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Genotypic dependent effect of exogenous glutathione on Cd-induced changes in proteins, ultrastructure and antioxidant defense enzymes in rice seedlings

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ABSTRACT

Greenhouse hydroponic experiments were conducted using Cd-sensitive (*cv.* Xiushui63) and tolerant (Bing97252) rice genotypes to evaluate how different genotypes responded to Cd toxicity in presence of glutathione (GSH). Results showed that GSH alleviates Cd-toxicity, ameliorates Cd-induced damages on leaf/root ultrastructures. Nine proteins in roots were identified, using 2-DE coupled with mass spectrometry, whose expression were down-regulated in Xiushui63, up-regulated/unchanged in Bing97252 by Cd; coinstantaneously enhanced/unchanged in Cd + GSH over Cd alone treatment in both genotypes. They are L-ascorbate peroxidase, putative short-chain dehydrogenase/reductase, Glycolipid transfer protein, elongation factor, Os04g0652700, carbonic anhydrase, Os08g0374000, chitinase, and putative disease resistance response protein. Eight proteins in leaves with expression of increase in Bing97252 but down-regulate/unchange in Xiushui63, categorized as four groups of their functions: carbon metabolism, TCA cycle, photorespiration and RNA processing. Furthermore, we identified eight proteins with repressed expression in Cd-treated and up-regulated in Cd + GSH-treated rice leaves of Xiushui63.

1. Introduction

Cadmium (Cd), widely recognized as a very dangerous environmental pollutant, principally occurs in human diet as a result of its uptake and accumulation from soil by food crops, although the whole pathway of Cd transfer into the food chain is also involved in input from atmosphere, water and aquatic life. Rice, a staple food crop for Japanese, was estimated to represent 36–50% of the total oral intake of Cd for Japanese population during 1998–2001 [1]. Correspondingly it is urgently necessary to elucidate the mechanism of rice in Cd accumulation/tolerance to develop approaches for preventing its accumulation in plants so as to alleviate health risks associated with exposure to highly Cd-contented food.

Reduced glutathione (γ-Glu–Cys–Gly, GSH), a unique redox and nucleophilic properties, is involved in the cellular defense against the toxic action of xenobiotics, oxyradicals, salinity, acidity and as well as metal cations [2]. GSH is also the direct substrate for the synthesis of phytochelatins (PCs). It has been suggested that GSH acts as a first defense line against metal toxicity through complexing metals before induced synthesis of PCs reaches to an effective level. Concerning cellular GSH levels under Cd stress, quite different results have been reported. Cellular GSH content has been reported to decrease [3] or to increase [4]. Our previous study revealed that GSH significantly alleviated Cd induced growth inhibition, which is related to significant improvement in chlorophyll content, photosynthetic performance and root GSH levels [5]. However, so far there is no study on genotypic difference in the effect of extracellular GSH on cellular responses concerning the changes in protein pattern and ultrastructure of rice plants under Cd stress. Thus further studies is needed to test the hypothesis that external GSH could act as a regulator or antioxidant intervention strategy in preventing Cd toxicity, and to get a better understanding of how plants adjust to an adverse environment in proteomic and ultrastructure levels. The present study reports genotypic difference in Cd-induced changes in protein pattern, antioxidative metabolism and ultrastructure, and the role of GSH in Cd tolerance using two rice genotypes varying in Cd tolerance and accumulation. These results would be useful to understand the mechanisms of Cd tolerance/accumulation in rice, and open novel prospective for the improvement of Cd tolerance in plants.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; γGT, γ-glutamyl transpeptidase; ACN, acetonitrile; APN, aminopeptidase N; APX, ascorbate peroxidase; CA, carbonic anhydrase; CAT, catalase; Cd, cadmium; CHCA, α-cyano-4-hydroxycinnamic acid; DLD, dihydrolipoamide dehydrogenase; EDG, electron dense granules; FTL, formate-tetrahydrofolate ligase; GLTP, Glycolipid transfer protein; Gly, glycine; GSH, reduced glutathione; HPR, hydroxypyruvate reductase; HSP, heat shock protein; IEF, isoelectrofocusing; PCs, phytochelatins; PEP, phophoenolpyruvate; POD, peroxidase; ROS, reactive oxidative species; Ser, serine; SOD, superoxide dismutase; TEM, transmission electron microscopy; TFA, trifluoroacetic acid.

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2. Materials and methods

2.1. Plant material and experimental design

Hydroponic experiment was carried out on Huajiachi Campus, Zhejiang University, Hangzhou, China. Two *Japonica* unwaxy rice genotypes were used: Bing97252 and Xiushui63 of relatively Cd-tolerant/low grain accumulation and sensitive/high accumulation, respectively [5].

Healthy seeds were surface sterilized by soaking in 1.5% H_2O_2 for 30 min, fully rinsed by deionized water. After soaked in deionized water at room temperature for 2 d, the seeds were germinated for 1 d at 35 °C. The germinated seeds were sowed in sterilized moist sand in an incubator at 30 °C-day/23 °C-night under 80% relative humidity. At the 2nd leaf stage (10 d old), the uniform healthy plants were selected and transplanted to 5-L plastic containers filled up with basal nutrient solution [5]. The container was covered with a polystyrol-plate with 7 evenly spaced holes (2 plants per hole) and placed in a greenhouse. On the 7th day after transplanting, Cd (as $CdCl_2$) and GSH were added to the corresponding containers to form 3 treatments: basal nutrient solution (control), 5 μM Cd (Cd), 5 μM Cd + 50 μM GSH (Cd + GSH). The experiment was laid in a split-plot design with treatment as the main plot and genotype as the sub-plot with seven replicates. The nutrient solution pH was adjusted to 5.1 ± 0.1 with NaOH or HCl as required, and renewed every 5 d. Samples for enzymatic analysis were taken every 5 d. Proteomic analysis and ultrastructure observation were carried after 15 d Cd exposure.

2.2. Observation of leaf and root ultrastructure

Fresh leaf fragments without veins (about 1 mm^2) and root tips of randomly selected plants were fixed overnight with 2.5% glutaraldehyde (v/v) in 0.1 M PBS (sodium phosphate buffer, pH 7.0) and washed three times with the same buffer. The samples were post fixed in 1% OsO_4 in the same PBS for 1 h. After fixing, the samples were dehydrated in a graded series of ethanol (50%, 70%, 80%, 90%, 95% and 100%) with 15 min interval and finally transferred to absolute acetone for 20 min. The samples were then infiltrated in 1:1 acetone-Spurr's resin mixture (v:v), then transferred to 1:3 mixture of acetone-Spurr's resin for 3 h, and finally to Spurr's resin in capsule overnight. After heating the specimens at 70 °C for 9 h, the specimen sections were stained by uranyl acetate and alkaline lead citrate for 15 min respectively and observed using a transmission electron microscopy (TEM, JEOL JEM-1230, Japan).

2.3. Proteome analysis

2.3.1. Protein extraction and two-dimensional gel electrophoresis analysis

Total protein extracts were prepared essentially according to the phenol extraction method described by Carpentier et al. [6] with minor modification [7]. Protein concentration was determined by standard Bradford assay using bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA).

Proteins were separated by two-dimensional gel electrophoresis (2-DE) [6,7], and the protein spots in analytical gels were visualized by silver staining [7]. For each sample, at least two independent protein extract each treatment and at least three 2-DE analyses each protein extract were performed. All chemicals used were, if not further specified in the text, p.a. or electrophoresis grade. All electrophoresis units employed were from Amersham Biosciences.

