukuyutaka

The high cadmium accumulating cultivar Harosoy and the RIL CDH-80 and low cadmium accumulating Fukuyutaka were used as plant materials. For better understanding of root proteins involved in the transportation of cadmium, proteomic analysis was carried out using roots of control plants. In total, 12 protein spots were differentially expressed among Harosoy, CDH-80 and Fukuyutaka (Figs. 2, 3). These differentially expressed protein spots were identified using MS (Table 1). Totally 15 proteins were identified from the 12 differentially expressed protein spots. Out of the 12 protein spots, two spots (spots R03 and R05) were accumulated in Fukuyutaka as compared to Harosoy and CDH-80. The proteins were NADP-dependent alkenal double bond reductase P1, chalcone-flavonone isomerase 1A and P34 probable thiol protease. The identified proteins were analyzed for functional classification. Six proteins were related to cellular metabolism, while remaining 9 proteins were involved in energy, cell

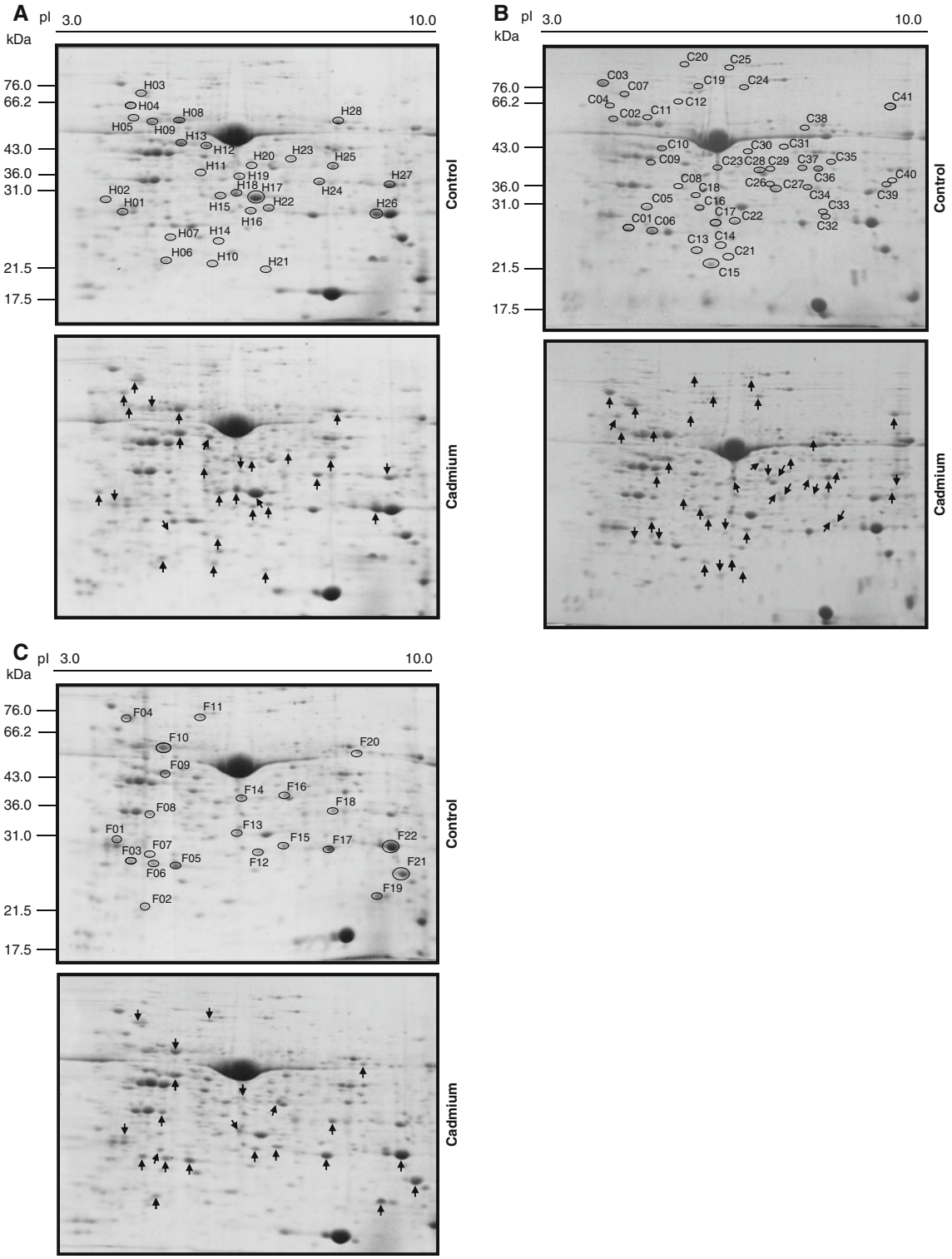


Fig. 5 2-DE gel images of soybean leaf proteins showing differential expression patterns in Harosoy, CDH-80 and Fukuyutaka under cadmium stress. Ten-day-old seedlings of Harosoy (**a**), CDH-80 (**b**) and Fukuyutaka (**c**) were treated with 100 μM $CdCl_2$. After 3 days of stress, proteins were extracted from uni-foliate leaves, separated by 2-DE, and visualized after CBB staining. The p*I* and M_r of each protein were determined using 2-DE markers. *Arrows* indicate change in protein expressions (*upward arrows* indicate increase and *downward arrows* indicate decrease) under cadmium stress and *circles* mark the position of the same proteins from the control