2.3.2. Protein visualization, image analysis, and quantification

Stained gels were scanned and calibrated using a PowerLook1100 scanner (UMAX), followed by analysis of protein spots using GE HealthCare Software (Amersham Biosciences). The spots were quantified using the % volume criterion. Only those with significant and reproducible changes ($P < 0.05$) were considered to be differentially accumulated proteins. The target protein spots were automatically excised from the stained gels and digested with trypsin using a Spot Handling Workstation (Amersham Biosciences). Peptides gel pieces were placed into the EP tube and washed with 1:1 mixture of 50 μL of 30 mM $K_3Fe(CN)_6$ and 100 mM NaS_2O_3 for 10–15 min until completely discolored then washed with 200 μL bi-distilled water (two times for 5 min each). The washed solution was drained and washed with 50% ACN (acetonitrile, Fisher A/0626/17) and 100% ACN rotationally, and then incubated in 25 mM NH_4HCO_3 (Sigma A6141) for 5 min at 37 °C. After absorbed out of the incubation solvent, 50% ACN and 100% ACN was rotationally added and dried at 40 °C for 5 min respectively. Trypsin digestion was carried out as follows: sequencing-grade porcine trypsin (Promega, Madison, WI, USA) was suspended in 25 mM NH_4HCO_3 at a concentration of 12.5 ng/μL to rehydrate the dried gel pieces. The trypsin digestion was carried out for 16 h at 37 °C. Peptides were extracted from the digest as follows for three times: 10 μL of 50% ACN containing 0.1% TFA (trifluoroacetic acid, GE HealthCare) was added to each tube and incubated for 5 min at 37 °C and transferred the supernatants to new EP tube. The extracts were pooled and then vacuum concentrated for about 2 h. A solution of peptides was filtrated via Millipore (Millipore ZTC18M096) and mixed with the same volume of a matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA. After the peptides were co-crystallized with CHCA by evaporating organic solvents, tryptic-digested peptide masses were measured using a MALDI-TOF–TOF mass spectrometer (ABI4700 System, USA). All mass spectra were recorded in positive reflector mode and generated by accumulating data from 1000 laser shots. The following threshold criteria and settings were used: detected mass range of 700–3200 Da (optimal resolution for the quality of 1500 Da), using a standard peptide mixture (des-Argl-Bradykinin Mr904.468, Angiotensin I Mr1296.685, Glul-Fihrinopeptide B Mr1570.677, ACTH (1–17) Mr2093.087, ACTH (18–39) Mr2465.199; ACTH (7–38) Mr3657.929) as an external standard calibration, with laser frequency of 50 Hz, repetition rate of 200 Hz, UV wavelength of 355 nm, and accelerated voltage of 20,000 V. Peptide mass fingerprint data were matched to the NCBInr database using Profound program under 50 ppm mass tolerance.

2.3.3. Peptide and protein identification by database search

Data was processed via the Data Explorer software and proteins were unambiguously identified by searching against a comprehensive non-redundant sequence database using the MASCOT software search engine (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS). The search parameters were as follows: (1) peptide quality of 800–4000 Da, mass tolerance for the fragment ion of 0.25 Da; (2) a minimum of seven matching peptides; (3) one missed cleavage; (4) Taxonomy: Viridiplantae (green plants, *Oryza sativa*); and (5) allowed modifications, carbamidomethylation of Cys (complete) and oxidation of Met (partial). Moreover, in order to evaluate protein identification, we considered the percentage of sequence coverage, the observation of distribution of matching peptides (authentic hit is often characterized by peptides that are adjacent to one another in the sequence and that overlap), the distribution of error (distributed around zero), the gap in probability and score distribution from

the first to other candidate; only matches with over 90% sequence identity and a maximum e-value of 10^{-10} were considered.

2.4. Assay of antioxidant enzymes

Fresh roots and leaves (0.3 g) were homogenized in 8 mL 50 mM PBS extraction buffer (pH 7.8). The homogenates were centrifuged at 10,000 g for 15 min and the supernatants used for enzyme activity assaying. Catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities were measured according to Wu et al. [8].

Data are the average of three independent replicates. Statistical analysis was carried out by two-way ANOVA and Duncan's multiple-range test (SSR) was used to test the significant treatment effects at significant level of $P \leq 0.05$.

3. Results

3.1. GSH protects the ultrastructure of chloroplasts and root-cells against Cd-induced damage

Cd stress (5 μM Cd alone treatment) markedly hindered shoot and root elongation, and induced brown spots and yellow necrotic patches in rice leaves. The sensitive Xiushui63 was more affected and Cd toxicity symptoms also appeared rapidly and severely. After 15 d of 50 μM GSH addition, Cd-induced destructive impact was significantly alleviated. For example symptoms of chlorosis and yellow necrotic patches in Xiushui63 were well recovered; plant height and root length increased significantly compared with Cd alone treatment in both genotypes (Supplemental Fig. S1).

As shown in Fig. 1, Cd stress affected ultrastructures of leaf mesophyll cells: disorganized parallel arrangement of lamellae and led to swollen grana/stroma lamellae and loose thylakoid membranes, reduced the number of starch grains and grana stacking per chloroplast, but increased the number of osmiophilic plastoglobuli. Cytoplasm was becoming sparsely, and membrane systems blurred in 5 μM Cd treatment. Ultrastructural deterioration caused by Cd was severer in Xiushui63 than in Bing97252. Besides, nuclear membrane was blurry or even disappeared in many cells of Xiushui63, which was rare observed in Bing97252. Addition of GSH (Cd + GSH) markedly alleviated the above structure damage of chloroplast. Compared with Cd alone treatment, chloroplasts in plants treated with Cd + GSH become better or in relatively normal shape with well structured thylakoid membranes and parallel pattern of lamellae, and unfolded less osmiophilic plastoglobuli (Fig. 1e and f). Furthermore, nuclear membrane, which was degraded by Cd in Xiushui63, was clear.

Electron micrographs of meristematic cells from 5 μM Cd stressed rice seedlings revealed obvious ultrastructural changes, compared with control, characterized by cracked karyotheca and increased vacuolar size and the distortion of cells (Fig. 2a–d). More but smaller Cd deposits formed as EDG presented in vacuoles. However, Cd-induced distortion of cells and nucleus and plasmolysis were only observed in Xiushui63, and its vacuolar became much larger than the control. Besides depositing in vacuoles, EDG were also found in nucleus of Xiushui63. Addition of GSH in 5 μM Cd, the number of vacuoles was increased, but with much smaller size, compared with Cd alone treatment (Fig. 2e and f). Moreover, more EDG were deposited in vacuoles. Dense of cytoplasm, structure of cells and nucleus were recovered almost similar to that of the control. In generally, Xiushui63 had more EDG both in vacuoles and nucleus.

3.2. Genotypic difference in protein patterns in response to Cd-stress and as affected by external GSH

Fig. 3 shows the entire image of the silver Blue-stained 2-DE gels of total extracted proteins from rice roots under normal, Cd and Cd + GSH conditions. The averages of root protein spots of 2-DE gels in control, Cd and Cd + GSH treated plants were 1505, 1617, 1924 in Bing97252, and 1851, 1569, 1921 in Xiushui63, respectively. Comparing 2-DE gels from control and from Cd and Cd + GSH treated samples showed many differences in protein presence. A 1.5-fold quantitative change was set as the criteria. Overall, 69, and 138 protein spots were found to be altered by Cd in Bing97252 and Xiushui63, respectively, with 50 and 47 up-regulated by Cd, respectively, while 19 and 91 down-regulated (Supplemental Fig. S2a). As to Cd + GSH *vs* Cd alone treatment, 35 and 51 protein spots were up-regulated, while 6 and 13 down-regulated in Bing97252 and Xiushui63, respectively (Supplemental Fig. S2b).