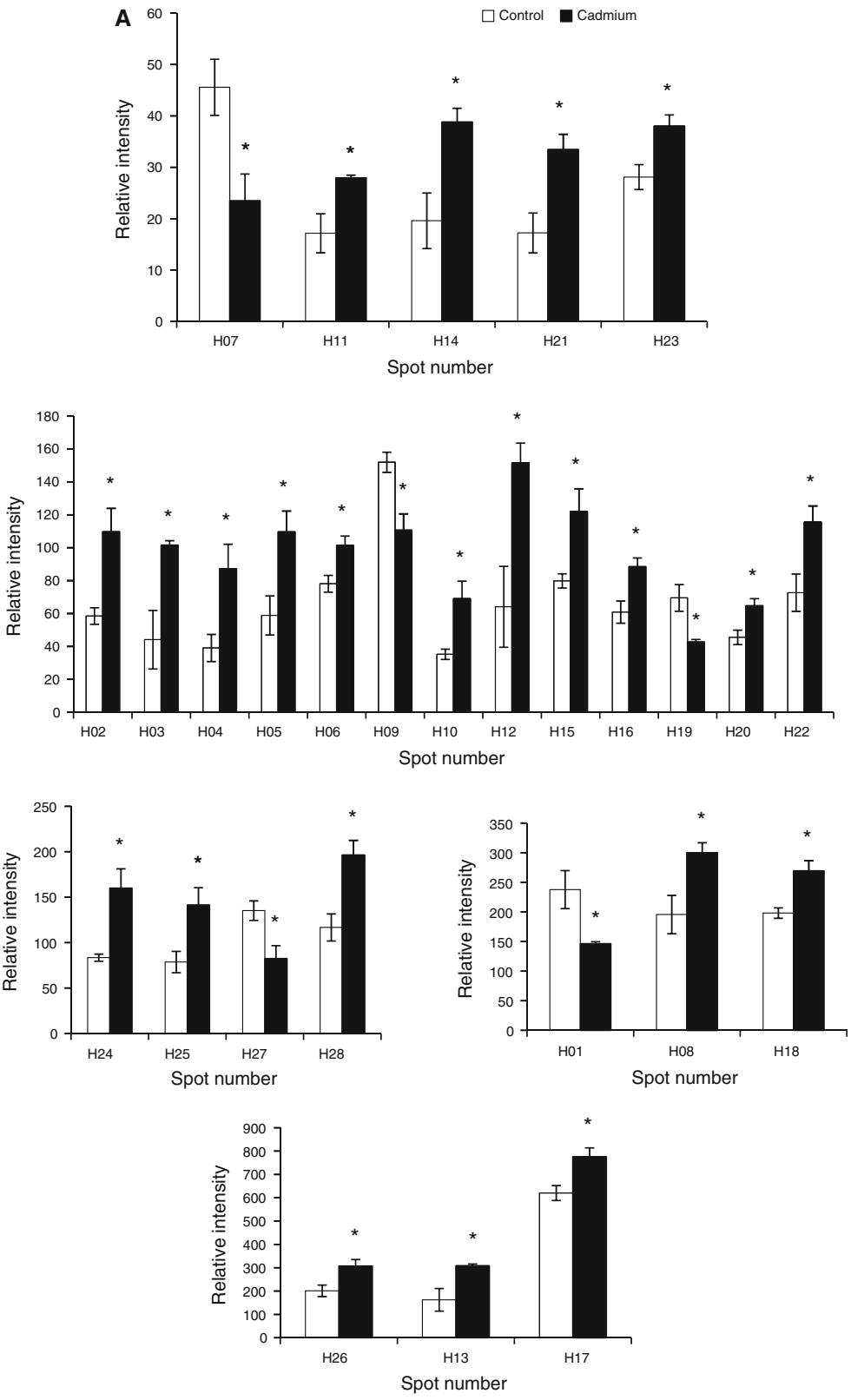
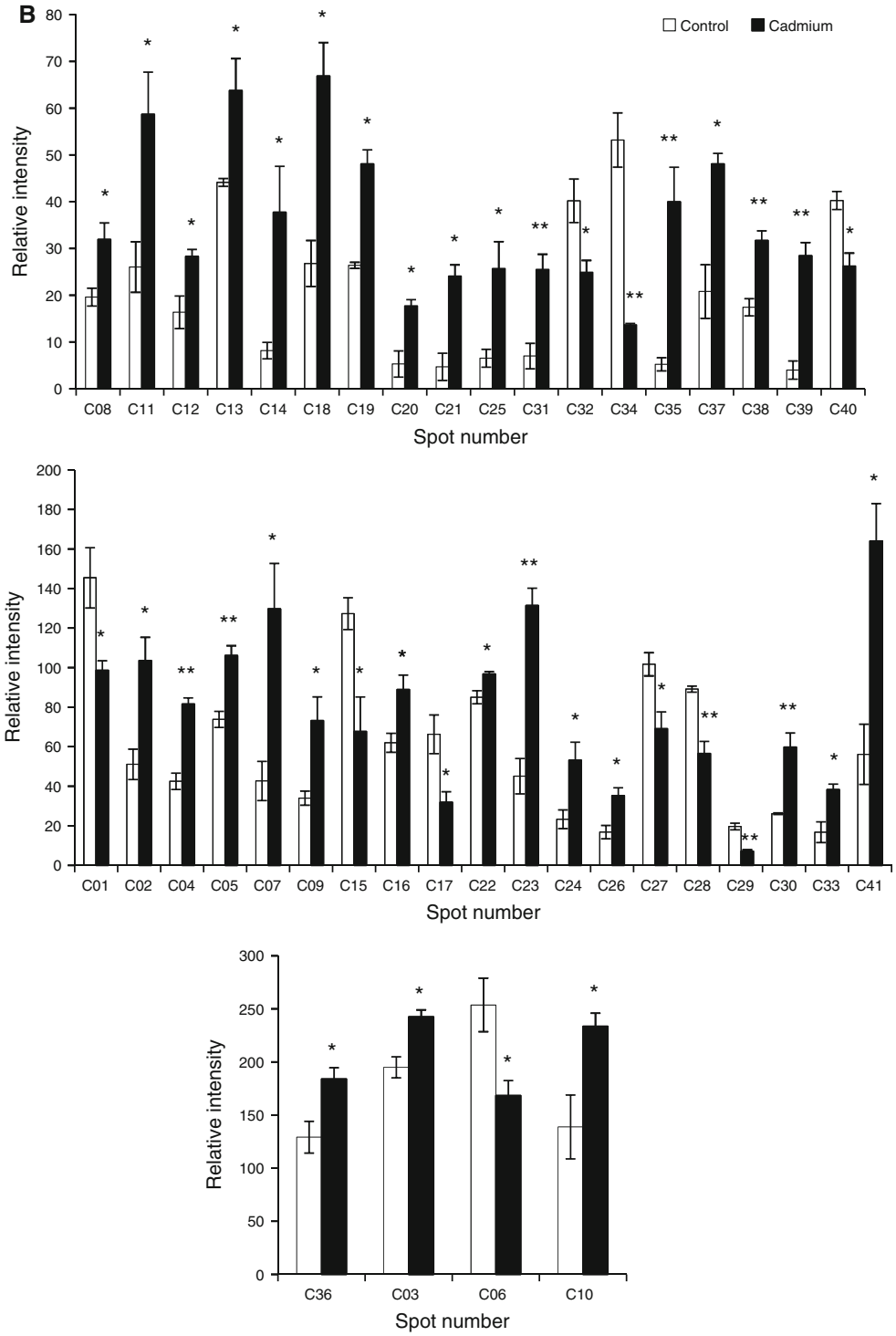


Fig. 6 Expression levels of cadmium stress-responsive proteins of soybean leaves. Ten-day-old seedlings of Harosoy (**a**), CDH-80 (**b**) and Fukuyutaka (**c**) were treated with 100 μM $CdCl_2$ for 3 days. Relative intensities of proteins were obtained using PDQuest software. *White* and *black bars* represent relative intensity of differentially expressed leaf proteins of control and cadmium stressed soybean seedlings, respectively. *Each column* represents the mean ± SE of relative intensity determined from gels of three biological replicates. The means were compared using the Student *t* test (*$P < 0.05$ or **$P < 0.01$). Standard errors are denoted by *error bars*

structure, protein destination and disease defense (Table 1; Fig. 4). The identified proteins were analyzed to predict their subcellular localization using WoLF PSORT, ESLpred, and SubLoc. Eleven proteins were predicted to be cytoplasmic in localization and remaining were related to extracellular and cytoskeleton (Table 1; Fig. 4).

Fig. 6 continued



Differentially expressed proteins induced in leaves of Harosoy, CDH-80 and Fukuyutaka under cadmium stress

To study the changes in protein expression level under cadmium stress, the proteins in leaves of control and cadmium stressed plants were compared. Extracted leaf proteins were separated by 2-DE and stained with CBB to evaluate their expression level (Fig. 5). The relative intensities of all protein spots from three independent biological replicates were analyzed using PDQuest software (Fig. 6). Out of the detected protein spots on 2-DE pattern of leaf proteome, 23, 31 and 16 protein spots were increased and 5, 10 and 6 protein spots were decreased in

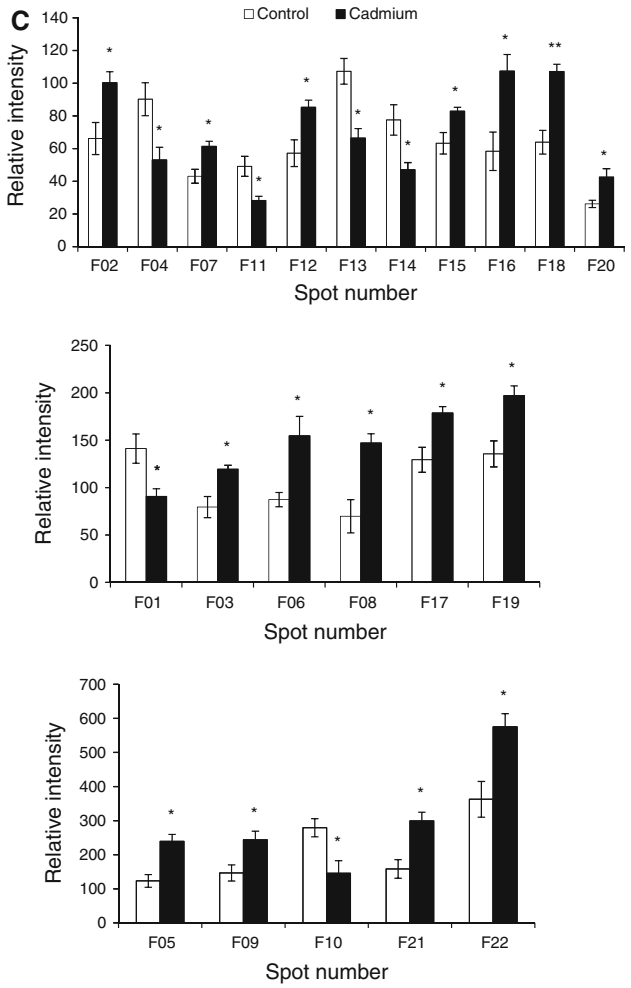


Fig. 6 continued

cadmium treated Harosoy, CDH-80 and Fukuyutaka, respectively (Fig. 6).