Total leaf proteins of control, Cd and Cd + GSH treated plants were resolved into 1929, 1925, 1744 in Bing97252, and 2005, 2137, 1978 in Xiushui63, respectively (Fig. 4, Supplemental Fig. S3). Comparative analysis of Bing97252 *vs* Xiushui63 under control condition showed 9 higher expressed spots and 19 lower expressed spots. Concerning control *vs* Cd treated samples, in Bing97252, 22 up-regulated spots were found (Supplemental Fig. S2), including 5 newly specifically synthesized spots, while 44 down-regulated; in Xiushui63, 21 up-regulated, including 1 specific expressed spots, and 15 down-regulated spots, containing 1 protein disappeared after Cd exposure (Supplemental Fig. S2). The effects of external GSH in proteomics differed between the two genotypes. Only 1 spot up-regulated in Bing97252, over Cd alone treatment, while 20 spots including 3 newly synthesized proteins up-regulated in Xiushui63. Furthermore, 17 spots in Bing97252 and 63 spots in Xiushui63 were down-regulated, respectively (Supplemental Fig. S2).

3.3. MALDI-TOF–TOF MS analysis and identification of differentially expressed proteins by Cd and GSH in the two rice genotypes

From the excised protein spots, 9 proteins of root samples were analyzed by MALDI-TOF–TOF MS (Table 1, Fig. 5). Some of identified proteins had different p*I* value with the theoretical ones. This phenomenon is a common observation in proteomic analyses, and is likely a consequence of post-translational modifications, proteolytic cleavage [9]. These 9 proteins were down-regulated by Cd in Xiushui63 but up-regulated/unchanged in Bing97252; and coinstantaneously enhanced/unchanged in Cd + GSH over Cd alone treatment in both genotypes (Table 1). For example glycolipid transfer protein-like was severely inhibited by Cd in Xiushui63 (reduced by 95% over control), and restored to appreciable level by GSH (10 folds higher than Cd alone treatment); while increased by 0.55/8.18 folds in Bing97252 of Cd/Cd + GSH treatments over control/Cd, respectively.

Concerning regulation factors above 1.5 between Cd and control in leaves, upregulated in Bing97252 and depressed/unchanged in Xiushui63, other 9 spots were chosen to be identified. One of them had no MS/MS data, although it could be identified by PMF data, its theoretical MW and p*I* did not fit well with the experimental ones. Their identities need to be further confirmed. The other 8 up-regulated proteins in Bing97252 were unambiguously identified by MS and data bank analysis via matching to proteins from *O. sativa* (Table 2, Supplemental Fig. S4).

In addition, we identified 8 proteins in leaves of Xiushui63, whose expression were significantly repressed in the comparision of Cd *vs* control but up-regulated of Cd + GSH *vs* Cd treated rice (Table 3 Supplemental Fig. S5). They are 2 protein and amino acid metabolism related enzymes (c.f. orthophosphate dikinase

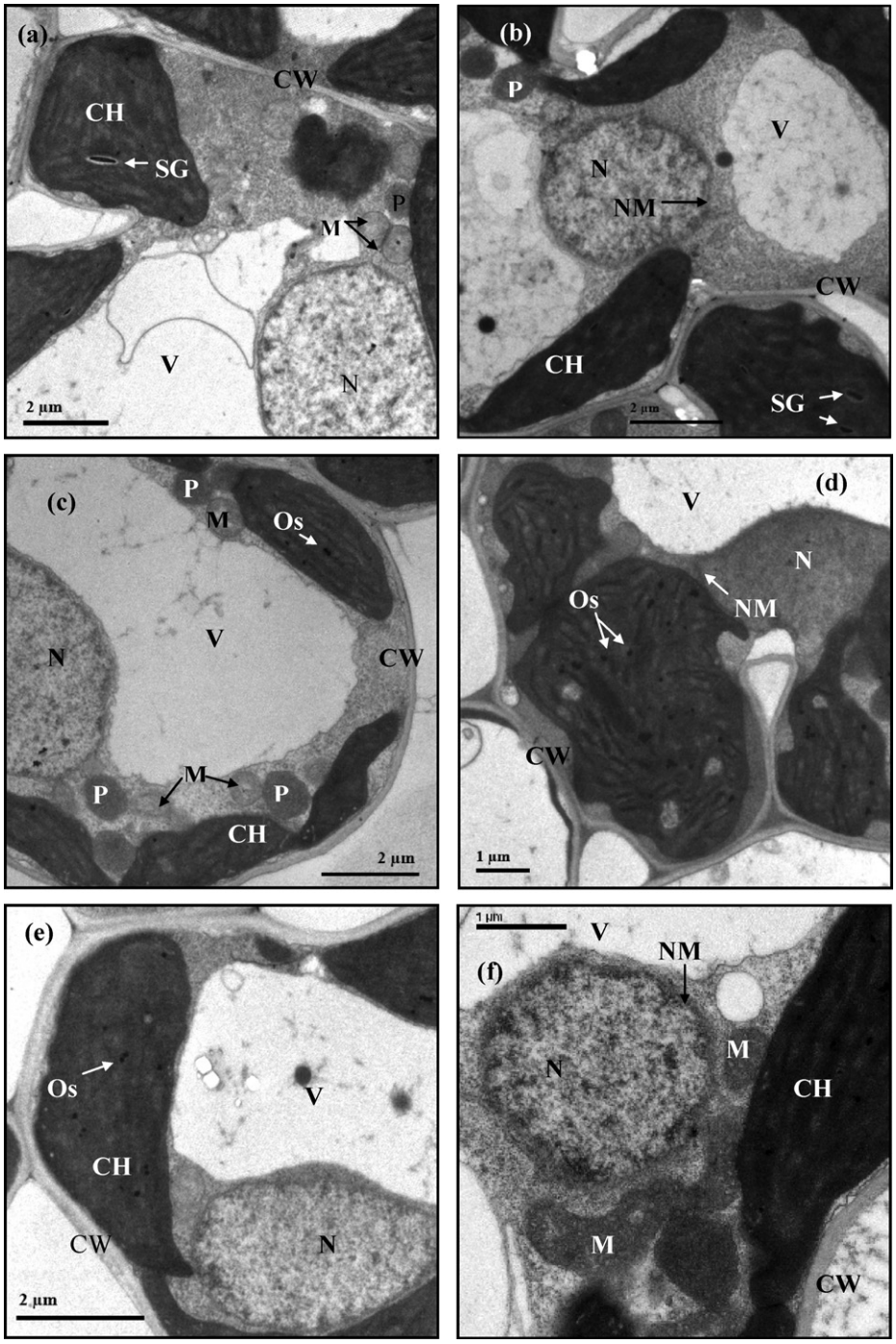

Fig. 1. Transmission electron micrograph of leaf mesophyll cells from Bing97252 (left panels) and Xiushui63 (right panels) cultured in basic nutrition solution (BNS; a and b), BNS + 5 μM Cd (c and d) and BNS + 5 μM Cd + 50 μM GSH (e and f).

and aminopeptidase N), Osjnba0039c07.4 (clpA/clpB family protein), and heat shock protein (spot ID: B-8). For example when Xiushui63 treated with Cd, expression of the heat shock protein was reduced by 86% compared with control; addition of exogenous GSH increased the expression of this protein over 5-fold than Cd alone treatment.