Differential expression of common leaf proteins among Harosoy, CDH-80 and Fukuyutaka under cadmium stress

To identify the proteins that were differentially expressed under cadmium stress, 2-DE protein spots were identified using MS (Tables 2, 3, 4). Venn diagram was prepared to compare the commonly expressed proteins among Harosoy, CDH-80 and Fukuyutaka (Fig. 7). Out of 71 differentially expressed protein spots, 3 were commonly expressed in leaves of Harosoy (Spots H03, H13 and H16), CDH-80 (Spots C07, C10 and C22) and Fukuyutaka (Spots F04, F09 and F12). The expressions of two protein spots (H13/C10/F09 and H16/C22/F12) were commonly enhanced under cadmium stress in all three. The expression of another protein spot was increased in Harosoy (H03) and CDH-80 (C07), but decreased in Fukuyutaka (F04).

A total of 32, 44 and 26 proteins were identified from 28, 41 and 22 differentially expressed protein spots of Harosoy, CDH-80 and Fukuyutaka, respectively, (Tables 2, 3, 4). Under cadmium stress, total 3 proteins were commonly expressed in Harosoy, CDH-80 and Fukuyutaka (Fig. 7). The expression of 70 kDa heat shock protein was increased in both high cadmium accumulating cultivar Harosoy (Spot H03) and RIL CDH-80 (Spot C07), whereas its expression was decreased in low cadmium accumulating Fukuyutaka (Spot F04) (Tables 2, 3, 4). However, expression of glutamine synthetase (Spots H13/C10/F09) and triosephosphate isomerase (Spots H16/C22/F12) were increased under cadmium stress in all three.

Cadmium stress induced leaf proteins common between Harosoy and CDH-80

Out of the 61 differentially expressed protein spots, 5 were common between Harosoy and CDH-80 (Fig. 7). Under cadmium stress, the expressions of all the 5 protein spots (H04/C04, H05/C02, H14/C13, H15/C16, H25/C36) were increased in both. Moreover, 15 and 29 protein spots were separately expressed in Harosoy and CDH-80, respectively. Fifteen protein spots in Harosoy might be considered as cadmium stress responsive cultivar-specific proteins (Fig. 7).

In total, 6 proteins were identified from 5 differentially expressed protein spots. The commonly expressed proteins between Harosoy and CDH-80 were RuBisCO large subunit-binding protein subunit alpha (H04 and C04), tubulin beta-3 chain (H05a and C02a), V-type proton ATPase subunit B 2 (H05b and C02b), stem 28 kDa glycoprotein (H14 and C13) and glyceraldehyde-3-phosphate dehydrogenase (H25 and C36) (Tables 2, 3).

Differentially expressed common proteins between Harosoy and Fukuyutaka

To find out the differences in cadmium stress induced changes in leaf protein expression level among high and low cadmium accumulating soybean cultivars, the spot intensities were analyzed. Among the 42 differentially expressed protein spots, 5 were common between Harosoy and Fukuyutaka (Fig. 7). Expressions of three protein spots were increased in both Harosoy (H06, H22 and H24) and Fukuyutaka (F02, F15 and F18). Two protein spots exhibited an increased expression in Harosoy (H08 and H18), but decreased in Fukuyutaka (F10 and F13) (Fig. 7). Moreover, cultivar-specific cadmium stress responsive proteins that were differentially expressed in Harosoy and Fukuyutaka were 15 and 10 protein spots, respectively (Fig. 7).

Table 2 Differentially expressed proteins of soybean cv. Harosoy leaf under cadmium stress

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	M_r (kDa)/pI		FC[i]	P value[j]	MS[k]	Func.[l]	Local.[m]
							Theo.[g]	Exp.[h]					
H01	Ribose-5-phosphate isomerase	XP_003521745.1	166	32	5	514	29.8/5.5	29.2/4.3	0.62	0.047	MS/MS	Ene	Chlo
H02	31 kDa ribonucleoprotein	XP_003525605.1	164	23	5	506	33.0/4.8	30.2/4.0	1.88	0.027	MS/MS	ProtDes	Chlo
H03	Heat shock cognate 70 kDa protein	XP_003554320.1	130	34	16	1165	71.8/5.0	72.8/4.8	2.30	0.033	MS/MS	Dis/Def	Cyto
H04	RuBisCO large subunit-binding protein subunit alpha	XP_003538152.1	124	17	8	1003	61.7/5.2	65.2/4.5	2.23	0.048	MS/MS	Ene	Chlo
H05a	Tubulin beta-3 chain	XP_003546276.1	187	21	7	852	50.7/4.7	59.4/4.6	1.86	0.044	MS/MS	Cell/Div	Chlo
H05b	V-type proton ATPase subunit B 2	XP_003534485.1	183	27	9	966	54.2/4.9	59.4/4.6	1.86	0.044	MS/MS	Ene	Cyto
H06	Cu/Zn superoxide dismutase II	CAA39819.1	200	47	5	312	20.9/6.0	22.6/5.1	1.30	0.038	MS/MS	Dis/Def	Chlo
H07	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase 4	XP_003536514.1	116	30	4	382	22.3/5.2	26.0/5.1	0.52	0.043	MS/MS	Met	Cyto
H08a	Enolase	NP_001237329.1	338	32	8	873	47.9/5.4	56.9/5.3	1.53	0.046	MS/MS	Met	Cyto
H08b	Granule-bound starch synthase	NP_001236366.1	146	14	7	1154	68.5/6.6	56.9/5.3	1.53	0.046	MS/MS	Met	Chlo
H09	ND	–	–	–	–	–	–	57.2/4.9	0.73	0.024	–	–	–
H10	Peroxiredoxin	CAQ56034.1	266	41	7	305	17.4/5.4	22.0/5.9	1.96	0.039	MS/MS	Dis/Def	Cyto
H11	Arginase	NP_001237121.1	101	22	8	682	38.9/6.0	36.9/5.6	1.63	0.048	MS/MS	Met	Chlo
H12	Glutamine synthetase precursor	NP_001237784.1	413	35	11	825	47.9/6.7	45.3/5.8	2.36	0.033	MS/MS	Met	Chlo
H13	Glutamine synthetase	XP_003546121.1	192	37	10	824	47.9/6.4	46.9/5.3	1.90	0.041	MS/MS	Met	Chlo
H14	Stem 28 kDa glycoprotein	NP_001238459.1	399	34	9	528	29.2/8.7	25.3/5.9	1.98	0.034	MS/MS	ProtDes	Vacu
H15	L-Ascorbate peroxidase 2	NP_001235587.1	227	38	8	442	27.1/5.6	29.9/6.0	1.53	0.044	MS/MS	Dis/Def	Cyto
H16	Triosephosphate isomerase	ABA86966.1	70	38	7	379	27.4/6.6	28.8/6.6	1.45	0.033	MS	Ene	Cyto
H17	Stem 31 kDa glycoprotein	P10743.1	110	52	11	500	29.2/6.7	29.8/6.6	1.25	0.035	MS	ProtDes	Chlo
H18	Stem 31 kDa glycoprotein	NP_001241536.1	717	41	12	500	29.4/6.7	30.3/6.3	1.36	0.021	MS/MS	ProtDes	Chlo
H19	Ferredoxin–NADP reductase	XP_003520228.1	543	49	15	713	40.6/8.3	34.4/6.4	0.61	0.032	MS/MS	Ene	Chlo
H20a	Malate dehydrogenase	AAD56659.1	516	26	8	585	36.3/8.2	39.1/6.5	1.42	0.038	MS/MS	Met	Mito
H20b	Cytosolic malate dehydrogenase	NP_001236661.1	401	25	7	631	35.7/5.9	39.1/6.5	1.42	0.038	MS/MS	Met	Cyto
H21	Nucleoside diphosphate kinase 1	NP_001235398.1	147	47	6	295	16.5/6.3	21.3/6.9	1.94	0.030	MS/MS	SigTrans	Cyto
H22a	Thylakoid lumenal 29 kDa protein	XP_003519178.1	124	16	4	691	38.0/6.2	29.0/7.0	1.59	0.046	MS/MS	Dis/Def	Chlo
H22b	Carbonic anhydrase	XP_003553834.1	122	38	8	634	37.0/8.0	29.0/7.0	1.59	0.046	MS/MS	Met	Chlo
H23a	mRNA-binding protein	XP_003590649.1	240	36	7	629	43.8/6.5	42.1/7.2	1.35	0.039	MS/MS	Trans	Chlo
H23b	Phosphoglycerate kinase	XP_003546820.1	150	16	5	779	50.0/7.7	42.1/7.2	1.35	0.039	MS/MS	Ene	Chlo