3.4. Response of antioxidant enzymes of CAT, POD and SOD to Cd and GSH addition

In leaves, there were significantly genotypic differences in Cd-induced changes in CAT activities: Cd enhanced CAT activity in Bing97252 after 15 d treatment, while decreased in Xiushi63 but at 15 d. GSH addition (Cd + GSH) reduced CAT activity in Bing97252 after 10 d treatment, while enhanced in Xiushui63 at 5 and 15 d compared with Cd (Fig. 6a and b). A time-dependent change in POD activity was observed. During 10 d Cd exposure, POD activity in both genotypes increased relative to control, and GSH addition brought it down towards control level after 5 d in both cultivars while at 10 d Bing97252 slightly decreased but the Xiushi63 is unchanged. Concerning SOD activity, there was no difference between Cd and control at 10 d in both genotypes. However, on day 15, SOD activity of Cd alone treatment was significantly higher than control in Xiushui63, and

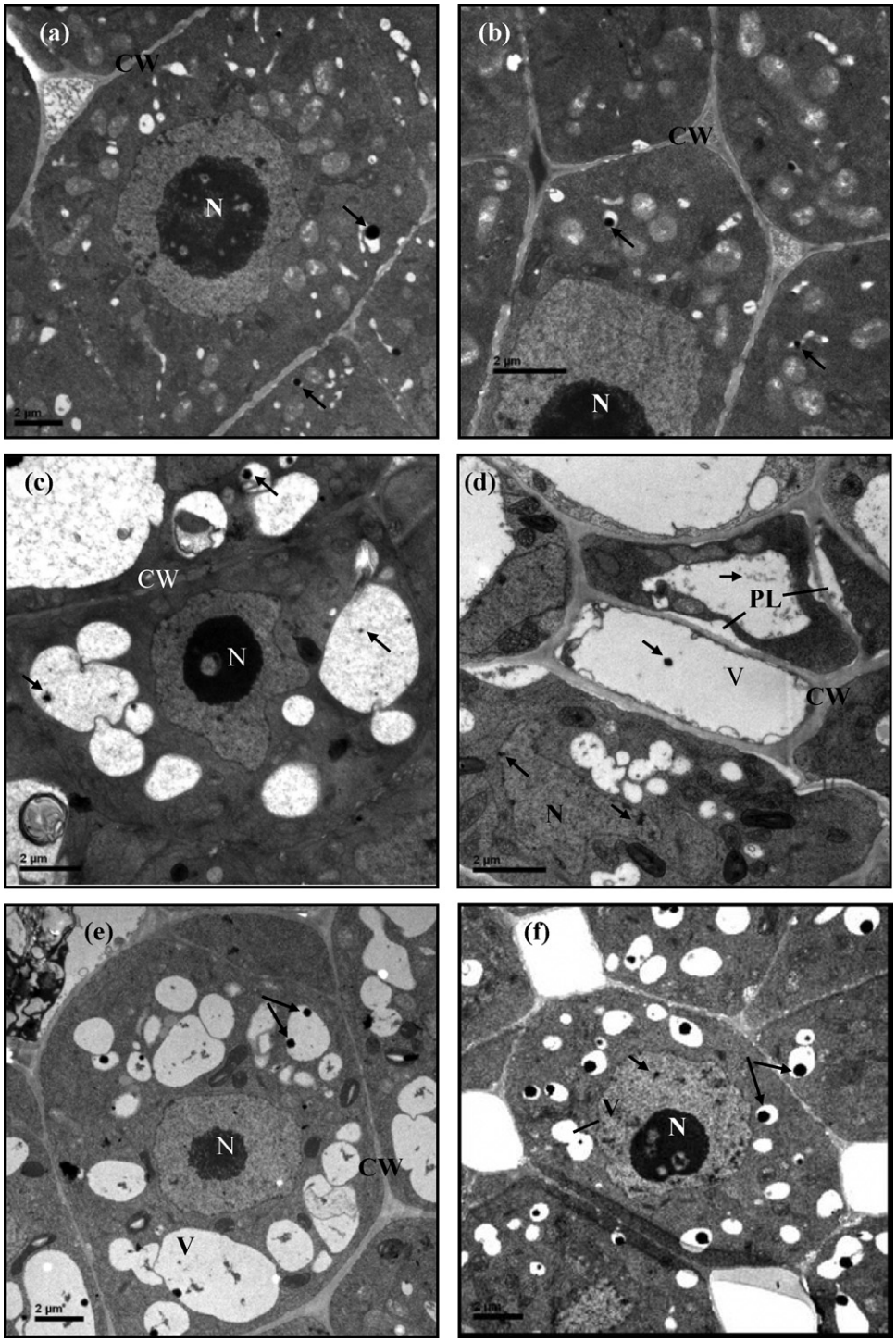

Fig. 2. Transmission electron micrograph of root tip cells from Bing97252 (left panels) and Xiushui63 (right panels) cultured in basic nutrition solution (BNS; a and b), BNS + 5 μM Cd (c and d) and BNS + 5 μM Cd + 50 μM GSH (e and f).

Cd + GSH decreased it, while no difference among 3 treatments in Bing97252.

In roots, CAT activity was seriously inhibited by Cd stress in both genotypes. Cd + GSH elevated CAT activity back to control level or even higher than control level (Fig. 6c and d). Change of POD activity in roots induced by Cd did not have genotypic difference on day 5 and 15. Cd + GSH enhanced POD activity significantly on day 15 in Bing97252, while no remarkable change in Xiushui63. During 10 d treatment, no difference in root SOD activity between Cd and control, on day 15, Cd decreased SOD activity in Bing97252, while no change in Xiushui63. Compared with Cd alone treatment, Cd + GSH did not change SOD activity except for day 15 in Xiushui63 (c.f. Cd + GSH induced a reduction by 20% in Xiushui63; Fig. 6k and l).

4. Discussion

Addition of 50 mM GSH in 5 μM Cd culture medium (Cd + GSH) significantly alleviated Cd induced growth inhibition for both rice genotypes (Supplemental Fig. S2) and dramatically reduced shoot/root Cd concentration [10,11]. The results suggested a prac-