Table 2 continued

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	M_r (kDa)/p*I*		FC[i]	*P* value[j]	MS[k]	Func.[l]	Local.[m]
							Theo.[g]	Exp.[h]					
H24	Gamma-glutamyl hydrolase	NP_001235549.1	473	47	16	619	38.2/6.7	34.0/7.7	1.92	0.024	MS/MS	Met	Chlo
H25	Glyceraldehyde-3-phosphate dehydrogenase	XP_003523131.1	486	46	13	640	36.9/7.1	40.3/8.0	1.80	0.048	MS/MS	Ene	Cyto
H26	Stem 28 kDa glycoprotein	NP_001238459.1	118	48	11	528	29.2/8.7	28.9/8.6	1.53	0.047	MS/MS	ProtDes	Vacu
H27	Gamma-glutamyl hydrolase	NP_001235549.1	365	41	15	619	38.2/6.7	33.7/8.8	0.61	0.042	MS/MS	Met	Chlo
H28	Catalase	NP_001240021.1	65	29	11	1005	55.2/6.5	56.6/8.1	1.68	0.023	MS	Dis/Def	Pero

[a] Spot number as given in Fig. 5a

[b] Accession number according to the NCBI database

[c] Ions score of identified protein using soybean genome sequence database

[d] Sequence coverage; proteins with <13 % sequence coverage were excluded from the result

[e] Number of query matched peptides; proteins with at least 2 matched peptides were considered

[f] The score of the high-scoring segment pair from that database sequence

[g] Theo. theoretical, M_r molecular weight, p*I* isoelectric point

[h] Exp. experimental, M_r molecular weight, p*I* isoelectric point

[i] Fold change. The protein spots showed a significant change in abundance compared to the control analyzed by Student *t* test

[j] *P* value indicates the significance of decrease or increase of abundance spots according to the student *t* test

[k] MS MALDI-TOF MS, MS/MS nanoLC MS/MS

[l] Func. function category using functional classification: ProtDes protein destination/storage, Met metabolism, Dis/Def disease/defense, Ene energy, Cell/Div cell growth/division, Sig/Trans signal transduction, Trans transcription

[m] Local. localization category using local classification, Chlo chloroplast, Mito mitochondria, Cyto cytoplasm, Vacu vacule, Pero peroxisome

Table 3 Differentially expressed proteins of soybean RIL CDH-80 leaf under cadmium stress

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	M_r (kDa)/pI		FC[i]	P value[j]	MS[k]	Func.[l]	Local.[m]
							Theo.[g]	Exp.[h]					
C01	Oxygen-evolving enhancer protein 2	P16059.1	329	25	7	440	28.3/6.9	26.6/4.7	0.68	0.043	MS/MS	Ene	Chlo
C02a	Tubulin beta-3 chain	XP_003546276.1	187	21	7	852	50.7/4.7	57.1/4.6	2.03	0.020	MS/MS	Cell/Div	Chlo
C02b	V-type proton ATPase subunit B 2	XP_003534485.1	183	27	9	966	54.2/4.9	57.1/4.6	2.03	0.020	MS/MS	Ene	Cyto
C03a	Stromal 70 kDa heat shock-related protein	XP_003548186.1	1021	45	32	1238	73.8/5.2	80.0/4.5	1.24	0.017	MS/MS	Dis/Def	Chlo
C03b	Acyl-peptide hydrolase	XP_003600065.1	524	32	13	1137	75.2/4.8	80.0/4.5	1.24	0.017	MS/MS	Met	Cyto
C04	RuBisCO large subunit-binding protein subunit alpha	XP_003538152.1	124	17	8	1003	61.7/5.2	64.6/4.5	1.92	0.002	MS/MS	Ene	Chlo
C05	NAD-dependent epimerase/dehydratase	NP_001148959.1	161	19	4	426	35.8/8.9	29.5/5.0	1.44	0.007	MS/MS	Met	Chlo
C06	PSII-P protein	BAE71271.1	422	25	7	420	28.5/7.6	26.4/5.0	0.66	0.042	MS/MS	Ene	Chlo
C07	Heat shock cognate 70 kDa protein	XP_003554320.1	130	34	16	1165	71.8/5.0	71.4/4.8	3.04	0.026	MS/MS	Dis/Def	Cyto
C08a	Cysteine synthase	XP_003554689.1	336	67	17	539	34.4/5.5	34.0/5.4	1.63	0.037	MS/MS	Met	Cyto
C08b	Oxygen-evolving enhancer protein 1	XP_003548008.1	113	46	9	562	35.0/6.4	34.0/5.4	1.63	0.037	MS/MS	Ene	Chlo
C09	ND	–	–	–	–	–	–	39.2/5.0	2.15	0.035	MS	–	–
C10	Glutamine synthetase	XP_003546121.1	192	37	10	824	47.9/6.4	43.7/5.2	1.68	0.044	MS/MS	Met	Chlo
C11a	RuBisCO large subunit-binding protein subunit beta	XP_003531529.1	753	60	25	1013	63.1/5.8	57.7/5.0	2.25	0.036	MS/MS	Ene	Chlo
C11b	Alanine aminotransferase 2	XP_003528736.1	293	26	11	957	60.4/6.3	57.7/5.0	2.25	0.036	MS/MS	Met	Mito
C12	V-type proton ATPase catalytic subunit A	XP_003531776.1	364	42	19	1219	69.0/5.4	66.6/5.4	1.73	0.035	MS/MS	Ene	Chlo
C13	Stem 28 kDa glycoprotein	NP_001238459.1	399	34	9	528	29.2/8.7	24.3/5.6	1.44	0.047	MS/MS	ProtDes	Vacu
C14a	Pro-hevein	XP_003554704.1	160	21	3	434	23.3/5.5	24.9/5.9	4.62	0.042	MS/MS	Dis/Def	Extr
C14b	Photosystem II 22 kDa protein	XP_003526631.1	114	24	5	323	28.9/6.4	24.9/5.9	4.62	0.042	MS/MS	Ene	Plas
C15	ND	–	–	–	–	–	–	22.8/5.8	0.53	0.036	MS	–	–
C16	L-Ascorbate peroxidase 2	NP_001235587.1	227	38	8	442	27.1/5.6	29.8/5.7	1.43	0.037	MS/MS	Dis/Def	Cyto
C17a	Ribulose-phosphate 3-epimerase	XP_003549685.1	268	27	5	519	30.0/7.7	27.7/5.8	0.48	0.037	MS/MS	Ene	Chlo

Table 3 continued

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	M_r (kDa)/pI		FC[i]	P value[j]	MS[k]	Func.[l]	Local.[m]
							Theo.[g]	Exp.[h]					
C17b	Triosephosphate isomerase	XP_003542557.1	170	25	6	530	32.7/6.2	27.7/5.8	0.48	0.037	MS/MS	Ene	Chlo
C17c	Carbonic anhydrase	XP_003553834.1	121	33	9	628	37.0/7.0	27.7/5.8	0.48	0.037	MS/MS	Met	Chlo
C18	Dihydroflavonol-4-reductase	XP_003538316.1	213	36	10	641	35.2/5.6	31.7/5.6	2.50	0.010	MS/MS	Met	Chlo
C19	Ribulose-phosphate 3-epimerase	XP_003524706.1	104	13	2	520	30.0/8.2	79.4/5.7	1.82	0.002	MS/MS	Ene	Chlo
C20	Aconitate hydratase 1	XP_003517155.1	183	22	15	1773	99.0/5.5	95.2/5.5	3.34	0.016	MS/MS	Ene	Cyto
C21	Metalloendoproteinase 1	NP_001235535.1	136	28	7	610	34.1/6.2	23.6/6.1	5.12	0.007	MS/MS	Met	Cyto
C22	Triosephosphate isomerase	ABA86966.1	70	38	7	379	27.4/6.6	28.1/6.1	1.14	0.030	MS	Ene	Cyto
C23	Ribulose bisphosphate carboxylase/ oxygenase activase	NP_001240245.1	183	21	8	818	48.8/6.2	38.1/6.0	2.91	0.002	MS/MS	Ene	Chlo
C24	Methionine synthase	NP_001235794.1	75	29	16	1491	84.4/5.9	77.4/6.3	2.28	0.044	MS	Met	Mito
C25	Elongation factor EF-2	XP_003596166.1	218	14	9	1625	94.9/5.8	91.9/6.2	3.93	0.034	MS/MS	ProtDes	Cyto
C26	Probable NAD(P)H-dependent oxidoreductase 1	XP_003548326.1	227	36	10	653	36.2/6.3	34.5/6.5	2.09	0.025	MS/MS	Ene	Cysk
C27	Gamma-glutamyl hydrolase	NP_001235549.1	164	18	6	619	38.2/6.7	33.2/6.5	0.68	0.034	MS/MS	Met	Chlo
C28	NAD dependent epimerase/dehydratase	XP_002512541.1	63	28	8	664	42.1/7.7	38.0/6.4	0.63	0.007	MS	Met	Chlo
C29	NAD dependent epimerase/dehydratase	XP_002512541.1	101	20	6	664	42.1/7.7	38.2/6.5	0.36	0.003	MS/MS	Met	Chlo
C30	Glyceraldehyde-3-phosphate dehydrogenase B subunit	NP_001237135.1	331	30	12	815	48.6/7.1	42.9/6.3	2.28	0.010	MS/MS	Ene	Chlo
C31	ND	–	–	–	–	–	–	44.9/6.7	3.64	0.012	MS	–	–
C32	Carbonic anhydrase	XP_003553834.1	152	34	8	628	37.0/7.0	28.6/7.4	0.62	0.045	MS/MS	Met	Chlo
C33	Lectin	ABW72645.1	305	25	9	520	30.0/5.9	29.2/7.3	2.28	0.022	MS/MS	ProtDes	Chlo
C34	Gamma-glutamyl hydrolase	NP_001235549.1	121	25	9	619	38.2/6.7	33.9/6.9	0.26	0.002	MS/MS	Met	Chlo
C35	Fructose-bisphosphate aldolase	XP_003521445.1	117	37	8	622	38.4/7.1	40.6/7.5	7.64	0.010	MS/MS	Ene	Cyto

Table 3 continued

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	M_r (kDa)/p*I*		FC[i]	*P* value[j]	MS[k]	Func.[l]	Local.[m]
							Theo.[g]	Exp.[h]					
C36	Glyceraldehyde-3-phosphate dehydrogenase	XP_003523131.1	486	46	13	640	36.93/7.1	38.3/7.2	1.42	0.041	MS/MS	Ene	Cyto
C37a	Fructose-bisphosphate aldolase	NP_001237315.1	149	37	7	664	38.5/6.7	38.5/6.9	2.31	0.011	MS/MS	Ene	Chlo
C37b	Glyceraldehyde-3-phosphate dehydrogenase	NP_001236129.1	111	13	3	640	36.8/6.7	38.5/6.9	2.31	0.011	MS/MS	Ene	Cyto
C38	NADP- dependent GA3PDH	XP_003549550.1	223	19	6	957	53.8/6.7	53.2/6.9	1.82	0.007	MS/MS	Ene	Cyto
C39	ND	–	–	–	–	–	–	35.4/8.98	7.11	0.002	MS	–	–
C40	ND	–	–	–	–	–	–	35.9/9.12	0.65	0.014	MS	–	–
C41	Subtilase family protein	NP_566888.2	625	49	26	610	79.9/8.9	65.4/9.0	2.92	0.011	MS/MS	Met	Chlo

[a] Spot number as given in Fig. 5b

[b] Accession number according to the NCBI database

[c] Ions score of identified protein using soybean genome sequence database

[d] Sequence coverage; proteins with <13 % sequence coverage were excluded from the result

[e] Number of query matched peptides; proteins with at least two matched peptides were considered

[f] The score of the high-scoring segment pair from that database sequence

[g] Theo. theoretical, M_r molecular weight, p*I* isoelectric point

[h] Exp. experimental, M_r molecular weight, p*I* isoelectric point

[i] Fold change. The protein spots showed a significant change in abundance compared to the control analyzed by Student *t* test

[j] *P* value indicates the significance of decrease or increase of abundance spots according to the Student *t* test

[k] MS MALDI-TOF MS, MS/MS nanoLC MS/MS

[l] Func. function category using functional classification: ProtDes protein destination/storage, Met metabolism, Dis/Def disease/defense, Ene energy, Cell/Div cell growth/division

[m] Local. localization category using local classification, Chlo chloroplast, Mito mitochondria, Cyto cytoplasm, Vacu vacule, Cysk cytoskeleton, Extr extra cellular, Plas plasma membrane

NADP-dependent GA3PDH, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase

Table 4 Differentially expressed proteins of soybean cv. Fukuyutaka leaf under cadmium stress

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	M.P.[e]	Blast score[f]	M_r (kDa)/p*I*		FC[i]	*P* value[j]	MS[k]	Func.[l]	Local.[m]
							Theo.[g]	Exp.[h]					
F01	Gamma-glutamyl hydrolase	NP_001235549.1	126	18	4	619	38.2/6.7	29.6/4.5	0.64	0.044	MS/MS	Met	Chlo
F02	Cu/Zn superoxide dismutase II	CAA39819.1	200	47	5	312	20.9/6.0	22.7/5.0	1.51	0.046	MS/MS	Dis/Def	Chlo
F03	Oxygen-evolving enhancer protein 2	P16059.1	329	25	7	440	28.3/6.9	28.4/4.7	1.50	0.028	MS/MS	Ene	Chlo
F04	Heat shock cognate 70 kDa protein	XP_003554320.1	130	34	16	1165	71.8/5.0	72.2/4.7	0.59	0.043	MS/MS	Dis/Def	Cyto
F05	Oxygen-evolving enhancer protein 2	P16059.1	259	32	10	393	28.1/7.6	27.9/5.4	1.94	0.014	MS/MS	Ene	Chlo
F06	PSII-P protein	BAE71271.1	422	25	7	420	28.5/7.6	28.2/5.2	1.77	0.037	MS/MS	Ene	Chlo
F07a	20 kDa chaperonin	XP_003609971.1	149	57	11	378	26.6/6.7	28.6/5.1	1.42	0.026	MS/MS	Dis/Def	Chlo
F07b	Triosephosphate isomerase	XP_002529248.1	107	14	4	509	33.3/6.3	28.6/5.1	1.42	0.026	MS/MS	Ene	Chlo
F08	Oxygen-evolving enhancer protein 1	XP_003548008.1	178	46	12	578	34.8/6.0	33.6/5.1	2.11	0.018	MS/MS	Ene	Chlo
F09	Glutamine synthetase	XP_003546121.1	192	37	10	824	47.9/6.4	44.4/5.3	1.66	0.049	MS/MS	Met	Chlo
F10a	Enolase	NP_001237329.1	338	32	8	873	47.9/5.4	55.2/5.3	0.52	0.043	MS/MS	Met	Cyto
F10b	Granule-bound starch synthase	NP_001236366.1	146	14	7	1154	68.5/6.6	55.2/5.3	0.52	0.043	MS/MS	Met	Chlo
F11	Transketolase	XP_003592793.1	290	26	13	1274	80.5/6.0	72.1/5.7	0.57	0.035	MS/MS	Ene	Chlo
F12	Triosephosphate isomerase	ABA86966.1	70	38	7	379	27.4/6.6	28.7/6.2	1.49	0.040	MS	Ene	Cyto
F13	Stem 31 kDa glycoprotein	NP_001241536.1	717	41	12	500	29.4/6.7	29.9/6.0	0.62	0.014	MS/MS	ProtDes	Chlo
F14	Ribulose bisphosphate carboxylase/oxygenase activase	NP_001240245.1	183	21	8	818	48.8/6.2	36.9/6.1	0.61	0.041	MS/MS	Ene	Chlo
F15a	Thylakoid lumenal 29 kDa protein	XP_003519178.1	124	16	4	691	38.0/6.2	29.0/6.5	1.31	0.049	MS/MS	Dis/Def	Chlo
F15b	Carbonic anhydrase	XP_003553834.1	122	38	8	634	37.0/8.0	29.0/6.5	1.31	0.049	MS/MS	Met	Chlo
F16	NAD dependent epimerase/ dehydratase	XP_002512541.1	63	28	8	664	42.1/7.7	37.4/6.5	1.84	0.035	MS	Met	Chlo
F17	Carbonic anhydrase	ACZ74707.1	72	43	8	501	28.3/6.1	28.8/6.9	1.38	0.029	MS	Met	Chlo
F18	Gamma-glutamyl hydrolase	NP_001235549.1	473	47	16	619	38.2/6.7	33.4/7.0	1.68	0.007	MS/MS	Met	Chlo
F19	Photosystem I reaction center subunit IV A	XP_003625462.1	123	38	4	147	13.7/9.6	23.6/8.1	1.45	0.023	MS/MS	Ene	Chlo
F20a	Serine hydroxymethyltransferase 5	NP_001237509.1	236	28	15	1011	57.4/8.6	51.1/7.6	1.63	0.042	MS/MS	Ene	Mito