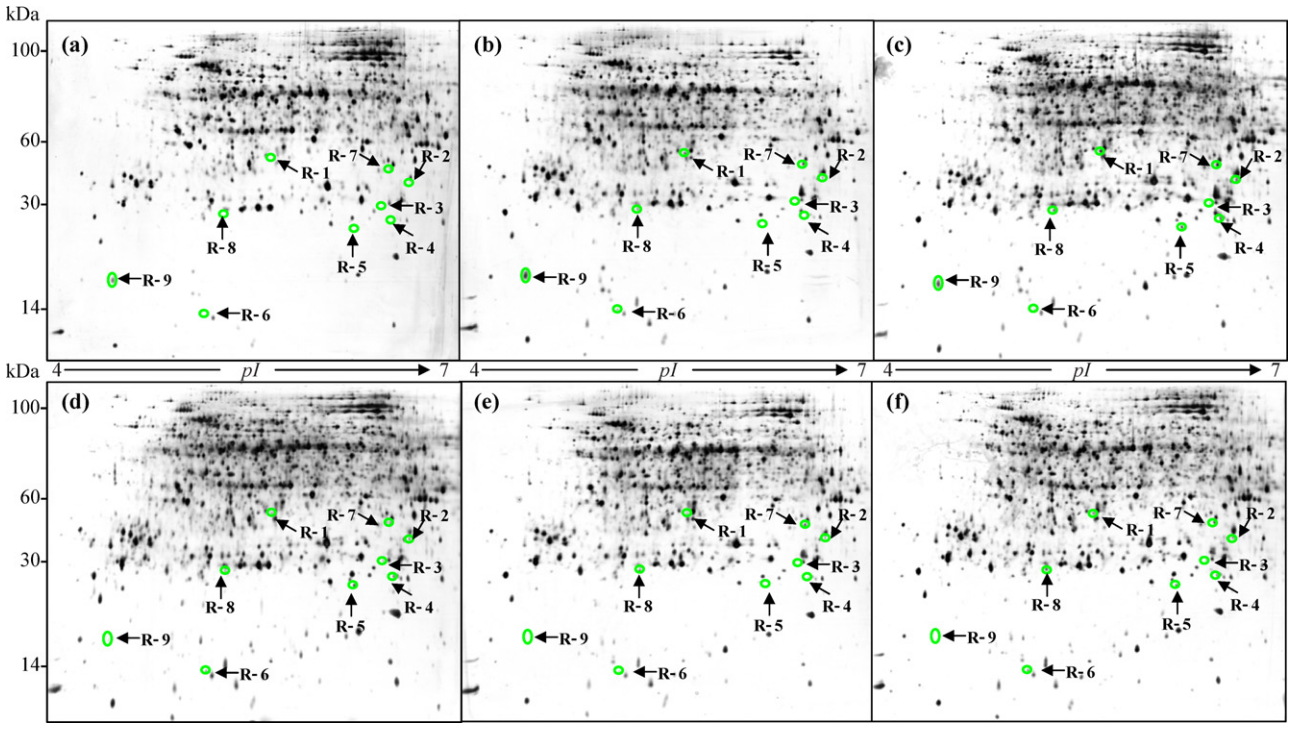

Fig. 3. Representative 2-DE maps of Bing97252 (upper) and Xiushui63 (below) root proteins isolated from normal (a and d), 5 μM Cd (b and e) and 5 μM Cd + GSH (c and f) treated plants.

Table 1
Proteins whose expression were significantly induced (+) in Cd, Cd + GSH-treated Bing97252 roots and down-regulated (−) in Cd-treated Xiushui63.

Spot No	Protein name	Accession number	MW (Da)	p*I*	Fold increase (+) or decrease (−) Cd *vs* control		Cd + GSH *vs* Cd	
					Xiushui63	Bing97252	Xiushui63	Bing97252
R-1	Os04g0652700 (nuclease family protein)	gi|115461002	32,425	5.57	−10,000	+1.72	+10,000	+0.85
R-2	L-ascorbate peroxidase 7	gi|75232661	38,302	8.76	−1.54	+0.45	+1.82	+1.18
R-3	Putative short-chain dehydrogenase/reductase	gi|50509737	26,608	6.30	−2.90	+0.68	+1.62	+1.08
R-4	Carbonic anhydrase	gi|606817	29,186	8.41	−3.82	+0.14	+2.14	+1.05
R-5	Glycolipid transfer protein-like	gi|47847652	22,687	6.10	−17.92	+0.55	+10.33	+8.18
R-6	Os08g0374000 (Bet v 1 allergen family protein)	gi|115476134	16,537	5.13	−2.56	+0.65	+2.24	+0.38
R-7	Elongation factor	gi|119395218	93,913	5.85	−1.08	+1.53	+0.74	+0.88
R-8	Chitinase 8	gi|75141289	27,534	6.09	−1.00	+3.04	+1.06	+0.96
R-9	Putative disease resistance response protein	gi|115481554	17,290	4.70	−10,000	+1.69	+10,000	-1.02

Protein spot ID refers to numbers in Fig. 3. Accession number of top database match from the NCBInr database. 'Cd *vs* Control' and 'Cd + GSH *vs* Cd' referred to fold variation of Cd exposed *vs* unexposed plants and Cd + GSH treated *vs* Cd exposed plants, respectively. Fold increase and decrease were calculated as Cd/control, Cd + GSH/Cd and −control/Cd, −(Cd/Cd + GSH) for up and down-regulated proteins respectively. All ratios shown are statistically significant ($P < 0.05$). +10,000 and −10,000 referred to the specific expressed and totally inhibited proteins, respectively.

Table 2
Proteins whose expression were significantly induced (+) in Cd-treated Bing97252 leaves but repressed (−) or increased by a factor <1 in Xiushui63.

Spot ID	Protein name	Accession number	MW (Da)	p*I*	Fold increase (+) or decrease (−)	
					Bing97252	Xiushui63
A-1	Putative formate-tetrahydrofolate ligase	gi|51536102	68,069	6.55	+1.73	−0.64
A-2	Putative dihydrolipoamide dehydrogenase precursor	gi|13365781	52,610	7.21	+2.22	−0.58
A-3	Hypothetical protein	gi|22773230	42,692	6.04	+2.00	−0.85
A-4	Putative hydroxypyruvate reductase	gi|41052893	42,048	6.56	+2.02	−0.82
A-5	Putative nucleic acid-binding protein	gi|52076131	35,403	4.41	+10000	−1.60
A-6	Putative nucleic acid-binding protein	gi|52076131	35,403	4.41	+10000	−0.29
A-7	Nucleic acid-binding protein-like	gi|42407940	14,683	5.48	+7.05	+0.63
A-8	Putative ZmEBE-1 protein	gi|115437470	44,623	6.25	+1.63	−0.94

Protein spot ID refers to numbers in Fig. 4. Accession number of top database match from the NCBInr database. Fold increase and decrease were calculated as Cd/control and −control/Cd for up and down-regulated proteins respectively. All ratios shown are statistically significant ($P < 0.05$). +10,000 and −10,000 referred to the specific expressed and totally inhibited proteins, respectively.