Table 4 continued

Sp. no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	M.P.[e]	Blast score[f]	M_r (kDa)/pI		FC[i]	P value[j]	MS[k]	Func.[l]	Local.[m]
							Theo.[g]	Exp.[h]					
F20b	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	XP_003519141.1	230	18	6	963	53.9/6.7	51.1/7.6	1.63	0.042	MS/MS	Ene	Cyto
F21	Photosystem I subunit PsaD	NP_001235355.1	361	52	10	366	23.0/9.6	26.3/8.9	1.89	0.020	MS/MS	Ene	Chlo
F22	Stem 28 kDa glycoprotein	NP_001238459.1	364	37	10	528	29.2/8.7	28.9/8.6	1.59	0.031	MS/MS	ProtDes	Vacu

[a] Spot number as given in Fig. 5c

[b] Accession number according to the NCBI database

[c] Ions score of identified protein using soybean genome sequence database

[d] Sequence coverage; proteins with <13 % sequence coverage were excluded from the result

[e] Number of query matched peptides; proteins with at least two matched peptides were considered

[f] The score of the high-scoring segment pair from that database sequence

[g] Theo. theoretical, M_r molecular weight, pI isoelectric point

[h] Exp. experimental, M_r molecular weight, pI isoelectric point

[i] Fold change. The protein spots showed a significant change in abundance compared to the control analyzed by Student t test

[j] P value indicates the significance of decrease or increase of abundance spots according to the Student t test

[k] MS MALDI-TOF MS, MS/MS nanoLC MS/MS

[l] Func. function category using functional classification: ProtDes protein destination/storage, Met metabolism, Dis/Def disease/defense, Ene energy

[m] Local. localization category using local classification, Chlo chloroplast, Mito mitochondria, Cyto cytoplasm, Vacu vacular

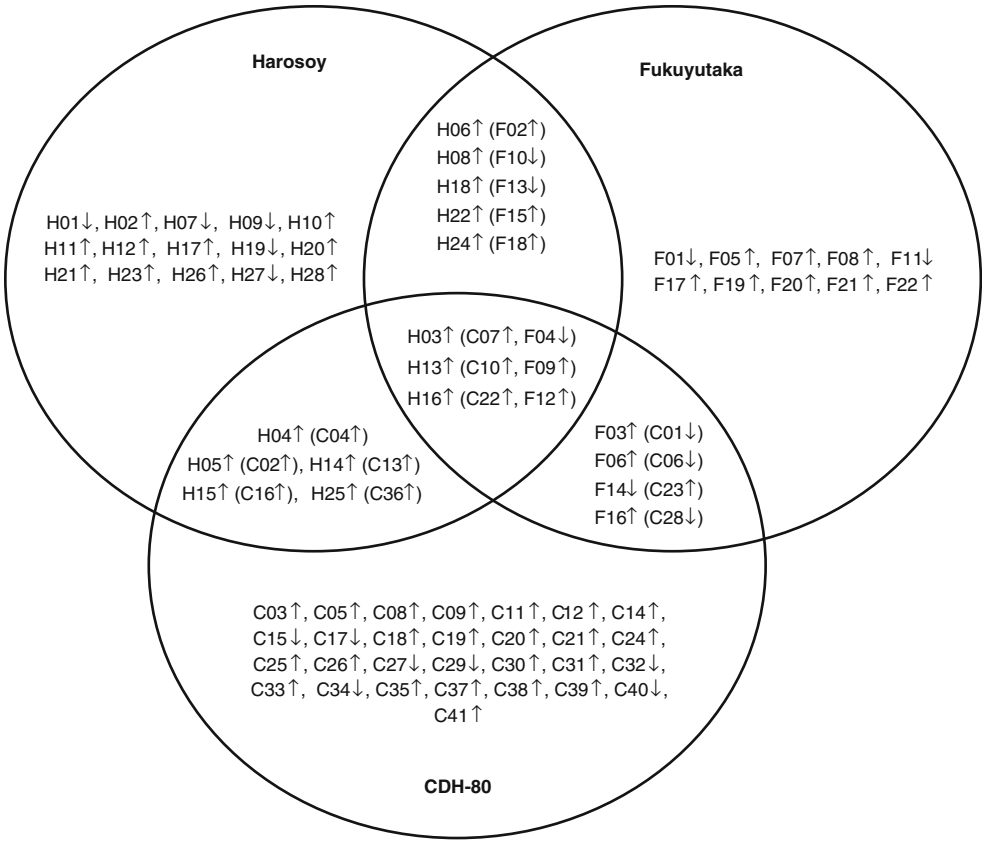


Fig. 7 *Venn diagram* showing differential expression of leaf proteins of Harosoy, CDH-80 and Fukuyutaka under cadmium stress. *Numbers* correspond to the protein spots present in the 2-DE patterns of Harosoy (H), CDH-80 (C) and Fukuyutaka (F). The *overlapped portions* represent commonly expressed protein spots. *Upward* and *downward arrows* denote increased or decreased protein expression under cadmium stress

Total 7 proteins were identified from the 5 commonly expressed protein spots between Harosoy and Fukuyutaka. Cadmium stress induced enhanced expression of Cu/Zn superoxide dismutase (SOD) II (H06 and F02), gamma-glutamyl hydrolase (H24 and F18), thylakoid lumenal 29 kDa protein and carbonic anhydrase (H22a,b and F15a,b) were detected in both Harosoy and Fukuyutaka (Tables 2, 4). Enhanced expressions of stem 31 kDa glycoprotein (H18), enolase (H08a) and granule bound starch synthase (H08b) were detected in high cadmium accumulating cultivar Harosoy, whereas their (F13 and F10) expressions were decreased in Fukuyataka under cadmium stress (Tables 2, 4).

Proteins commonly expressed in leaves of Fukuyutaka and CDH-80 under cadmium stress

Under cadmium stress, 4 out of 56 protein spots were commonly expressed in both Fukuyutaka and CDH-80 (Fig. 7). Among them, expressions of 3 protein spots (F03, F06 and F16) were increased in cadmium stressed Fukuyutaka, while those (C01, C06 and C28) were decreased in CDH-80. However, expressions of protein spots (C23 and F14) were increased and decreased in CDH-80 and Fukuyutaka, respectively, (Fig. 7). Ten protein spots were detected as cultivar-specific cadmium stress responsive proteins in Fukuyutaka (Fig. 7).

Functional analysis and subcellular localization of identified proteins

The identified proteins were grouped into functional classes based on their presumed biological function, as described by Bevan et al. (1998). In both high and low cadmium accumulating cultivars (Harosoy and Fukuyutaka, respectively) and their RIL CDH-80, majority of the differentially expressed identified proteins were grouped into functional categories of metabolism and energy (Fig. 8a). Out of the 32, 44 and 26 identified proteins 11 (34.3 %), 15 (34.0 %) and 8 (30.7 %) proteins were involved in the cellular metabolism of Harosoy, CDH-80 and Fukuyutaka, respectively. However, energy related

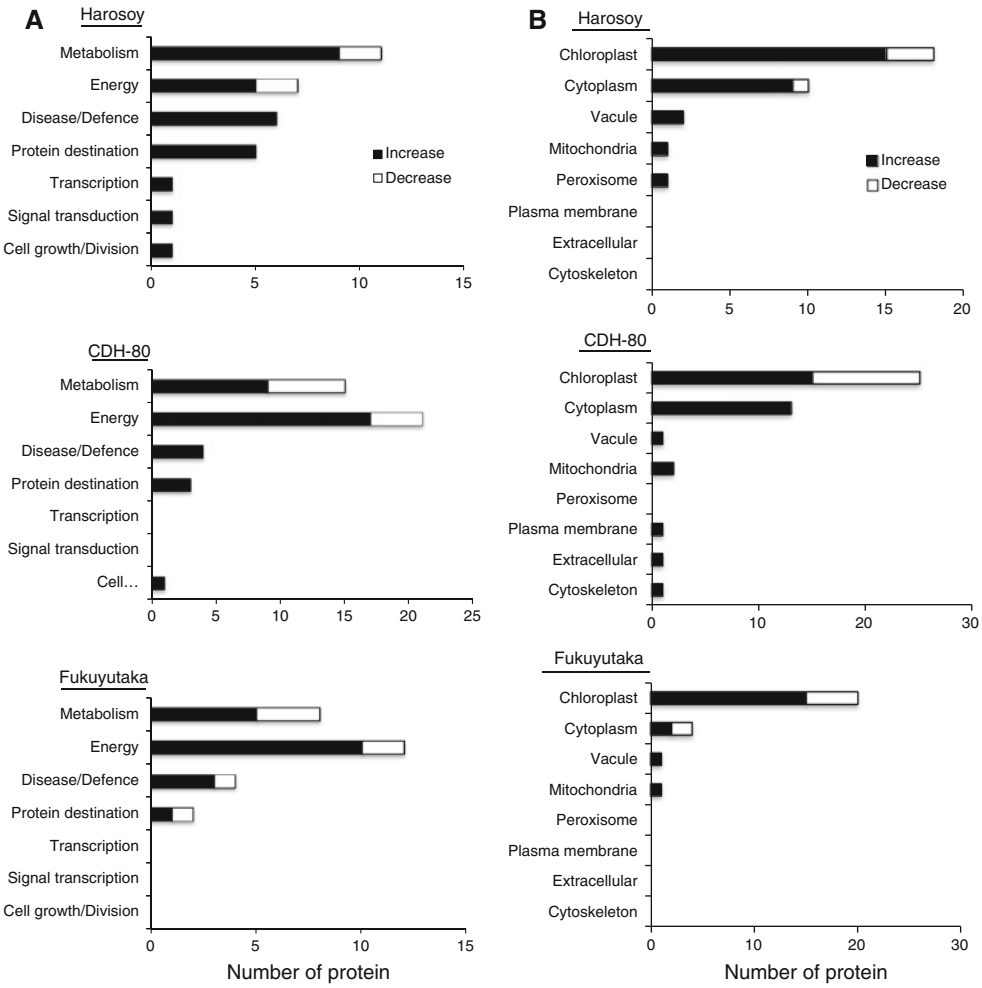


Fig. 8 Classification of proteins that were differentially expressed in soybean leaves under cadmium stress. For cadmium exposed Harosoy, CDH-80 and Fukuyutaka, the functions of differentially expressed proteins (**a**) and subcellular localization (**b**) were determined. *Black* and *white bars* represent increased and decreased protein expressions, respectively

proteins were much abundant in CDH-80 (47.7 %) and Fukuyutaka (46.1 %) as compared to Harosoy (21.8 %). Differential expressions of plant disease resistance/defense related proteins were higher in Harosoy (18.7 %) as compared to Fukuyutaka (15.3 %) and CDH-80 (9.0 %). Moreover, proteins associated with signal transduction and transcription were only detected in Harosoy (Fig. 8a).

All the identified proteins were analyzed with WoLF PSORT, ESLpred, and SubLoc prediction to predict their subcellular localization. Majority of the cadmium stress-responsive proteins were predicted to be chloroplastic in localization (Fig. 8b). As compared to Fukuyutaka, cytoplasmic proteins were much abundant in Harosoy and CDH-80. Among Harosoy, CDH-80 and Fukuyutaka, chloroplastic localized proteins were primarily increased. In both Harosoy and CDH-80, increased abundance of cytoplasmic localized proteins was detected, while in Fukuyutaka, only four proteins were related to cytoplasm (Fig. 8b). The other low abundant proteins were predicted to be localized in vacuole, mitochondria, peroxisome, plasma membrane, extracellular and cytoskeleton.

Discussion

Cadmium had significant effects on root growth in all studied soybean cultivars. However, the magnitude of decrease in root length and weight was much higher in Fukuyutaka as compared to Harosoy and CDH-80. Low root-to-shoot Cd^{2+} translocation in Fukuyutaka might be responsible for higher accumulation of cadmium in their root part. This could be the probable reason of much decreased root biomass under cadmium stress. Among the differentially expressed root proteins, NADP-dependent alkenal double bond reductase P1 was much abundant in low cadmium accumulating Fukuyutaka as compared to high cadmium accumulating Harosoy and RIL CDH-80. This protein plays a distinct role in plant antioxidant defense and is possibly involved in NAD(P)/NAD(P)H homeostasis (Mano et al. 2005). Higher expression of NADP-dependent reductase might help Fukuyutaka in better scavenging the reactive oxygen species (ROS) and thus protects the cells. Although no symptoms of cadmium leaf injury was detected after 3 days of stress, however, it is assumed that leaf had already sensed the stress at

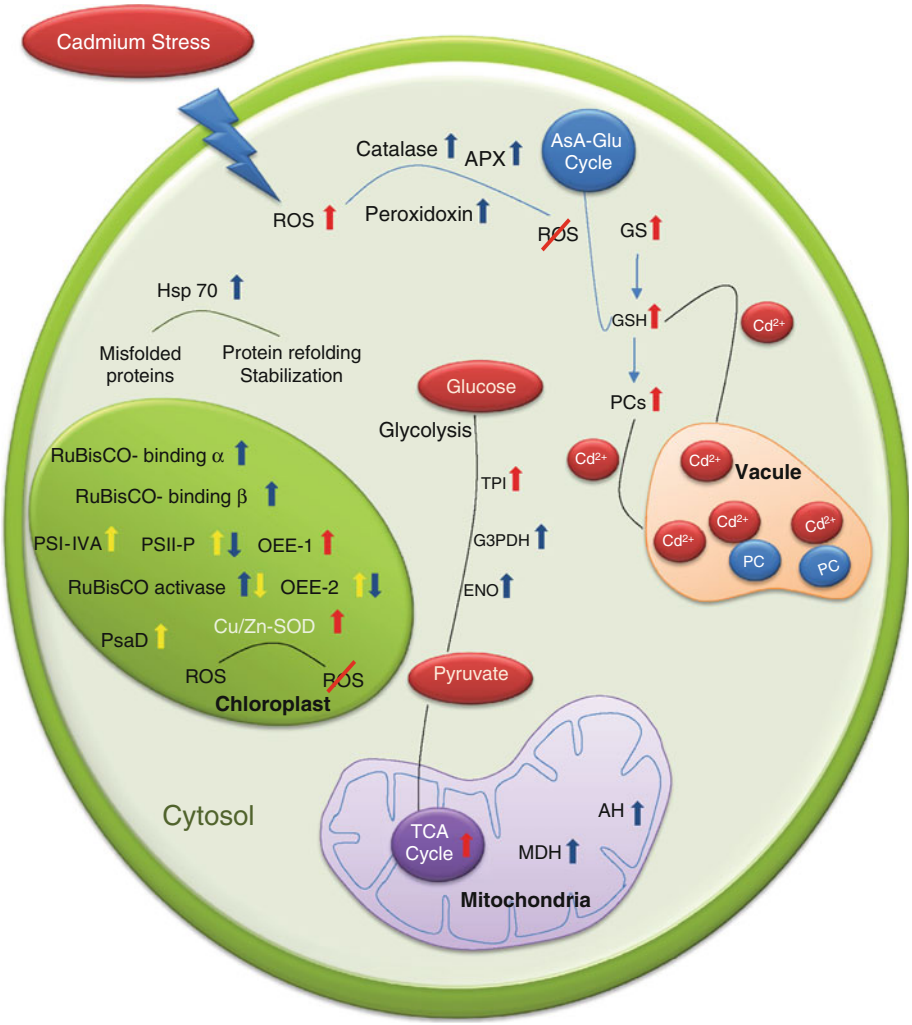


Fig. 9 Proposed model showing various cellular mechanisms operating in soybean leaf to mitigate cadmium stress. The scheme is based on the obtained proteomic results of present investigation. Only those functional proteins/enzymes, whose expressions were changed under cadmium stress, are presented here. *Upward* and *downward arrows* indicate increased and decreased expression of the concerned protein, respectively. *Red arrows* denote the commonly expressed proteins among Harosoy, CDH-80 and Fukuyutaka, while *blue arrows* depict proteins expressed in high cadmium accumulating Harosoy and/or CDH-80. *Yellow arrows* indicate proteins expressed in low cadmium accumulating cultivar Fukuyutaka. *TPI* triosephosphate isomerase, *G3PDH* glyceraldehydes-3-phosphate dehydrogenase, *ENO* enolase, *TCA* tricarboxylic acid, *MDH* malate dehydrogenase, *AH* aconitate hydratase, *GS* glutamine synthetase, *GSH* glutathione, *PCs* phytochelatins, *AsA-Glu* ascorbate glutathione, *ROS* reactive oxygen species, *APX* ascorbate peroxidase, *OEE* oxygen-evolving enhancer protein, *PS* photosystem (Color figure online)

molecular level and subsequently activated their defense mechanisms as evident from the present proteomic study.