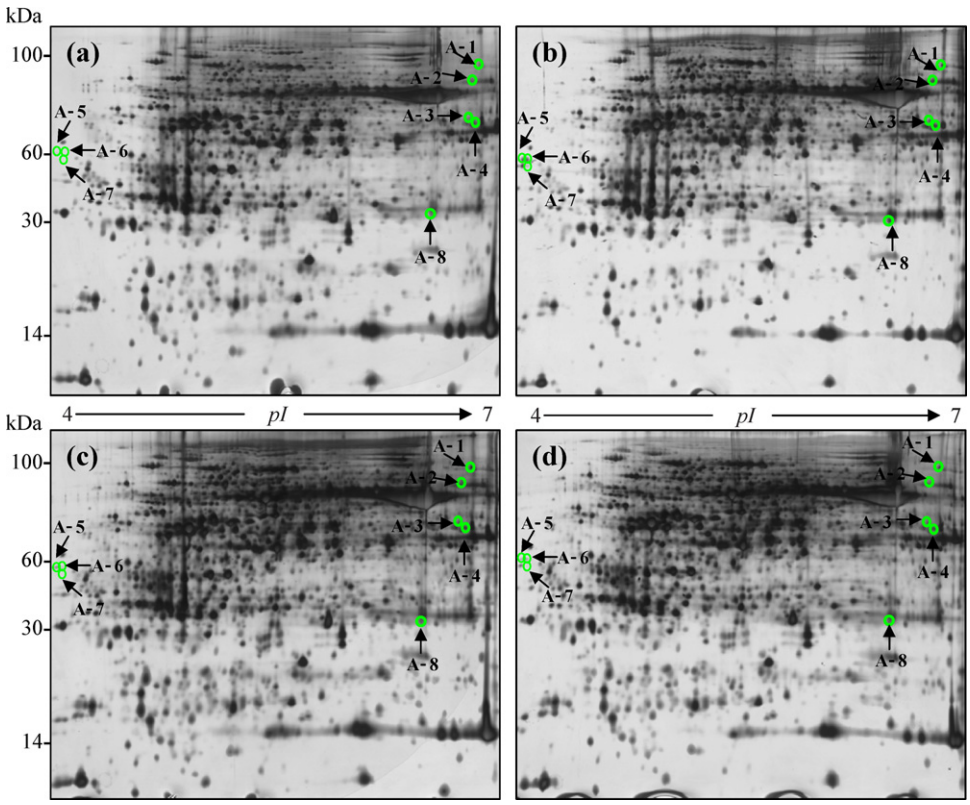

Fig. 4. Representative 2-DE maps of Bing97252 (upper) and Xiushui63 (below) leave proteins isolated from normal (a and c) and 5 μM Cd (b and d) treated plants.

tical potential for exogenous GSH application as an intervention strategy in mitigating Cd stress and reducing Cd uptake and translocation in rice plants. The reduced Cd uptake might also attribute to the complex between GSH and Cd hindered Cd uptake by root cells. Thus, we speculated that in our experiment, Cd–GSH compounds might be arrested outside cells due to heavy molecular weight. In order to prove that intracellular defense mechanism, rather than Cd–GSH compounds outside cells, was responsible for the GSH-induced alleviation effect on Cd-toxicity, rice seedlings were exposed to the following 3 treatments: pre-GSH + Cd (i.e., pretreated with 100 μM GSH for 24 h, and then transferred to 50 μM Cd solution), Cd (50 μM Cd), and Control (basal solution), separately. The results indicated that 24 h pretreated with GSH had an amelioration effect on Cd-induced inhibition of plant growth and reduced Cd uptake (Supplemental Table 1). This evidence proves the hypothesis that intracellular defense mechanism accounts for the alleviation effects of GSH on Cd toxicity. The present study demonstrates, at ultrastructural and proteomic level, the role of GSH in promoting acclimative and adaptive responses in rice seedlings to cope with Cd stress and its genotypic difference.

Vázquez et al. [12] reported that a low concentration of Cd is sufficient to cause the formation of cell wall ingrowths in hypodermis of bean roots. Increased vacuolation is a common feature induced by heavy metal at low concentration [13]. The results from the present experiment were consistent with these observations. It was assumed that vacuolation play a role in Cd detoxification and tolerance by preventing the circulation of Cd^{2+} in cytosol and forces them into a limited area [14]. Furthermore, Cd stress in present study even resulted in plasmolysis and distortion of cells and nucleus in Xiushui63. Addition of GSH, a donor of phytochelatins, significantly increased the depositing of EDG in vacuole, and the number of vacuoles but in much smaller size, compared with Cd alone treatment. Clemens [15] reported Cd ions bind PCs and form PCs–Cd complexes, which are transported across tonoplast and sequestered in vacuoles. Therefore, detailed study is needed to verify if Cd deposit formed as EDG or PCs–Cd complexes in vacuoles as a mechanism for Cd detoxification.

Leaf ultrastructural investigation revealed that Cd stress damaged the ultrastructure of chloroplasts and nucleus, as manifested by the disturbed shape and the dilation of thylakoid membranes, accumulation of osmiophilic plastoglobuli (Fig. 1c and d), with Xiushui63 being much severer. These ultrastructural alterations suggest that Cd induced important disturbances in metabolic functions and lipid composition of chloroplast membranes. Genotypic difference in the ultrastructural damage could partially account for the genotypic difference in Pn reduction [11] and biomass accumulation. Our previous study observed a much more decrease in chlorophyll content, *Fv/Fm* ratio and more increase in ROS accumulation in Xiushui63 than that in Bing97252 [11]. Increased osmiophilic plastoglobuli under Cd stress was also reported in *Chara vulgaris* [16]. Furthermore, Cd stress lead to cracked nuclear membrane in Xiushui63. Similar ultrastructural changes of radish leaf were observed [17]. The detrimental effects of Cd on leaf and root ultrastructure were almost completely reverted when GSH was added (Fig. 1e and f), indicating the membrane-integrity effect of GSH addition to alleviate the damage on the ultrastructure.

Concerning the root quantitative proteomic analyses, two of the proteins identified are involved in anti-oxidative system: L-ascorbate peroxidase (APX, Spot R-2) and putative short-chain dehydrogenase/reductase (R-3). The function of APX is well known as an antioxidant involves in scavenging H_2O_2. Lee et al. [18] reported APX and a NADH-ubiquinone oxidoreductase were induced by 100 μM Cd in rice root proteomic analysis. Short-chain dehydrogenase/reductase superfamily contains over 3000 members with a substrate spectrum ranging from alcohols, sugars, steroids and aromatic compounds to xenobiotics, and they all catalyze NAD(P)(H)-dependent oxidation/reduction reactions [19]. In the present study, elevated expression of the two proteins identi-

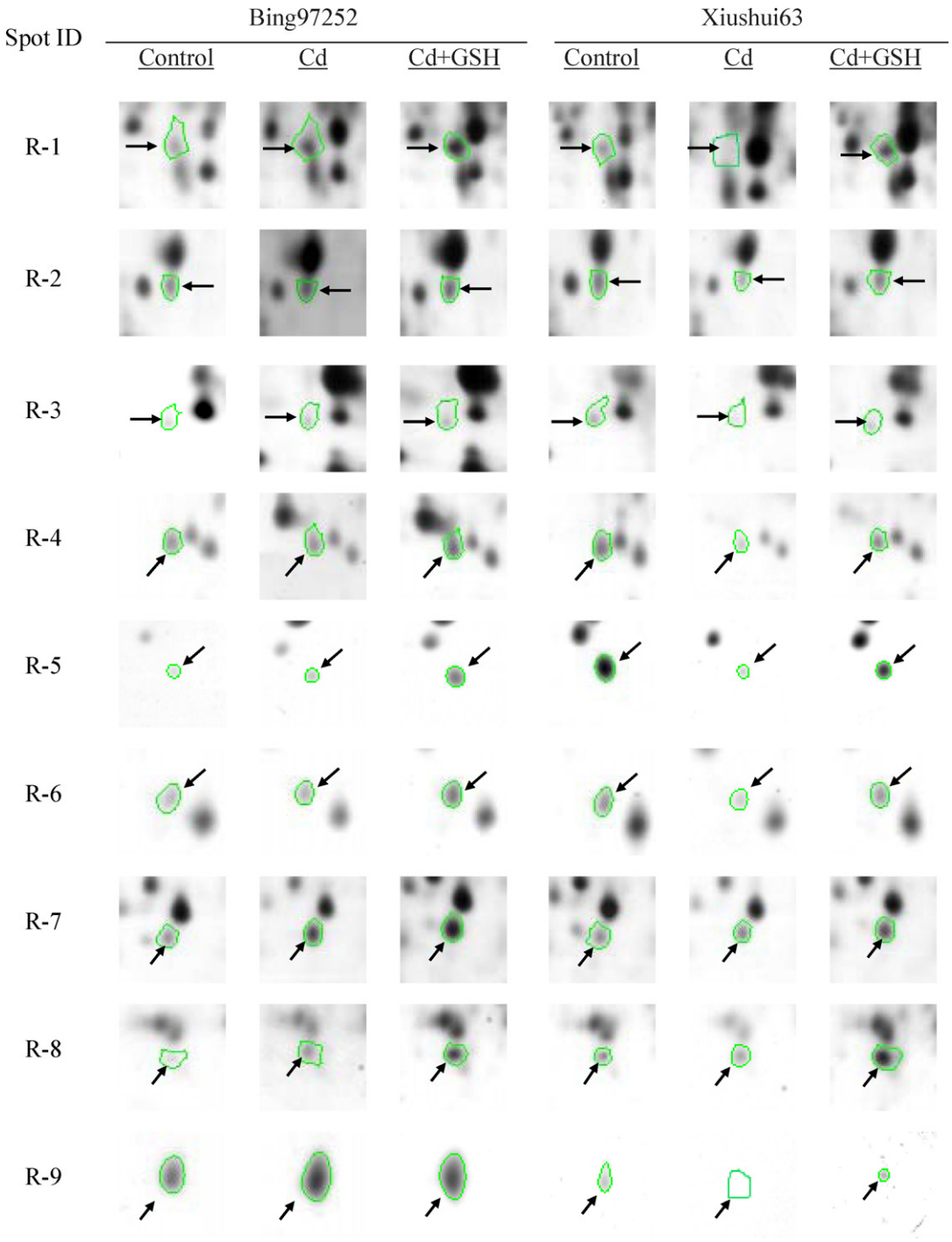

Fig. 5. The 'spot view' of identified proteins in roots.

fied in tolerant genotype after Cd exposure, and in both genotypes under Cd + GSH, may be in favor of efficaciously protection on ROS against Cd-toxicity.

Another protein, glycolipid transfer protein (GLTP), is one of lipid transfer proteins which are major membrane components. Garcia-Olmedo et al. [20] reported lipid transfer proteins are involved in plant defense and have been suggested as being important in several types of plant stress responses towards pathogens and various environmental conditions. Moreover, it has been demonstrated that Cd stimulates expression of certain lipid-transfer protein genes in barley [21]. Combing with the results observed in electronic microscope, stabilization of membrane may play a critical role in preventing root cell death induced by Cd. Significantly upregulated GLTP induced by GSH in both genotypes indicated the alleviating effect of exogenous GSH in membrane protection.

The other 6 sports are elongation factor (R-7), Os04g0652700 (nuclease family protein; R-1), carbonic anhydrase (R-4), Os08g0374000 (Bet v 1 allergen family protein; R-6), chitinase 8 (R-8), and putative disease resistance response protein (R-9). Elongation factors (R-7) are a set of proteins that facilitate the translational elongation, the steps in protein synthesis from the formation of the first peptide bond to the formation of the last one. Ge et al. [22] found an elongation factor Tu whose

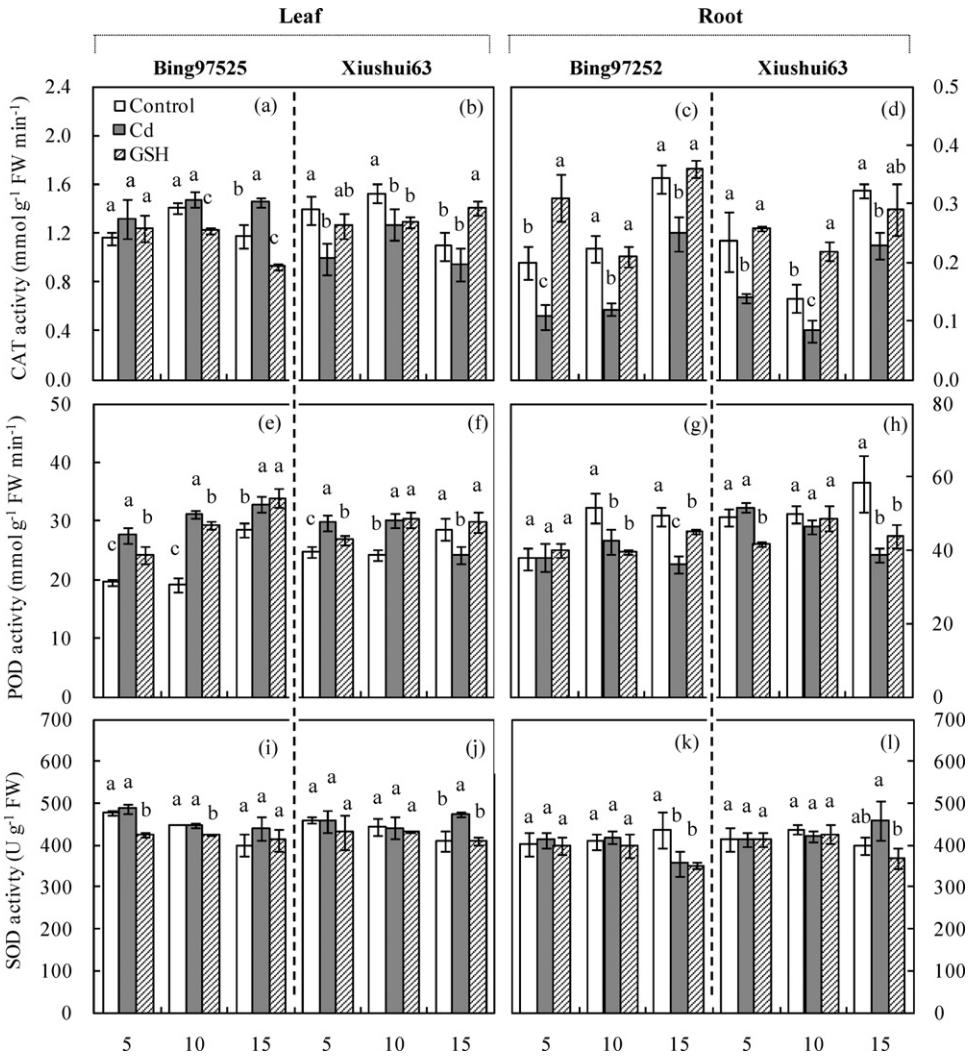

Fig. 6. Changes in CAT, POD and SOD activities of Bing97252 and Xiushui63 in leaves (left panels) and roots (right panels).

expression was up-regulated in rice roots under 100 μM Cd stress.

Os04g0652700 (R-1), a nuclease family protein, vanished in Xiushui63 under Cd stress. Carbonic anhydrase (CA, R-4) is a Zn^{2+} metalloenzyme interconverting of CO_2 and bicarbonate [23]. Nocito et al. [24] found an enhanced CO_2 fixation accompanied with increased CA activity in maize root cell. They supposed that the metabolic significance of the glycolytic activation under Cd stress could be related to the need to maintain adequate carbon fluxes through anaplerotic pathways involved in the production of TCA cycle intermediates essential to sustain GSH and PCs biosynthesis.