Dysfunction of protein is a common consequence of abiotic stress. Molecular chaperones/heat-shock proteins are responsible for proper protein folding, assembly and translocation (Wang et al. 2004). Cadmium often causes disturbances in the cellular metabolism by inactivating vital enzymes and protein function. Under stressed condition, heat-shock proteins help in refolding and stabilization of misfolded proteins (Vierling 1991; Wang et al. 2004). Thus, they play a key role in combating stress by re-establishing normal protein conformation and hence cellular homeostasis. Heat-shock protein 70 is generally induced in response to various heavy metal stresses including cadmium (Rodriguez-Celma et al. 2010; Hradilová et al. 2010; Kieffer et al. 2009a; Ahsan et al. 2007; Sarry et al. 2006). In high cadmium accumulating cultivar Harosoy and RIL CDH-80, increased expression of heat-shock protein 70 (more than twofold) was detected under cadmium stress, while low cadmium accumulating cultivar Fukuyutaka exhibited decreased expression (Tables 2, 3, 4; Fig. 7). This enhanced expression might help Harosoy and CDH-80 to stabilize their protein structure and function, thus maintain cellular homeostasis.

One of the important strategies to detoxify heavy metal within the plant cell is to synthesize specific low molecular

weight chelators to minimize the bindings of metal ions to functionally important proteins (Verbruggen et al. 2009). Phytochelatins (PCs), synthesized from glutathione (GSH) by the enzyme PC synthase readily form complexes with cadmium in the cytosol and to facilitate their transport into vacuoles (Grill et al. 1989). In the present proteomic study, under cadmium stress glutamine synthetase (GS) was commonly expressed in Harosoy, CDH-80 and Fukuyutaka (Tables 2, 3, 4; Fig. 7). Although, increased expressions were evident in all, Harosoy exhibited maximum expression (1.9-fold) in response to cadmium. The enzyme GS is involved in the synthesis of GSH through glutamate biosynthesis pathway (Semane et al. 2010; Sarry et al. 2006). Thus, the enhanced expression of GS leads to more GSH formation. Up regulation of GSH synthesis implies higher metal binding capacity as well as enhanced cellular defense mechanism against oxidative stress (Verbruggen et al. 2009). Since GSH is the precursor of PC, enhanced expression of GS also helps the cell to synthesize and accumulate more PC to detoxify cytosolic Cd^{2+}. This result is in agreement with previous findings of up regulation of GS in response to cadmium (Ahsan et al. 2012; Semane et al. 2010; Hradilová et al. 2010; Kieffer et al. 2008). Although Harosoy, CDH-80 and Fukuyutaka differ in cadmium accumulation, but they share this common strategy to immediately synthesize GSH and PCs to minimize cadmium induced cellular damages.

To maintain the normal growth and development under stressed environment, plants need to up regulate metabolic pathways such as glycolysis and tricarboxylic acid (TCA) cycle. Triosephosphate isomerase, one of the key enzymes of glycolysis that catalyzes the inter-conversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, play an essential role in efficient energy production. In this study, Harosoy, CDH-80 and Fukuyutaka all exhibited increased abundance of triosephosphate isomerase under cadmium stress. Moreover, higher abundance of another important glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase was detected in cadmium challenged leaves of Harosoy and CDH-80. The function of glyceraldehydes-3-phosphate dehydrogenase is to convert glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate, thus in turn helps in glucose breakdown to generate energy. Interestingly, expression of enolase, the metalloenzyme that catalyzes penultimate step of glycolysis- conversion of 2-phosphoglycerate to phosphoenolpyruvate, was increased in high cadmium accumulating cultivar Harosoy, while decreased in low cadmium accumulating Fukuyutaka. Previous proteomic study in poplar plants subjected to cadmium stress revealed over expression of triosephosphate isomerase, glyceraldehydes-3-phosphate dehydrogenase, and enolase (Kieffer et al. 2009a). Under cadmium stress enzymes of TCA cycles, such as malate dehydrogenase and aconitate hydratase were also observed to be up regulated in Harosoy and CDH-80, respectively. Up regulation of glycolysis and subsequent TCA cycle might help the plant to produce more reducing power to compensate high-energy demand of cadmium challenged cell.

Excess generation of ROS is an inevitable outcome of any abiotic stresses including cadmium toxicity. Cadmium instead of being a non-redox-active metal could able to exacerbate the basal ROS level. Cadmium is believed to inactivate or down regulate the ROS scavenging antioxidant enzymes (Kieffer et al. 2008; Sanita di Toppi and Gabrielli 1999). The excess intracellular ROS level alters protein structure by inducing oxidation of both protein backbone and amino acid side chain residues (Villiers et al. 2011). Among the cadmium induced defense related proteins, enhanced expressions of antioxidant enzymes namely ascorbate peroxidase, catalase and Cu/Zn superoxide dismutase II were evident in the present investigation. The primary function of ascorbate peroxidase and catalase is to decompose peroxides and hence protecting cell membrane from hydroxyl radical induced lipid peroxidation (Barber and Thomas 1978). SOD is another important antioxidant enzyme of ascorbate GSH cycle and potent quencher of superoxide radicals. Increased ascorbate peroxidase expression was detected in leaves of cadmium challenged Harosoy and CDH-80, while Harosoy and Fukuyutaka exhibited higher abundance of superoxide dismutase (Fig. 7; Tables 2, 3, 4). In addition, expressions of peroxiredoxin and thylakoid lumenal 29 kDa protein were found to be increased in Harosoy. Peroxiredoxins are basically thiol peroxidase that detoxify peroxides and also play essential role in enzyme activation and redox sensoring (Dietz 2003; Barranco-Medina et al. 2009). High abundance of peroxiredoxins was reported in cadmium exposed *Populus* (Durand et al. 2010) and rice (Ahsan et al. 2007). However, proteomic study on cadmium-induced changes in root proteins of *Brassica juncea* revealed significant decreases in ascorbate peroxidase and Cu/Zn SOD protein abundance (Alvarez et al. 2009). In our study, among the contrasting cadmium accumulating cultivars abundance of antioxidant enzymes was much higher in Harosoy. These increased expressions of antioxidant enzymes might help them to cope with the cadmium induced oxidative stress.

Down regulation of photosynthetic machinery is a known phenomenon of cadmium stress. Low abundance of proteins involved in photosynthetic electron transport chain and Calvin cycle has been reported in cadmium exposed *Populus* (Durand et al. 2010; Kieffer et al. 2008, 2009a) and *Thlaspi* (Tuomainen et al. 2006). However, in the present study, increases in abundance of RuBisCO large subunit-binding protein subunit alpha and beta, RuBisCO activase, oxygen-evolving enhancer protein 1 and 2,

NAD(P)H-dependent oxidoreductase, photosystem I and II related proteins were evident. Enhanced expressions of proteins involved in photosystem I, II and Calvin cycle might be an adaptive feature to overcome the cadmium injury. In other words, 3 days of cadmium stress may not be that lethal to affect the photosynthesis in these studied cultivars. Semane et al. (2010) also reported increase of photosynthetic protein abundance in leaves of *Arabidopsis thaliana* treated with mild cadmium stress. In our opinion, contribution of high photosynthetic assimilates into respiration would help plants to yield more energy needed to combat the cadmium stress.

Based on the obtained results and above discussion, a scheme has been proposed to elucidate the various mechanisms operating at cellular level to nullify cadmium stress effects in soybean leaf (Fig. 9). In summary, both high and low cadmium accumulating soybean cultivars although experiences different levels of stress in the aerial part because of the differential root-to-shoot Cd^{2+} translocation, however, they share the following common defense strategies to cope with the stress: (1) high abundance of enzymes involved in glycolysis and TCA cycle to produce more energy necessary to meet the high energy demand of cadmium challenged cells, (2) increased synthesis of GSH and PCs to detoxify cytosolic Cd^{2+} and subsequent compartmentalization into vacuoles, (3) increased expressions of proteins involved in photosynthetic electron transport chain and Calvin cycle for balancing photosynthetic activity with quick utilization of photoassimilates in energy generation, (4) increased abundance of antioxidant enzymes to scavenge the excess ROS, which in turn protects the cellular components from oxidative stress damages. Enhanced expression of molecular chaperones in high cadmium accumulating soybean cultivars is another additional defense mechanism for refolding of misfolded proteins and to stabilize protein structure and function, thus maintain cellular homeostasis.

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Conflict of interest The authors declare that they have no conflict of interest.

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