Chitinase is such a protein degrading chitin, which is a major component of fungal cell wall. Békésiová et al. [25] found enhanced accumulation of several chitinase isoforms in roots of different plant species exposed by Cd. In the present study, much more increase in chitinase 8 was observed in Cd-tolerant genotype Bing97252 in response to Cd stress than that in Xiushui63 (c.f. 3 and 1-fold increase in Bing97252 and Xiushui63, respectively). Os08g0374000 belongs to Bet v 1 allergen family which has evolved to bind with cytokinins [26]. The Bet v 1 allergen family protein, as well as another putative disease resistance response protein, was totally inhibited by Cd in Xiushui63, but significantly enhanced in Bing97252. This result might indicate that the absence of these pathogensis/disease related proteins decrease Cd tolerant capacity of rice roots. Ge et al. [27] also found similar responses of chitinase and Bet v 1 allergen family protein within 100 μM and 1 mM Cd treated rice. The mechanism of chitinase and Bet v 1 allergen family protein acting in plant cells still remain unknown, but the induction of them may be important for rice Cd tolerance.

The results of leaf proteomic analysis showed differences between treatments and genotypes. We identified 8 proteins whose expression was increased significantly in Bing97252 but down-regulated/unchanged in Xiushui63. They can be categorized as four groups according to their functions: carbon metabolism, TCA cycle, photorespiration and RNA processing (Table 2).

In one-carbon metabolism of C3 plant, formate-tetrahydrofolate ligase (FTL, A-1) is a key enzyme mediating conversion of glycine (Gly) to serine (Ser) through photorespiration [28]. Sarry et al. [29] found enzymes participating in one-carbon metabolism, including FTL, were up-regulated in *Arabidopsis* cells exposed to Cd. Hydroxypyruvate reductase (HPR, A-4) is a photorespiratory enzyme in leaf peroxisomes. Romero-Puertas et al. [30] found enhanced activity of HPR in pea plants exposed to 50 μM Cd. The enhanced expressions of FTL and HPR indicated an increase of photorespiration. Photorespiration metabolism is usually regarded as a wasteful process inevitably resulting from the kinetic properties of rubisco. However, photorespiration is an important pathway by maintaining electron flow to prevent photoinhibition under stress condition [31]. A recent study revealed engineered chloroplastic photorespiration increases photosynthesis and biomass in C3 plant [32].

Table 3
Proteins whose expression were significantly repressed (−) in Cd-treated and up-regulated (+) in Cd + GSH-treated rice leaves of Xiushui63.

Spot ID	Protein name	Accession number	MW (Da)	p*I*	Fold increase (+) or decrease (−)	
					Cd *vs* Control	Cd + GSH *vs* Cd
B-1	Putative aminopeptidase N	gi|42408435	97,970	5.42	−2.44	+1.70
B-2	Putative aminopeptidase N	gi|42408435	97,970	5.42	−1.95	+2.49
B-3	Orthophosphate dikinase	gi|2443402	102,736	5.98	−4.80	+2.15
B-4	Osjnba0039c07.4 (clpA/clpB family protein)	gi|38347158	98,436	5.79	−1.55	+4.53
B-5	Osjnba0039c07.4 (clpA/clpB family protein)	gi|38347158	98,436	5.79	−3.33	+3.67
B-6	Osjnba0039c07.4 (clpA/clpB family protein)	gi|38347158	98,436	5.79	−2.19	+1.69
B-7	Osjnba0039c07.4 (clpA/clpB family protein)	gi|38347158	98,436	5.79	−1.89	+3.05
B-8	Putative heat shock protein	gi|37718900	82,531	5.43	−6.11	+5.34

Protein spot ID refers to numbers in Supplemental Fig. S3. Accession number of top database match from the NCBInr database. 'Cd *vs* Control' and 'Cd + GSH *vs* Cd' referred to fold variation of Cd exposed *vs* unexposed control plants and Cd + GSH treated *vs* Cd exposed plants, respectively. Fold increase and decrease were calculated as Cd/control, Cd + GSH/Cd and −control/Cd, −(Cd/Cd + GSH) for up and down-regulated proteins respectively. All ratios shown are statistically significant ($P < 0.05$).

The protective function of photorespiration has been found under drought [33] and salt stress [34], but its role in Cd tolerance still remained unknown.

Dihydrolipoamide dehydrogenase (DLD, A-2) mediates the formation of Acetyl Coenzyme A in TCA cycle [35]. Similar enhancement was observed in *Arabidopsis* treated with Cd [29]. There are evidences that DLD can function as a part of a NADH-dependent peroxidase and peroxynitrite reductase system capable of providing protection intermediates [36]. Moreover, DLD can catalyze the reduction of ubiquinone to ubiquinol, a lipid-soluble antioxidant that protects against lipid peroxidation, and this reaction is enhanced by exposure to Cd and Zn [37]. Besides, exogenous GSH increased the expression of DLD by 2-fold and one nucleic acid-binding protein by 3.5-fold in Xiushui63, compared with Cd treatment. Nucleic acid-binding proteins (A-5, A-6, A-7) are typically cytoplasmic and nuclear proteins that associate with RNA recognition motif. The increase of these proteins in Bing97252 may help to regulate the translation and post-transcription of RNA which will provide enzymes in response to Cd toxicity.

In addition, we identified eight proteins whose expression were significantly repressed in Cd-treated and up-regulated in Cd + GSH-treated rice leaves of Xiushui63 (Table 3) to elucidate alleviate effect of GSH. Two of them were predicted to be an aminopeptidase N (APN, B-1 and B-2), which is involved in hydrolyzing peptide bonds at the N-terminus of proteins and polypeptides on membrane [38]. Dringen et al. [39] demonstrated in mammal neuron membrane that APN hydrolyze CysGly, which is product of GSH degradation reaction catalyzed by γ-glutamyl transpeptidase (γGT). However, little is known about the function of APN and its role in response to stress in plants. Storozhenko et al. [40] suggested analogous catabolism of GSH may occur in plants. Our current results suggest that exogenous GSH may also undergo such cycle to reach specifically target. According to the proteomic analysis of Ryu et al. [41], during a fungus pathogen stress (*Magnaporthe oryzae*), APN was induced in susceptible rice mutants.

There were other four spots identified as Osjnba0039c07.4 (Spot ID: B-4, B-5, B-6, B-7). From a BLAST search, Osjnba0039c07.4 is homologue of ATP-binding subunit of the ATP-dependent Clp protease. Significant induction of this protein was also found under cold and salt stress [42,43].

The other two proteins were orthophosphate dikinase (B-3) and heat shock protein (HSP, B-8). The function of orthophosphate dikinase still remains blurry in C3 plants. However, study of Moons et al. [44] showed induction of orthophosphate dikinase genes (*osppdka* and *osppdkb*) under low-oxygen stress and water deficit in rice roots. Chastain and Chollet [45] hypothesized that cytoplasmic orthophosphate dikinase in C3 plants may function to supplement the stromal pool of phophoenolpyruvate (PEP), which is of pivotal importance as a substrate for aromatic amino acid biosynthesis and phenolic metabolism, such as isoflavonoids. Ge et al. [22] also found the increase of orthophosphate dikinase in rice roots as a response to 1,2,4-trichlorobenzene stress. Heat shock proteins are well known to be induced by all kinds of stress conditions and are efficient to protecting cells against these stresses. The Cd-induced severely inhibition of HSP in Xiushui63 leaves may contribute to its Cd sensitivity, while significant increase of HSP in Cd + GSH suggesting the protective role of exogenous GSH on the expression of HSP. However, detailed studies on this post-translational modification may facilitate a better understanding of the mechanisms involved in Cd-tolerance of rice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.06.011.

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